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Determination Of Ricin Content In Castor (Ricinus Communis L.) Tissues And Comparison Of Detoxification Methods

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DETERMINATION OF RICIN CONTENT IN CASTOR (*Ricinus communis* L.) TISSUES AND COMPARISON OF DETOXIFICATION METHODS

By

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DETERMINATION OF RICIN CONTENT IN CASTOR (*Ricinus communis* L.)

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Experiments were conducted to test for ricin content in tissue samples from four castor cultivars, developing castor seed, germinating castor seedlings, and chemically and heat treated seed meal. Ricin content of each sample was examined via Western blotting with ricin A-chain specific antibodies. Results indicate that ricin is present solely within castor endosperm and is not present any other tissues. Samples from developing seed and germinating seedlings indicate ricin production begins around day 28 post pollination, and ricin is absent from the seedling 6 days after the onset of radicle emergence. This would seem to indicate that the purpose of ricin is to protect the seed and not the entire plant. Ricin content of seed meal treated separately with heat and chemicals was tested. It was found that hot-pressing of the seed was sufficient to denature ricin in the seed meal.

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CHAPTER I

INTRODUCTION

For millennia mankind has cultivated the castor plant (*Ricinus communis* L.) for the oil found in its seed. The average castor seed can contain up to 60% oil by weight, and most of that oil is in the form of ricinoleic acid (Fig. 1.1). This unique fatty acid imparts properties to castor oil that make it unique among plant seed oils. The characteristics of castor oil make it useful for a wide range of industrial applications. Unfortunately the endosperm of castor seed contains relatively large amounts of the deadly toxin, ricin (RCA_{60}), as well as the hemeagglutinin *Ricinus communis* agglutinin (RCA_{120}) and 2S albumins that are known to cause allergic reactions. Deaths from ricin handling are most commonly the result of anaphylaxis caused by allergic reactions with the 2S albumins (Chen et al., 2004). However, the presence of ricin is commonly highlighted due to its highly toxic nature. In short, even though ricin is not the source of most of the deaths from castor exposure, its presence still provides a perceived nation security issue. Therefore a comprehensive study regarding ricin content within the castor plant is needed.

The castor plant is a member of the spurge family (*Euphorbiaceae*). The genus *Ricinus* is monotypic, however castor is found to have a wide variety of phenotypes. The genus name *Ricinus* relates to the resemblance of castor seed to a tick; 'ricinus' is the

Latin word for 'tick' (Weiss, 2000). The specific epithet, castor, comes from *Castor*, the genus name of the beaver. It bears this name presumably because the scent given off by the plant resembles the musk produced by beavers when marking their territory. Castor is commonly referred to as the castorbean plant, and its seed are commonly called castor beans. This is incorrect, as castor is not a member of the *Leguminosae* (*Fabaceae*) family, and is therefore not a true bean (Weiss, 2000).

The castor plant is believed to have originated in tropical Africa. In its native tropical clime, castor behaves as a perennial; however, in sub-tropical and even temperate climes it is grown as an annual, producing winter-hardy seed that volunteer in spring (Brigham, 1993). Castor is typically considered a short-day plant (Baldwin and Cossar, 2008). However, it flowers over a large range of day lengths with some loss in seed yield (Weiss, 2000). Each raceme on the castor plant typically bears male and female flowers. In most genotypes the female flowers are distal with the male flowers proximal on the raceme. However, there are some genotypes in which the positions of the flowers are reverse, or on separate racemes, and therefore separate plants all together. The individual female flowers pollinate and ripen sequentially from the bottom of the raceme to the top (White, 1918). This is directly opposed to most domestic species in which all flowers are pollinated and ripen synchronously, producing all of the seed at once.

Castor has been bred by mankind for many centuries to produce a more suitable crop for cultivation. Wild-type castor can grow as tall as 12 meters; however, contemporary industrial cultivars have been bred to have shortened internodes and typically grow to around one meter tall to allow for machine harvesting (Brigham, 1993). Wild type castor also suffers from shattering (undesirable dehiscence) wherein as the fruit

ripens, the seed is spontaneously scattered or drops to the ground. This renders the seed unharvestable by mechanical harvesters and has therefore been selected against in modern industrial cultivars (Weiss, 2000). Most industrial cultivars have also been bred for resistance to disease and fungus such as; *Alternaria ricini* and *Xanthomonas ricinicola* (Brigham, 1993). The seed yield of modern industrial cultivars can be as much as 2,250 kg/ha with the seed containing 50-60% oil by weight; generating as much as 1,200 kg/ha of castor oil (Domingo and Crooks, 1945; Baldwin and Cossar, 2008).

Castor is a diploid with ten chromosomes (2n=2x=20). It is morphologically similar to cross-pollinated crops; however it suffers no inbreeding depression when self-pollinated. The successful breeding of castor is enhanced by this ability to self-pollinate. This allows breeders to screen and utilize single plants that exhibit desirable phenotypes and generate new cultivars relatively quickly. This has led to a vast number of phenotypically separate castor varieties (White, 1918; Weiss, 2000).

Regardless of the cultivar, the main commodity produced by castor has always been the oil found in its seed. Castor oil has been used by mankind for thousands of years. Archeological findings have dated castor oil use as far back as 6,000 years ago when ancient Egyptians used it as lamp fuel (Weiss, 1971; Brigham, 1993; Weiss, 2000). The oil has since been adapted for a variety of purposes. For centuries, it has been used for its laxative or purgative properties. Contemporary uses of castor oil range from pharmaceutical and cosmetic additives to industrial and aviation lubricants and hydrolytic fluids. Most of the industrial applications for castor oil stem from a unique hydroxy fatty acid, ricinoleic acid (Zimmerman, 1958). Castor oil contains approximately 90% ricinoleic acid; making it the only commercially available source of significant amounts

of the fatty acid (James et al., 1965; Broun and Somerville, 1997). Ricinoleic acid imparts several unique properties to castor oil, making it one of the most important commercial plant oils. First, castor oil blends easily with polar alcohols, important additives in many industrial processes. The most notable example of this is the transesterification of castor oil into biodiesel in which raw, filtered castor oil has to be mixed with methanol or ethanol and a base catalyst then heated for a period of time to produce crude biodiesel (Conceição et al., 2007). Second, ricinoleic acid stabilizes the gelling temperature of castor oil. This means that castor oil stays fluid at lower temperature compared to other vegetable oils; a useful characteristic for use as a cold-weather lubricant (Zimmerman, 1958). Finally ricinoleic acid does not rancify unless heated excessively, an important characteristic for oils to be stored for long periods of time without breaking down and becoming unusable (Ogunniyi, 2006).

Castor oil is produced by expressing the oil from the endosperm of the castor seed (Fig. 1.2). Mechanical pressing only recovers 45-50% of the oil within the seed (25% of seed weight). In order to obtain the remaining oil, solvent extraction procedure must be used (Ogunniyi, 2006). In solvent extraction, the ground seed meal is typically mixed with an organic solvent (usually hexane) and both the solvent and the oil are separated from the meal. The solvent is then recovered by distillation (Gardner et al., 1960). Both mechanical pressing and solvent extraction produce seed meal as a byproduct. This resulting seed meal is high in nitrogen and can therefore be sold for use as fertilizer (Spies et al., 1962). The meal as well also exhibits nematocidal activity when used as fertilizer for plants that are susceptible to nematode infection (Akhtar, 1997).

Individual castor seed are generally large, with the endosperm making up the majority of the volume and weight. The cotyledons occupy a small area between the two prominent lobes of the endosperm (see Fig. 1.2). The entire endosperm is coated in a thin membrane that separates the endosperm from the testa (seed coat). The caruncle (elaiosome) is a small bump on the end of the seed that regulates the uptake of water by the seed. Directly interior to the caruncle is the radical (Weiss, 2000). The radicle is the first part of the embryo that expands during germination to break through the seed coat. The radicle expands throughout germination to become the root and hypocotyl of the seedling. As germination takes place, the endosperm is absorbed as an energy source by the heterotrophic embryo (Ahn and Chen, 2007). Once the endosperm is nearly completely absorbed, it separates from the cotyledons, allowing them to expand.

Unfortunately the endosperm of the castor seed contains the potent cytotoxin, ricin. Ricin is not soluble in oil; therefore, when the oil is removed from the meal, the concentration of ricin is effectively doubled (Spies et al., 1962). Literature suggests that ricin is present within all parts of the castor plant (Weiss, 1971). Other research has stated that all parts of the plant are toxic(Knight, 1979; Weiss, 2000). However no research has definitively stated that the entire plant contains significant amount of ricin. Literature has also identified day five of germination as the point at which ricin disappears from the seed (Harley and Beevers, 1986). However, no known literature has provided the time at which ricin begins to accumulate within the seed. This information is paramount for handlers of castor to know in order to safely manage and harvest their fields. However ricin is not the only hazardous material found within castor seed. The endosperm also contains RCA_{120}, a relatively weak cytotoxin, as well as 2S albumins,

which are known to cause severe allergic reactions (Chen et al., 2004). This makes castor seed meal dangerous to handle by susceptible individuals. Therefore the meal must be treated to inactivate the toxins and allergens before it can be safely handled or used as feed for livestock (Bris and Algeo, 1970; Vilhjalmsdottir and Fisher, 1971).

Ricin and RCA_{120} are cytotoxic proteins from a family of carbohydrate-binding, plant proteins called lectins. Ricin is classified as a type II ribosome-inactivating protein (RIP). Proteins of this group are heterodimeric, containing an A-chain and a B-chain, with only A-chain having toxicity. The A-chain functions by enzymatically depurinating the adenine residue from position A_{4324} on the rRNA of the 28s ribosomal subunit in eukaryotic cells (Endo et al., 1987). This irreversibly destroys the binding site for elongation factors on the ribosome; effectively rendering the entire ribosome inactive. A single A-chain molecule is capable of depurinating approximately 1,500 ribosomes per minute within a eukaryotic cell (Pinkerton et al., 1999). This removes the ability of the cell to synthesize new proteins, so the cell eventually dies. The A-chain alone, however, is not able to enter the cell (Harley and Beevers, 1982; Lord et al., 2003). The B-chain of ricin allows for the entry of the entire molecule into the target cell. The B-chain is the lectin element of the ricin molecule. It binds galactosides residues on the surface of the cell. Once bound, the cell absorbs the ricin molecule via endocytosis. Inside the cell, the disulphide link joining the A-chain and B-chain is broken, releasing the active A-chain into the cytosol (Endo et al., 1987). RCA_{120} shares approximately 90% homology with ricin; however it is a tetramer, containing two A-chains and two B-chains (Harley and Beevers, 1982; Roberts et al., 1985). Compared to ricin, RCA_{120} is a weak cytotoxin and

does not absorb through the digestive tract as readily. However, RCA_{120} does show more hemeagglutinin activity than ricin (Harley and Beevers, 1982).

Ricin is perceived as the most dangerous element within castor seed. It has an LD_{50} (lethal dose for 50% of a population) in mice of 30 mg/kg ingested and 3-5 μg/kg inhaled or injected, making it one of the most toxic natural compounds (Audi et al., 2005). However, most deaths from exposure to castor seed or seed meal result from anaphylaxis due to the 2S albumins, not ricin (Knight, 1979; Chen et al., 2004). The presence of ricin is still a major concern because there is no known antidote for ricin poisoning. Ricin is lethal to everyone, not only those allergic to it, providing a much wider range of potential casualties. Thus, concerns about the use of ricin for bioterrorism have become an issue that impacts domestic commercial production (Audi et al., 2005).

Historically, methods for identifying ricin containing samples involved a live animal bioassay. In live animal bioassays, animals, usually rats or mice, are injected with a sample (Spies et al., 1962). If the animal dies or exhibits certain ailments, the sample is considered to have contained ricin. This methodology is expensive, time-consuming, and prone to false positives. The animal may die from other toxic elements within the sample and not specifically ricin, leading to an inaccurate assessment of the sample. Current methods employ antibodies that are specific to ricin (Harley and Beevers, 1982; Harley and Beevers, 1986; Alderton and Paddle, 1997; Pinkerton et al., 1999). Assays such as enzyme-linked immunosorbent assays (ELISA), Western blotting, and dot-blotting rely on the binding of ricin within a sample by the ricin-specific antibodies. Horseradish peroxidase (HRP) bound secondary antibodies that are specific to the ricin-bound antibodies are then added and the samples are then assayed for HRP activity. The HRP

activity directly correlates to the amount of ricin within the sample. This method of detecting specific proteins in a sample was first described by Towbin (1979). It has since been modified to allow for greater sensitivity and ease of use. Modern methods utilize a semi-dry technique for transferring the proteins from the gel to the membrane. The basics of the assay however, remain intact.

Other assays, such as radial immunodiffusion (RID), involve the immuno-precipitation of ricin from a sample using the ricin-specific antibodies (Harley and Beevers, 1986; Pinkerton et al., 1999). The amount of precipitation can then be measured, directly correlating to the amount of ricin present in the sample. All of these methods can be run using whole protein extract or purified ricin. RID assays can also make use of solid materials such as dissected seed tissue or seed meal (Pinkerton et al., 1999). Western blotting provides the most information from a single assay because individual proteins are separated via polyacrylamide gel electrophoresis (PAGE) before the ricin is detected. This allows the researcher to see any other protein that may be reacting with the ricin-specific antibodies as well as identify the samples that contain ricin.

Therefore, the objectives of this research were to identify plant parts containing ricin, identify when ricin was produced in the developing seed, confirm that ricin levels decrease within the seedling around day five of germination, and identify viable strategies that could be applied in the oil industry to detoxify castor meal.

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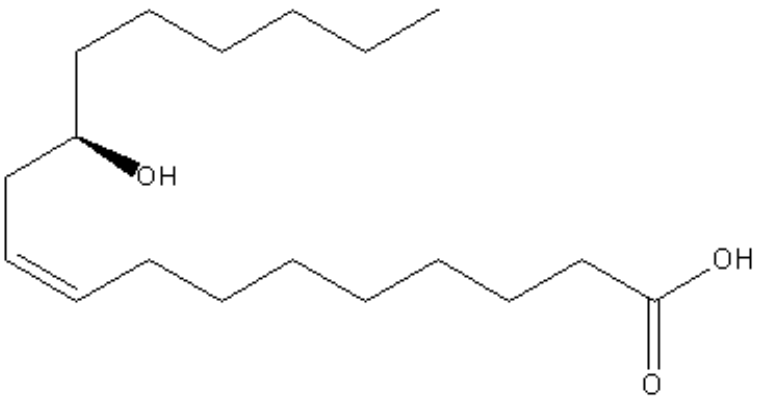

Fig. 1.1 Ricinoleic acid

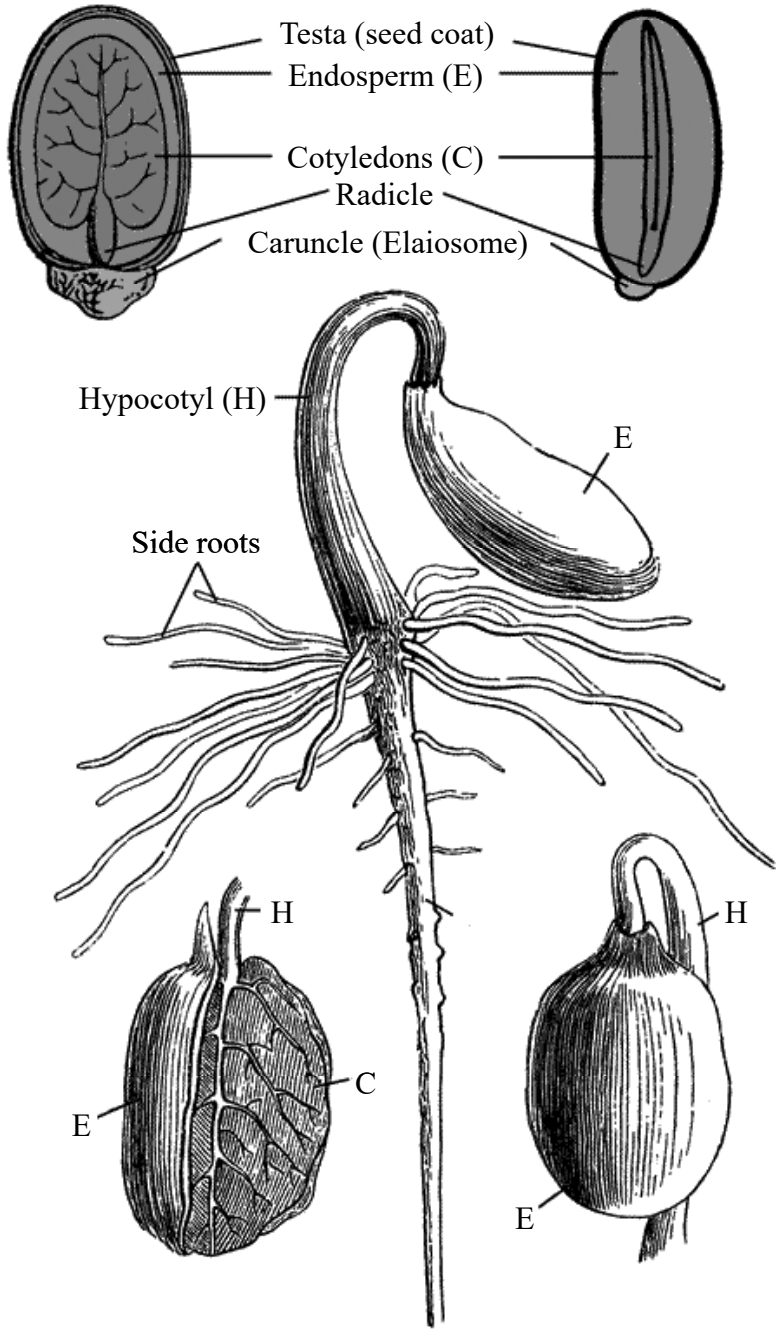

Fig. 1.2 Illustration of castor seed and seedling.

Modified from: Sachs, J. 1887. *Vorlesungen über Pflanzen-Physiologie*. Verlag Wilhelm Engelmann, Leipzig. From: Leubner, G. 2007. The Seed Biology Place. <http://www.seedbiology.de>.

CHAPTER II

DEVELOPMENTAL ACCUMULATION OF RICIN IN CASTOR SEED AND SUBSEQUENT DEGRADATION OF RICIN DURING GERMINATION

Abstract

The castor plant (*Ricinus communis* L.) is a euphorb, commonly grown for the high oil content found in its seed. The seed unfortunately also contains a deadly toxin know as ricin (RCA_{60}) as well as an allergen called ricin agglutinin (RCA_{120}). Using antibody screening (Western blotting) techniques, quantification of ricin content within seed, cotyledon, root, hypocotyl, and leaf tissues was performed. This analysis revealed that ricin is only present in the seed. In order to determine when the ricin accumulates in the seed, tissue samples were harvested as the seed developed and upon germination. Developing seed were collected from inflorescences in 4 day intervals. Total protein was extracted and assayed via Western blotting with a ricin A-chain specific antibody. Ricin was absent from the developing seed throughout development until the 28th day post pollination. Ricin content quickly increased with seed ripening. Seed were also germinated and collected at 2 day intervals once radicle emergence began, until the 12th, day when seedlings were fully expanded. Due to inconsistent germination, day 1 was set at the first appearance of the radicle. The germinating seedlings, from the 6th day onward, were dissected into: root, hypocotyl, and cotyledonary tissue. Total protein from

each tissue from each sample was extracted. Western blot analysis indicated that after the 6th day, ricin is degraded beyond levels of detection. Ricin is only detectable in the seed tissue from day 0 (ungerminated seed) through the 6th day samples and is not detected in any of the root, hypocotyl, or cotyledonary tissue. These data suggest ricin is generated and stored in the seed for the specific purpose of protecting the seed from predation in its quiescent state.

Introduction

The castor oil plant (*Ricinus communis* L.) has been cultivated for centuries specifically for the high oil content found in its seed. Castor oil commonly comprises as much as 50-60 percent of the weight of the seed, making it one of the highest yielding oil-seed crops (James et al., 1965; Weiss, 1971; Weiss, 2000; Baldwin and Cossar, 2008). The high oil content of castor seed makes it an attractive candidate for biodiesel production. More oil per hectare means cheaper oil for biodiesel production and therefore cheaper biodiesel. The composition of the castor oil also plays an important role in its use as a biofuel. Castor oil is 90 percent ricinoleic acid, a fatty acid for which castor seed is currently the only commercial source (James et al., 1965). Ricinonleic acid is good for use in biofuels because it is miscible in alcohol. The transesterification of raw oils into biodiesel requires the oils to be mixed with alcohol. Since castor oil is miscible in methyl and ethyl alcohols, the transesterification process would potentially be more efficient than with non-miscible oils (Conceição et al., 2007).

Unfortunately, besides large amounts of oil, castor seed also contains concentrated amounts of the cytotoxic lectin, ricin (RCA_{60}) and an allergen, *Ricinus*

communis agglutinin (RCA_{120}). Ricin and RCA_{120} are almost identical in their protein sequence. However, ricin is a heterodimer consisting of a single A-chain disulphide bonded to a separate B-chain, whereas the agglutinin is a tetramer consisting of two A-chains bond to two B-chains. The differences between the two proteins are evident in their relative toxicities. Ricin is a highly toxic protein whereas ricin agglutinin is a weak toxin (Roberts et al., 1985). Ricin functions by disrupting the protein synthesis mechanism of the cell. The A-chain of ricin is the toxic portion of the protein. The A-chain functions by depurinating the 28S subunit of the infected cell's ribosomes. The B-chain of ricin is responsible for getting the A-chain into the target cell. It consists of a galactose-binding region capable of binding a target cell's membrane and initiation cell-entry (Endo et al., 1987). Without the B-chain's functionality, the A-chain cannot enter the cell (Harley and Beevers, 1982). However, once it is in the cell, the A-chain can disrupt up to 1,500 ribosomes per minute (Pinkerton et al., 1999). This unique action makes ricin highly toxic, with an LD_{50} (lethal dose for fifty percent of a population) of 3-5 µg/kg when inhaled and 1-20 mg/kg when ingested (Audi et al., 2005). While ricin is the major toxin in castor seed, the 2S albumins also found in the seed can cause a severe allergic reaction in sensitive individuals, and anaphylaxis caused by these allergens is the most common cause of death in the castor processing industry (Chen et al., 2004).

Castor is typically monoclinous monoecious and bears its female flowers distal to the male flowers on the same inflorescence. In the United States, castor typically emerges 10-13 day after sowing and flowers at around 50 days. The fertilized flowers typically bear seed in spiny, trilobial capsules on the raceme. Seed are fully ripened 40-50 days post-pollination (dpp) depending on environmental conditions (Weiss, 2000;

Baldwin and Cossar, 2008). Oil accumulates within the seed starting 20 days after pollination. Protein synthesis occurs throughout development, but accelerates at about 20 dpp until maturity (Weiss, 2000). Castor seed is often used as an example of a non-cereal endospermic seed. Mature seed is comprised of a testa (seedcoat), a relatively large endosperm, and an embryo. Upon resumption of growth by the embryo (germination), nutrients in the endosperm are consumed, providing energy for the rapidly expanding seedling.

Literature has reported that all parts of the castor plant are toxic, and some literature claims that ricin itself is present throughout the plant (Weiss, 1971; Knight, 1979; Weiss, 2000). If ricin is in fact, not the toxic element found throughout the plant and is only found in the ripe seed and early seedlings, then the castor plant itself would not be as dangerous as previously thought. Therefore it is important to determine the tissues that contain ricin as well as what concentration of ricin those tissues may contain. This data would be helpful in informing castor farmers of the risks associated with field plants. Hopefully this information could then be used to make castor cultivation and handling a safer enterprise.

The objective of this research is to determine the tissues of the mature castor plant that contain ricin, the time-points at which ricin is accumulating within the seed during development, and when the ricin is degraded in the germinating seed. This knowledge may aid in future efforts to better understand the mechanism under which ricin is generated as well as the role that ricin plays in castor seed development and germination, which may provide a protocol for safe handling and methods of silencing ricin in castor seed.

Materials and Methods

Sample Preparation

Seed of four genetically diverse castor cultivars (Hale and Lynn from Browning Seed, Plainview, TX; Carmencita from Dr. Brian Baldwin; and Bay Farm ornamental from Bay Farms, Bay City, MI) were planted and allowed to reach maturity. Mature root, cotyledon, hypocotyl, and leaf tissues were then harvested from the field and placed at -20°C for short-term storage. Non-germinated seed was also stored at -20°C until all samples were ready for co-processing. These samples were then used to identify the tissues containing ricin from each cultivar.

For the ricin accumulation portion of the study, mature castor plants [cv. Hale] were monitored and developing ovules as whole seed were harvested at 4 day intervals from 0 to 44 days. Three ovules were then immediately stored at -80°C until all samples could be co-processed.

Seed for the ricin degradation throughout germination study were incubated at 30°C on filter paper moistened with a 1-2g a.i/L Captan (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide) solution (Southern Agricultural Insecticides Inc.; Palmetto, FL). Three seed/seedlings were harvested on 2 day increments throughout the 12 days of germination beginning with first emergence of the radicle. The germinating seedlings were then dissected into endosperm, root, hypocotyl, and cotyledon samples and frozen at -80°C. When all samples were ready, they were co-processed.

Protein Extraction

Samples were submerged in liquid nitrogen then milled with an A11 Basic S1 analytical mill (IKA; Wilmington, NC) until homogenous. The milled samples were then weighed and separated into 0.2g aliquots and placed in 1.5mL Eppendorf tubes. Next 1mL of complete protein extraction buffer [50mM Tris Base, 120mM sodium chloride, 0.5% Nonidet P-40, 1mM Na-EDTA, 1mM DTT, 10mM ß-glycerophosphate, 0.1mM sodium fluoride, 0.1mM sodium orthovanadate, 1 tablet/25mL Complete™ protease inhibitor cocktail tablets (Roche; Basel, Switzerland) pH 7.4] was added to each sample tube (Draetta et al., 1989). The tubes were then vortexed for 1h at 20ºC. After vortexing, the tubes were centrifuged at 10,000 x g for 5 min at 20ºC in order to pellet solids. The resulting supernatant was then carefully removed via micropipet and placed in a Nanosep® MF 0.45µm pore-size centrifugal-filter (Pall Life Sciences; East Hills, NY) spin-filter tube and centrifuged at 14,000 x g for 1 min at 20ºC to remove any remaining solid material. The protein content of each sample was then calculated using a Micro BCA™ Protein Assay kit (Pierce; Rockford, IL) as per the manufacturer's instructions.

Western Blotting

Protein samples were reduced by heating aliquots to 90ºC for 10 min in the presence of loading dye (2% SDS, 12.5% glycerol, 0.002% bromophenol blue, 0.002% acid orange G, 50mM dithiothreitol, 62.5mM Tris-HCl, pH 6.8). The reduced samples were then loaded (2-35µg per well) onto a 0.75mm thick 12.5% Next Gel™ SDS-PAGE gel (Amresco, Solon, OH) with a 6% Protogel® stack (National Diagnostics; Atlanta, GA) for separation. An RCA_{60} positive control (Vector Labs; Burlingame, CA) was

loaded into empty lanes to serve as a positive identifier of ricin content. Gels were run at a constant 220V for 3 hours or until the bromophenol blue loading dye was within 1cm of the bottom of the gel. Separated proteins within the gel were transferred to an Immobilon™-FL PVDF membrane (Millipore; Billerica, MA) using a Panther™ semi-dry transfer apparatus (Owl; Rochester, NY) and Towbin transfer buffer (25mM Tris base, 192mM glycine, 20% v/v methanol pH 8.3) for 1h at 0.8mA/cm^2 (Towbin et al., 1979). After transfer membranes were blocked overnight (12-16 h) in NZAB-Teleostean gelatin blocking buffer [2% casein enzymatic hydrolysate, 2% Teleostean gelatin, 6ppm Kathon™ CG/ICP II (Supelco; Bellefonte, PA) in DPBS pH 7.4] on a rocker (25rpm) at 20ºC. Blocking buffer was removed, and a 1:1,000 solution of RCA-52B anti-RCA60 mouse IgG primary antibodies (US Biological; Swampscott, MA) diluted in Tris-buffered saline containing 0.5% Tween-20 (TTBS) at pH 7.4 incubated with the membrane for 1h at 20ºC. These antibodies bind specifically to the A-chain of ricin (RCA_{60}) although they also exhibit some cross-reactivity to ricin agglutinin (RCA_{120}). After incubation the primary antibody solution was removed, and the membrane was washed in TTBS for 5 min 4 times at 20ºC. A 1:100,000 solution of horseradish peroxidase conjugated Fc specific anti-mouse goat IgG (Sigma-Aldrich; St. Louis, MO) diluted in TTBS was added placed on the membrane and allowed to incubate for 1h at 20ºC. After incubation, the membrane was washed again in TTBS for 5 min 4 times at 20ºC. Residual TTBS was removed from the membrane, and a 1:1 solution of luminol and enhancer solution from the Supersignal© West Femto kit (Pierce; Rockford, IL) was overlaid onto the membrane. The membrane was then transferred to a film cassette and exposed onto CL-XPosure™ film (Pierce; Rockford, IL) in a dark room to imprint the signal onto the film. The

exposed film was then developed using an M35A X-Omat™ processor (Kodak; Rochester, NY).

Results and Discussion

Four diverse cultivars were selected to determine whether or not ricin is truly present throughout the entire castor plant. Ricin was determined to be present only in the seed and in no other tissues of the mature castor plant in all four cultivars tested (Fig. 2.1 and 2.2). These data conflict with previous literature that has suggested that ricin is found in all parts of the castor plant (Weiss, 1971). It is possible that the ricin is present in very low quantities in the other tissues of the plant, but it was not detectable using the Western blotting procedures described in this paper. Using data from this study and the sensitivity of this procedure, the ricin concentration of non-endospermic tissues would be lower than the nanogram per gram range (parts per billion, ppb). This would require more than 270 – 450kg of non-endospermic castor tissues to contain enough ricin to kill a 90kg human via injection or inhalation. At these amounts, even relatively nontoxic compounds exhibit harmful effects.

The response of protein extracted from developing castor ovules to ricin-specific antibodies is summarized in Figure 2.3. The antibody response to ricin is not detected prior to 28 days post pollination, indicating that ricin is only present in the last 16 days of seed development (Fig. 2.3 Lanes 9-13). This is especially interesting considering that the majority of seed protein development begins around 20 days post pollination (Weiss, 2000). This indicates that ricin production is specifically delayed by the plant during the majority of early seed development. A reason for this could be to protect the plant from

its own ricin during the early stages of development, when the developing embryo is likely vulnerable. During the early stages of seed development, cells within the ovary and accompanying tissues are rapidly dividing and differentiating (Weiss, 2000). At this point any stray ricin molecules would likely result in a nonviable stem cell, giving rise to large areas of void where there should be cells in the seed.

There is a slight decrease in the visual signal generated by the antibodies at day 40 compared to days 32 and 36 (Fig. 2.3 Lane 12). This variation is likely due to the increase of total protein synthesis in the seed at this time. As the other proteins increase in concentration the "apparent" concentration of ricin in the sample decreases through dilution effect though the actual amount of ricin in the whole seed may stay the same or even increase. There are also bands representing proteins of approximately 14kDa in the lanes corresponding to days 40 and 44 (Fig. 2.3 Lanes 12 & 13). These proteins have not been directly identified. However, it is possible that these are fragments of the ricin A-chain or RCA_{120} that accumulate as protein levels increase.

Ricin content of the different tissues of germinating castor seed is shown in Figure 2.4. A positive response to ricin-specific antibodies was present in dissected endosperm tissue up to day 6. From day 8 and on, none of the tissues examined showed any detectable response to the ricin-specific antibodies. Based on these findings it appears that the ricin is only present in the endosperm of the seed, and only until day 6, after which it is either used by the developing seedling putatively as a nitrogen source or enzymatically degraded to avoid poisoning the germinating seedling (Ahn and Chen, 2007). Literature suggests that ricin is no longer present in the germinating seed after day 5 (Harley and Beevers, 1986). However germination timing is dependent on a number of

factors including; temperature, moisture availability, and lighting conditions, making multiple reports difficult to compare. It is also important to highlight that the previous research labels the day of planting as day 0 whereas this research labels the first day of radical emergence as day 1. This is an important distinction because different seed will germinate at different rates.

Since ricin is only found in seed tissue and only during a certain period of seed development and germination, the role of ricin in the castor plant's lifecycle becomes a critical question. It has long been hypothesized that ricin serves as a general defense mechanism for the whole castor plant (Peumans and Damme, 1995). This makes sense if the toxin were present throughout the entire plant. However, since the ricin is only present in the nearly mature seed and early seedling, it cannot serve as a very effective general plant defense mechanism. There has also been speculation that ricin is one of the compounds responsible for the nematicidal activity of castor (Akhtar, 1997). However since the roots do not contain ricin, this cannot be the case, and the point can be made that a silencing of the ricin gene should not affect the nematicidal benefits of plants castor in crop rotation.

Instead it would appear that the ricin is serving to protect only the mature seed before germination. The seed is very high in oil and is therefore potentially a source of nutrition for birds, small mammals, and insects. If the seed lacked any sort of defense from predation, there would be very little seed left to continue the castor lifecycle. Ricin is also unique in that it can affect any eukaryotic cell regardless of cell origin; some insect, bird, reptile, or mammal (Peumans and Damme, 1995). So, ricin appears to be present in the seed to act as a deterrent from predation. Ricin is so toxic that very little of

the seed would be consumed before the offending predator is dispatched. However, if ricin were present in the early development of the seed and in the developing seedling, it is possible that there could be contamination of the rapidly dividing cells resulting in damage to the plant.

Conclusion

Castor oil has historically been and continues to be a valuable commodity. Unfortunately the growing, harvesting, and handling of the castor oil plant is made difficult by the presence of the cytotoxin ricin (RCA_{60}) as well as the less toxic *Ricinus communis* agglutinin (RCA_{120}). It is therefore useful to know at what developmental stage ricin is present so that industrial producers of castor oil can be aware of the risks involved in the different stages of cultivation. This research has shown that ricin is only present in significant amounts in castor seed after 28 days of seed development, and ricin dissapears from the plant approximately 6 days after radicle emmergence. This information provides insight into when castor is ultimately most safe to handle and when precautions need to be taken to avoid ricin exposure. It also provides information that allows one to discern the biological function that ricin holds in the castor lifecycle. Because of its late sythesis in the seed and early disappearance from the seedling it can be assumed that ricin does not play a role in the defense of the mature plant, nor in the nematicidal effects of the plant. It can thereby be assumed that the ricin only provides protection of the mature seed from predators after development but before germination. This knowledge may help in further research involving the effects of possibly removing the ricin from castor in order to provide a more industrially efficient crop.

This research is based on the premise that the absence of an antibody signal indicates the absence of the intact ricin A-chain and, therefore, the absence of active ricin. This premise has not been investigated or tested in any animal, tissue culture, or cell culture toxicity model.

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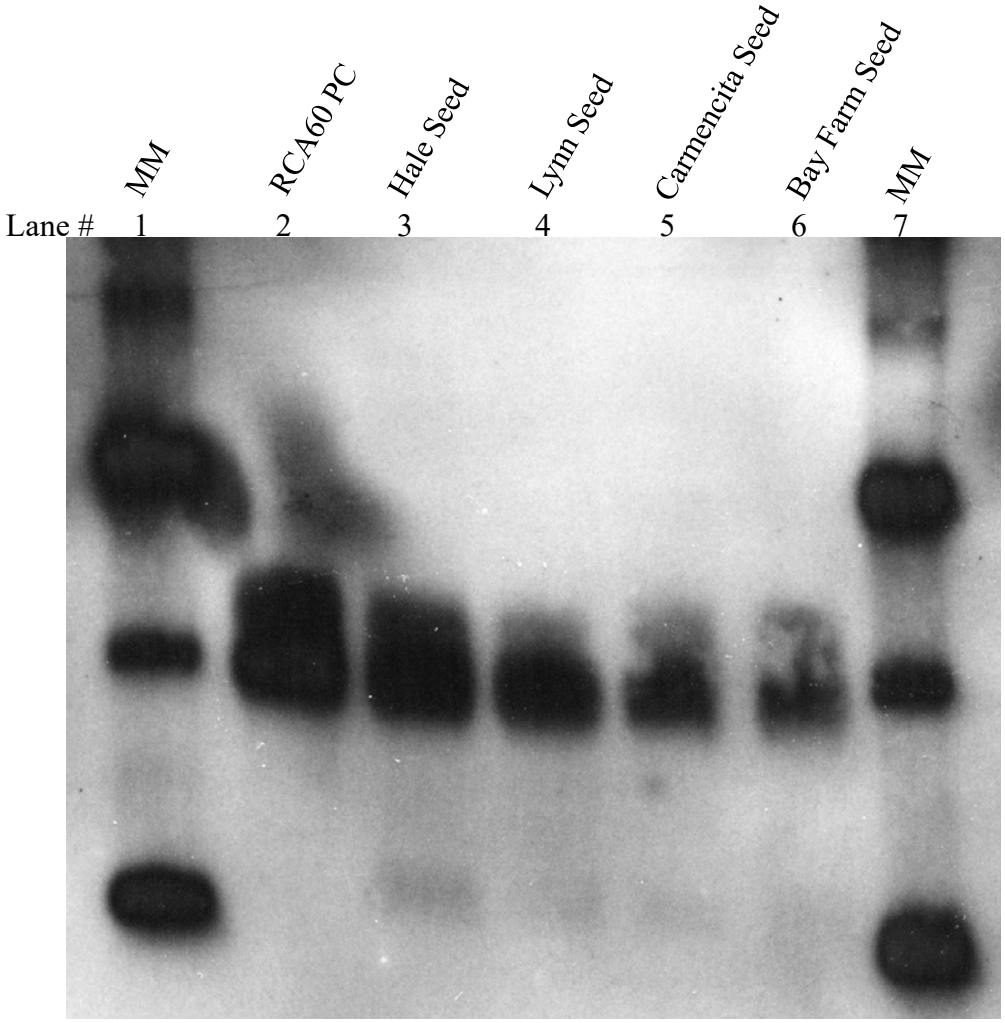

Figure 2.1 Seed protein samples of four castor cultivars.

All experimental samples were loaded at a total protein concentration of 2µg per well. RCA60 positive control lanes were loaded at a concentration of 100ng per well. The three bands in lanes 1 and 7 are 40kDa, 30kDa, and 20kDa. MM – MagicMark™ XP Western standard (Invitrogen, Carlsbad, CA); RCA60 PC – ricin A-chain positive control (Vector Labs; Burlingame, CA)

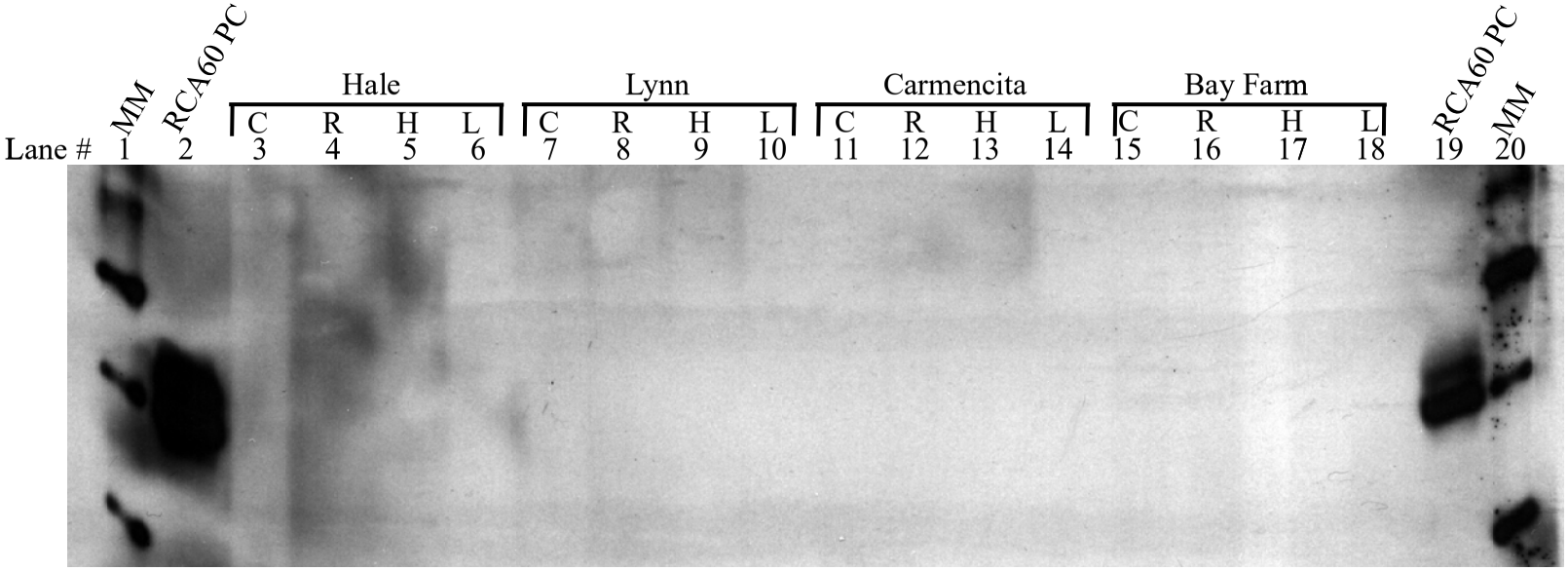

Figure 2.2 Protein samples of non-endospermic tissue of four castor cultivars.

All experimental samples were loaded at a total protein concentration of 35µg per well. RCA60 positive control lanes were loaded at a concentration of 500ng per well. The three bands in lanes 1 and 20 are 40kDa, 30kDa, and 20kDa. C – cotyledon; R – root; H- hypocotyl; L – mature leaf; MM – MagicMark™ XP Western standard (Invitrogen, Carlsbad, CA); RCA60 PC – ricin A-chain positive control (Vector Labs; Burlingame, CA)

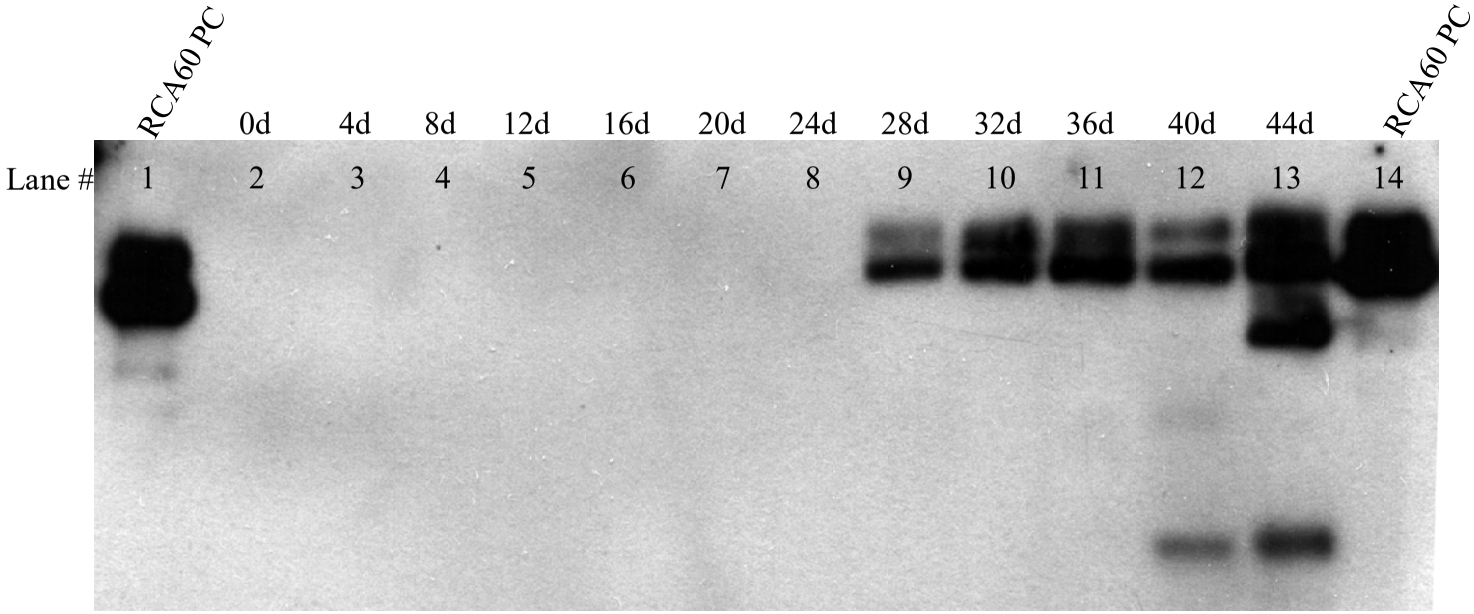

Figure 2.3 Protein samples from developing castor seed capsules.

All experimental samples were loaded at a total protein concentration of 10µg per well. RCA60 positive control lanes were loaded at a concentration of 500ng per well. RCA60 PC – ricin A-chain positive control (Vector Labs; Burlingame, CA)

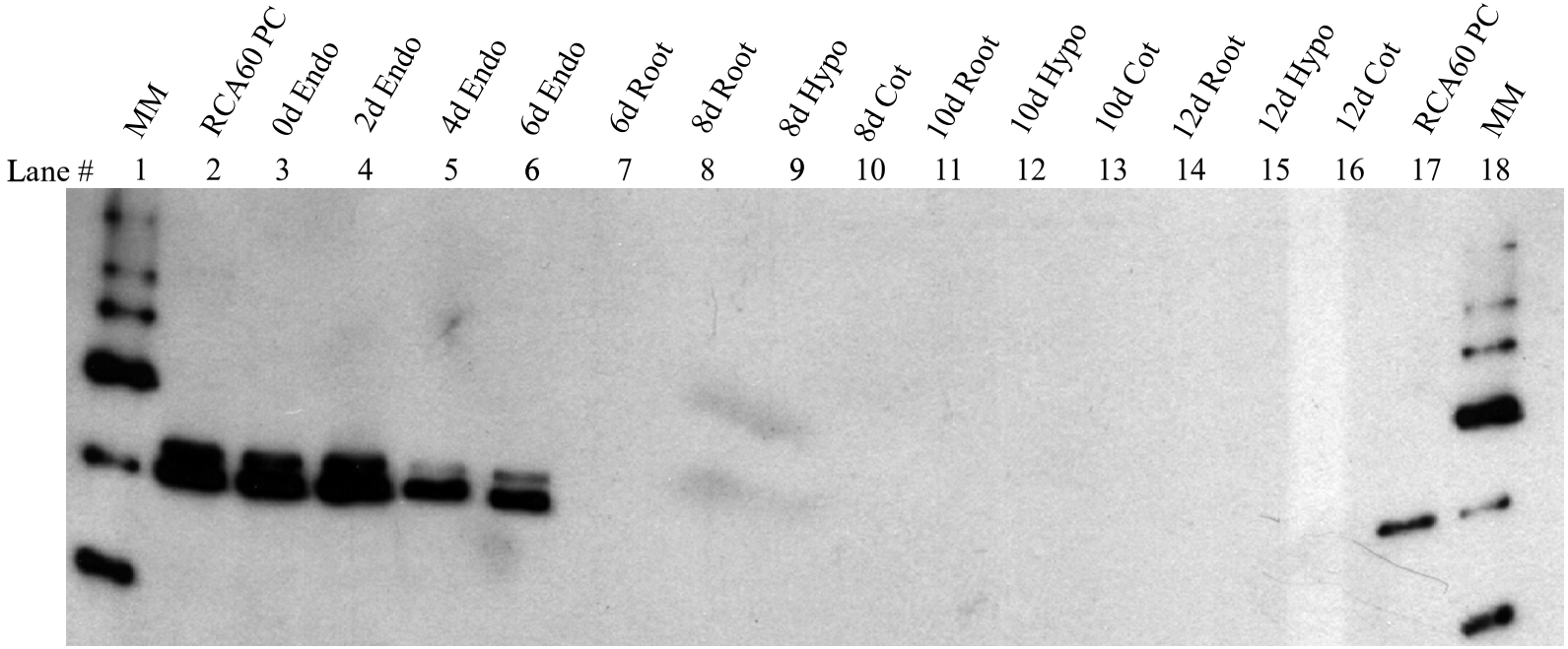

Figure 2.4 Protein samples from germinating castor seed and seedlings.

All experimental samples were loaded at a total protein concentration of 1µg per well. RCA60 positive control lanes were loaded at a concentration of 31.25ng per well. The six bands in lanes 1 and 18 are 80kDa, 60kDa, 50kDa, 40kDa, 30kDa, and 20kDa. MM – MagicMark™ XP Western standard (Invitrogen, Carlsbad, CA); RCA60 PC – ricin A-chain positive control (Vector Labs; Burlingame, CA)

CHAPTER III

DEGRADATION OF RICIN IN CASTOR SEED MEAL BY TEMPERATURE AND CHEMICAL TREATMENT

Abstract

Oil from the seed of the castor plant (*Ricinus communis* L.) is an important commodity for a number of industries, ranging from pharmaceuticals to renewable energy resources. However, the seed and subsequent seed meal contain ricin (RCA_{60}), a potent cytotoxin, making it an unusable product. In order to investigate the efficiency of previously researched methods of reducing the toxicity of the meal, cold-pressed oil extracted seed meal known to contain ricin was boiled in the presence of 50mM calcium hydroxide (pH 12.5). However, boiling of this seed meal in the presence of calcium hydroxide produced no significant difference from boiling alone. Therefore, heat and chemical treatments were performed to determine their effects on the denaturation of the ricin within whole seed, milled un-extracted seed, and cold-pressed extracted seed. Boiling and autoclaving showed varying degrees of effectiveness depending on the sample type. Ricin within the cold-pressed extracted meal was rendered unresponsive to antibody probing after ten minutes of boiling or autoclaving. In contrast, treatment of cold-pressed extracted meal with 8M urea and 6M guanidine-HCl for sixty minutes produced no observable reduction in the response of the ricin to the anti-body. Critically,

hot-pressing of the castor seed produced meal that exhibited no reactivity with the antibody, indicating that the ricin had been denatured during the oil extraction. By removing the toxic component of the castor meal, this by-product could create a new commodity from the production of castor oil, thereby making castor oil production more profitable.

Introduction

Castor (*Ricinus communis* L.) is a perennial oilseed plant native to the tropical regions of Africa (Brigham, 1993). Although native to tropical climates, it has been adapted to a wide range of sub-tropical and temperate climates where it is grown as an annual. Former areas of cultivation in the United States range from Texas north to Nebraska (Domingo and Crooks, 1945). Castor has been cultivated in the southern United States specifically for the oil found in its seed, one of the most desirable chemurgical oils. Castor seed oil has been utilized for centuries with evidence of its cultivation and use dating back 6,000 years (Weiss, 2000). As late as the 1890s, in the United States, castor oil was an important source of lamp fuel for non-coastal communities where whale oil was not available. There are many industrial uses for castor oil ranging from automotive lubricants to laxatives (Knight, 1979). However, current interests have focused on conversion of castor oil into biodiesel. Castor is a high-yield oilseed crop producing around 50 percent oil by weight, out-yielding soybean and cottonseed in U.S. production schemes (Weiss, 2000; Baldwin and Cossar, 2008). Castor not only produces significant quantities of oil, it also produces a unique fatty acid. Approximately 85 percent of the total lipids found within a castor seed is ricinoleic acid

(James et al., 1965). This composition gives the resulting oil the unique characteristic of being miscible with methyl and ethyl alcohols, which in turn enhances the efficiency of the transesterification of castor oil to biodiesel (Conceição et al., 2007).

The acceptance of castor as an oilseed crop has been discouraged due to its toxic nature. Ricin (RCA_{60}) is a class II ribosome-inactivating protein; a heterodimeric protein consisting of two chains linked by a disulfide bond (Endo et al., 1987). The A-chain of the ricin molecule is the effective toxin. It works by depurinating specific residues on the rRNA of the 28s subunit of the ribosome, halting translation (Endo et al., 1987). The B-chain of the ricin molecule is responsible for cell entry. It is a lectin that preferentially binds to galactosides on the cell membrane. The binding of the B-chain then induces the endocytotic uptake of the ricin molecule into the cell. Studies have shown that the disulfide link between the chains is not essential for the enzymatic activity of the A-chain, but it is necessary for toxicity since the A-chain cannot enter the cell without the B-chain (Harley and Beevers, 1982; Lord et al., 2003). Ricin has relatively low toxicity when orally consumed, but when injected or inhaled, the LD_{50} can be as little as 3-5μg/kg body weight. This high level of toxicity not only makes ricin difficult to deal with, but it also makes it a potential weapon for bio-terrorism (Audi et al., 2005).

The meal produced as a by-product of oil extraction, however, has little use, as it contains the cytotoxin ricin and ricin agglutinin (RCA_{120}), a potentially harmful allergen. Ricin and ricin agglutinin share around 90 percent homology within the A-chain of the proteins, meaning that detection of the ricin A-chain is directly linked to detection of the agglutinin when using A-chain specific antibodies (Pinkerton et al., 1999). This is important because safe handling and use of castor meal depends on neutralization of both

the toxic and the allergenic components. Once detoxified the meal can be used as feed for livestock, providing a source of profit on top of the oil production (Bris and Algeo, 1970; Vilhjalmsdottir and Fisher, 1971). Previous research has shown that the toxicity of the meal is reduced by heating a solution of the meal in water to boiling, or even as low as 70°C; the temperature at which the ricin protein irreversibly unfolds (Gaigalas et al., 2007). Current oil extraction procedures utilize solvent extraction, which does not involve heating the meal, leaving the ricin and agglutinin mostly intact (Ogunniyi, 2006). Complete denaturation of the toxic and allergenic components of castor meal would make it possible to more effectively utilize this by-product as an additional source of revenue from castor oil production.

This paper describes methodologies that can be implemented in an existing industrial infrastructure for neutralizing the toxic properties of castor seed meal. The use of hot-press technology, as well as guanidine, urea, and calcium hydroxide on ricin activity in the meal are investigated. The response of each material to treatment is relevant due to its mirroring of industrial procedures. If ricin in the seed could be denatured after harvest and before pressing, it would greatly reduce the danger inherent to working with the seed. Alternately if the milled material were subjected to elevated temperatures during processing, the meal cake would be non-toxic, which would provide more potential uses for this by-product and enhance revenue.

The objective is to treat the cold-pressed meal after processing in order to reduce toxicity and allow for safer downstream use. Reducing the toxicity of the meal would ultimately make the extraction of castor oil safer, and would allow for the use of the meal as a by-product, making production and processing more profitable.

Materials and Methods

Castor Seed

Seed of three cultivars (Hale, Lynn, and Brigham) were tested for gross ricin concentration. While Brigham tested lower in ricin, presence was still detected. Since Hale seed was readily available, it was used for these experiments. Castor seed (cv. Hale) were obtained from 2007 field production at the RR Foil Plant Research Facility at Mississippi State University, MS. Seed was stored at -20°C until needed. The same seedlot was used as the control to monitor differences between treatments. Care was taken to ensure the experimental sample was completely exposed to treatment, either temperature or chemical. Samples consisted of whole seed, milled seed, or screw-press oil extracted seed that was or was not heated. Milled seed was fragmented and homogenized using an A11 Basic S1 analytical mill (IKA; Wilmington, NC) prior to any treatment. Whole seed was treated intact.

Cold and Hot Pressing

Castor seed was driven through an IBG Monforts Model CA59G3 screw press (IBG Monforts; Mönchengladbach, Germany) to extract oil from the meal. The press includes a heating attachment that slips over the screw sleeve to heat the crushed meal during the procedure. This enhances the extraction of viscous oils such as those from castor. The heating ring reaches a temperature of 275ºC during the extraction process. Meal was expelled at a volume of 0.19 cm^3/sec. These conditions expose the 0.1 cm diameter meal plug to 275ºC for a period of 2.1 sec., resulting in expelled meal at a

temperature of 130ºC and the expressed oil at 70ºC. Samples of the meal were taken from both cold pressing and hot pressing procedures. These samples were maintained in 50mL plastic centrifuge tubes at -80°C for long term storage and -20°C for short term storage.

Boiling and Autoclaving

Whole seed, milled meal, and cold-pressed meal were subjected to boiling and autoclaving. Whole seed was boiled (100ºC at 1 atm) for 10-60 min at 10 min increments by adding the seed to boiling water and removing an aliquot at each time point. Milled and cold-pressed meal was boiled by inserting 0.2g of the material into a 1.5mL Eppendorf tube and adding 1mL Dulbecco's phosphate-buffered saline (DPBS) purchased from Sigma-Aldrich (St. Louis, MO) to each tube. The tubes were then submerged in a boiling water bath. Tubes were removed from the bath and placed directly into an ice bath at 0ºC at the appropriate time points. The tubes were then centrifuged and the supernatant was discarded. Whole seed, milled seed meal, and cold-pressed seed meal were placed in vented 50ml centrifuge and autoclaved (121ºC at 15 psi) for 10-60 min at 10 min increments for the autoclaved treatments.

Chemical Treatments

Solutions of 6M guanidine-HCl, 8M urea, and 50mM calcium hydroxide (pH 12.5) were individually used as protein denaturants. For the guanidine-HCl and urea treated samples, 0.2g of cold-pressed oil extracted seed meal was placed in a 1.5mL Eppendorf tube and 1mL of the appropriate solution was added to the tube. The tubes

were then placed on a rotary mixer for one hour. For the calcium hydroxide treatment, 75mg of cold-pressed oil extracted seed meal was placed in a 0.6mL Eppendorf tube and 375mL of the calcium hydroxide solution was added. Corresponding control samples were made by adding 375mL of DPBS to 75mg of cold-pressed oil extracted seed.

The seed meal samples were then placed in a Mastercycler™ gradient thermal block (Eppendorf; Hamburg, Germany) that had been pre-heated to 99°C. Seed meal samples were exposed in the block for 1, 2, 3, 4, 5, or 10 minutes. After removal from the block, the samples were immediately cooled to 0ºC in an ice bath. All chemically treated samples were centrifuged after treatment and the supernatant was removed, and protein was extracted from the pellet by the method described below. The calcium hydroxide samples were washed once with DPBS to neutralize residual calcium hydroxide before protein extraction, and the control samples were washed once as well to replicate the treatment of the test samples.

Protein Extraction

Following treatment, samples of whole, milled, or pressed oil-extracted seed from each treatment were homogenized using an A11 Basic S1 analytical mill (IKA; Wilmington, NC) until the particles in the sample were uniform. Each sample was then extracted by vortexing 0.2g of sample material with 1mL of complete protein extraction buffer [50mM Tris Base, 120mM sodium chloride, 0.5% Nonidet P-40, 1mM Na-EDTA, 1mM dithiothreitol, 10mM ß-glycerophosphate, 0.1mM sodium fluoride, 0.1mM sodium orthovanadate, 1 tablet/25mL Complete™ protease inhibitor cocktail tablets (Roche; Basel, Switzerland) pH 7.4] for 1 h at 20ºC (Draetta et al., 1989). The resulting milieu

was then centrifuged at 10,000 x g for 5 min at 20ºC. The pellet was then discarded and the supernatant was transferred to a Nanosep® MF 0.45µm pore-size centrifugal-filter (Pall Life Sciences; East Hills, NY) spin-filter tube and centrifued 14,000 x g for 1 min at 20ºC. The clarified sample was then assayed using the Micro BCA™ Protein Assay kit (Pierce; Rockford, IL) to quantify the total protein concentration. Protein samples were equalized (250ng – 2.5µg per lane) by dilution in SDS-PAGE loading dye (2% SDS, 12.5% glycerol, 0.002% bromophenol blue, 0.002% acid orange G, 50mM dithiothreitol, 62.5mM Tris-HCl, pH 6.8) to allow the same volume of protein to be loaded into each lane of the gel. All protein samples were heated to 90ºC for 10 mins to reduce disulfide bonds to prevent occlusion of the A-chain of ricin and ricin agglutinin by the B-chain and allow clear resolution on the gel.

Western Blotting

Protein samples were separated on a 0.75mm thick 12.5% Next Gel™ SDS-PAGE gel (Amresco, Solon, OH) with a 6% Protogel® stack (National Diagnostics; Atlanta, GA). An RCA_{60} positive control (Vector Labs; Burlingame, CA) was run on the same gel as the samples to provide a positive marker for ricin. The separated proteins were then transferred to an Immobilon™-FL PVDF membrane (Millipore; Billerica, MA) using a Panther™ semi-dry transfer apparatus (Owl; Rochester, NY) with Towbin transfer buffer (25mM Tris base, 192mM glycine, 20% v/v methanol pH 8.3) for 1 h at a constant current of $0.8mA/cm^2$ (Towbin et al., 1979). The membrane was blocked overnight (12-16 hours) on a rocker (25rpm) at 20ºC with NZAB-Teleostean gelatin blocking buffer [2% casein enzymatic hydrolysate, 2% Teleostean gelatin, 6ppm

Kathon™ CG/ICP II (Supelco; Bellefonte, PA) in DPBS pH 7.4]. The membrane was then incubated with a 1:1,000 dilution of RCA-52B anti-RCA60 mouse IgG primary antibodies (US Biological; Swampscott, MA) in Tris-buffered saline containing 0.5% Tween-20 (TTBS) at pH 7.4 for 1 h at 20ºC. These antibodies bind specifically to the full-length A-chain of ricin and cross-react with the A-chain of the ricin agglutinin. Any disruption of the active site of the test protein prevents binding by the antibody. After removing the primary antibody solution the membrane was washed four times in TTBS to remove any unbound antibody and subsequently incubated with a 1:100,000 dilution of horseradish peroxidase conjugated Fc specific anti-mouse goat IgG (Sigma-Aldrich; St. Louis, MO) in TTBS for 1 h at 20ºC. After washing with TTBS four times, the membrane was incubated for 1 min at 20ºC in a 1:1 mixture of luminol and enhancer solution from the Supersignal© West Femto kit (Pierce; Rockford, IL). The membrane was then placed in a film cassette and exposed to CL-XPosure™ film (Pierce; Rockford, IL) until the signal from the membrane was imprinted on the film. The film was finally developed on an M35A X-Omat™ processor (Kodak; Rochester, NY).

Results and Discussion

The effect that boiling cold-pressed oil extracted castor seed meal in the presence of calcium hydroxide versus DPBS control had on the ability of the antibody indicator to bind to the A-chain of ricin is compared in Table 3.1. The ricin A-chain activity is eliminated by 10 minutes of boiling in the presence of calcium hydroxide (pH 12.5) as well as DPBS. Calcium hydroxide treatments were performed because past literature suggests that it is a common and efficient method for inactivating the ricin, as well as the

allergenic components from the seed meal (Spies et al., 1962). The data in 3.1 suggest that calcium hydroxide does, in fact, have an effect on the denaturation of ricin. This denaturation causes a physical change in the conformation of the protein, preventing the antibody from binding to its specific epitope. Lanes 10-16 of Figure 3.1, representing the calcium hydroxide boiled samples, show a reduced signal compared to lanes 3-9 (Fig. 3.1), representing the DPBS control boiled samples, indicating that there is less ricin in the calcium hydroxide treated samples. This stands to reason as the pH itself should have a denaturing effect on the protein in the sample. The high pH of the sample may have also saponified the residual oil in the meal allowing more of the protein to be subjected to the hydroxide and therefore more easily denatured rather than being protected within an oil barrier. Alternatively more of the protein could have dissolved into the supernatant and subsequently removed from the sample due to the lack of the protective oil. The calcium in the calcium hydroxide treated samples may have also played a role in preventing the protein from refolding properly after boiling. Since DPBS is designed to be calcium-free, it is logical that the denatured proteins in the control samples would not have been as inhibited from refolding as the calcium hydroxide treated samples. Another point of interest is the decrease of ricin in the one minute boiled samples and the subsequent increase at 2 minutes of boiling. One explanation of this is that the proteins are being liberated by driving the oil off, allowing for better extraction of the ricin in subsequent steps. The presence of this phenomenon in both the control and the calcium hydroxide treated meal suggests that it is not a quality of the calcium hydroxide treatment, but rather a feature of the boiling of the sample. The most important observation from Figure 3.1 is that absence of a signal corresponding to ten minutes of

boiling in both calcium hydroxide as well as the control (lanes 9 and 16 Fig. 3.1). These data suggest that boiling in the presence of calcium hydroxide has little significant advantage over boiling by itself.

The effects of heating whole castor seed, milled un-extracted castor seed, and cold-pressed extracted castor seed are shown in Figure 3.2. These data indicate that as little as 10 min of boiling or autoclaving is sufficient to eliminate the presence of intact ricin A-chain in cold-pressed castor meal (lanes 4 and 7 Fig. 3.2). Similar to cold-pressed meal, whole seed requires longer exposure, taking greater than ten minutes of boiling and greater than twenty minutes of autoclaving to eliminate the detection of the ricin A-chain by the antibodies. Milled un-extracted seed samples show an interesting response as no amount of boiling tested here removed the A-chain, but as little as ten minutes of autoclaving removed the A-chain's response. This could be because of the high oil-content of the milled samples. During boiling (99ºC), the oil could be providing a barrier between the heat and the proteins within the seed meal. This barrier initially prevents the heat from fully denaturing the proteins, allowing the ricin to remain intact. Alternatively, the oil also provides an organic environment in which the proteins become trapped during the boiling treatment. In this environment the protein may still unfold, but may also refold properly once the temperature has been reduced. Previous research has shown that proteins may refold more successfully in a biphasic environment (Rariy and Klibanov, 1997). It is also known that proteins undergo kinetic trapping when placed in organic solvents. This kinetic trapping results in stronger hydrogen bonding and a more rigid protein backbone making the protein less likely to denature (Mattos and Ringe, 2001). The autoclaved samples likely do not show the same behavior because the heat and

pressure (121ºC 15psi) during autoclaving is much greater than that of boiling, creating a harsher environment for the ricin within the milled seed meal to denature. Therefore any treatments performed on seed milled before oil extraction will require either autoclaving or the application of a chemical such as a surfactant or hexane, such as with solvent extraction, to break-up the oil. Unfortunately the application of surfactant would likely hinder subsequent oil extraction process, rendering the product useless.

Chemical protein degradation methods applied to the cold-pressed un-extracted meal are compared to the heat degradation methods applied to the cold meal in Table 3.2 and Figure 3.3. Urea and guanidine treatments were selected because they are often added to feed to enhance nitrogen levels for ruminant feeds (Belasco, 1954). If they were to show a significant effect on the degradation of ricin within the meal, this treatment might provide an economical alternative to the heat treatments. Exposure to 8M urea or 6M guanidine for 60 minutes provides only marginal degradation of the ricin A-chain (lanes 8 and 9 Fig. 3.3). This is likely due to the inability of these chemicals to completely infiltrate the seed meal in order to denature the protein. Additional bands can be seen in Figure 3.3 indicating that the ricin was denatured into several smaller peptides that each still expressed the binding epitope for the antibody. However the presence of the full ricin A-chain band in lanes 8 and 9 indicates that 8M urea and 6M guanidine-HCl are not capable of completely denaturing ricin from cold-pressed seed meal.

The data from Figure 3.3, does however support hot-pressing as a viable method of both extracting the oil from the castor seed while simultaneously neutralizing ricin (lane 10 Fig. 3.3). The hot-pressing is a preferred method for extraction of highly viscous oils from meal, such as castor oil in the absence of solvents, according to the oil mill

manufacturer. It is interesting that this procedure which increases oil extraction also denatures the toxin within the meal because it makes hot-pressing an attractive alternative to current hexane/propane extraction methods. By integrating hot-pressing into industrial castor oil production not only will oil-yields increase, but the resulting meal will be free of ricin, making it a much more attractive by-product.

Conclusion

Castor oil has been an important agricultural commodity for thousands of years. Its production, however, has always been plagued by the presence of the deadly toxin ricin as well as the ricin agglutinin allergen. Several methods for reducing the presence or activity of the toxic portion of ricin have been discussed. The effect of boiling in the presence of calcium hydroxide on the denaturation of ricin was tested based on previous research which suggests that this treatment removes the toxicity from castor seed meal. However, boiling with calcium hydroxide showed removal of ricin from the sample was not significantly better when compared to a control. Boiling or autoclaving the castor seed for twenty minutes before solvent extraction, or heating the meal cake after pressing produced promising reductions in the activity of the ricin A-chain. Solvent extraction alone is less desirable as it would leave the ricin as a functional toxin within the meal unless solvents were heated to drive them from the meal to be recycled. Treatment of the meal with urea and guanidine showed no substantial reduction in the presence of the ricin as visualized by the antibody reaction. However, using a hot-press approach to the extraction of the oil showed very promising signs of significantly reducing the ricin in the resulting meal. Eliminating the toxicity of the castor seed meal, would enhance the value

of this by-product, making it useful and therefore enhancing the profitability of castor production. Making castor more profitable would increase production in the United States, making it more likely to be harnessed as an alternative fuel.

This research offers a starting point for industrial denaturation of ricin and ricin agglutinin. Ricin detection was based on the presence or absence of an antibody signal indicating the presence or absence of intact ricin A-chain (the toxic portion of the protein). The results of this study have not been tested with animal, tissue, or cell culture bioassay. Conditions of these treatments were uniformly applied to the samples tested. In an industrial setting involving larger quantities care would have to be taken to ensure every part of the seed or seed product was exposed to the treatment.

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Table 3.1 Detection of ricin A-chain from cold-pressed seed meal samples boiled in DPBS versus 50mM calcium hydroxide (pH 12.5).

	Boiling Duration						
Treatment	0 min	1 min	2 min	3 min	4 min	5 min	10 min
DPBSs	+	+	+	+	+	+	-
Calcium-hydroxide	+	+	+	+	+	+	-

+ Presence; - Absence

Table 3.2 Detection of ricin A-chain in chemical or heat denatured processed seed samples.

Initial Treatment	Secondary Treatment	Duration						
		0 min	10 min	20 min	30 min	40 min	50 min	60 min
Cold- pressed	Boiled	+	-	-	-	-	-	-
	Autoclaved	+	-	-	-	-	-	-
	Urea	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	+
	Guanidine	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	+
Hot-pressed	None	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Whole Seed	Boiled	+	+	-	-	-	-	-
	Autoclaved	+	+	+	-	-	-	-
	Urea	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	+
	Guanidine	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	+
Milled Seed	Boiled	+	+	+	+	+	+	+
	Autoclaved	+	-	-	-	-	-	-

+ Presence; - Absence; n.t. not tested

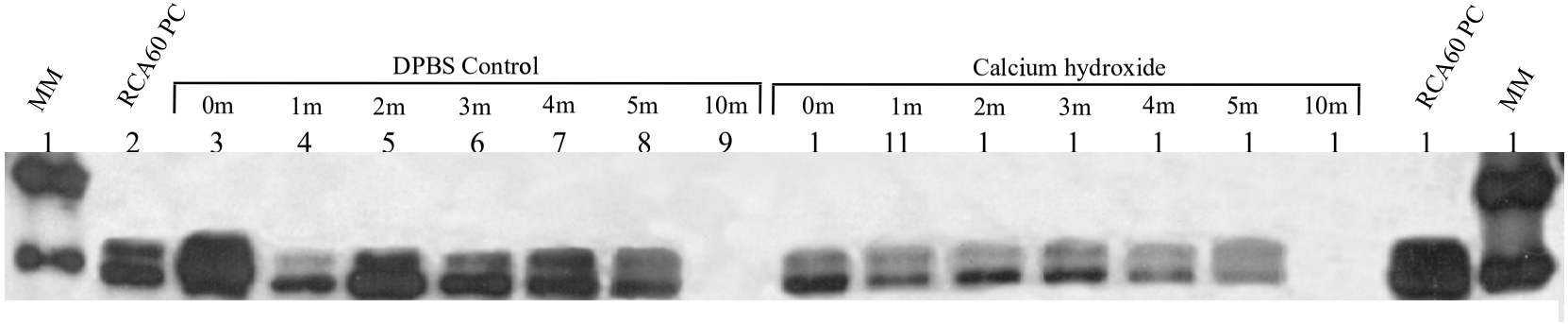

Figure 3.1 Calcium hydroxide boiling of cold-pressed seed meal compared to boiling in DPBS.

All experimental samples were loaded at a total protein concentration of 1µg per well. RCA60 positive control lanes were loaded at a concentration of 31.25ng per well. The two bands in lanes 1 and 18 are 40kDa and 30kDa. MM – MagicMark™ XP Western standard (Invitrogen, Carlsbad, CA); RCA60 PC – ricin A-chain positive control (Vector Labs; Burlingame, CA)

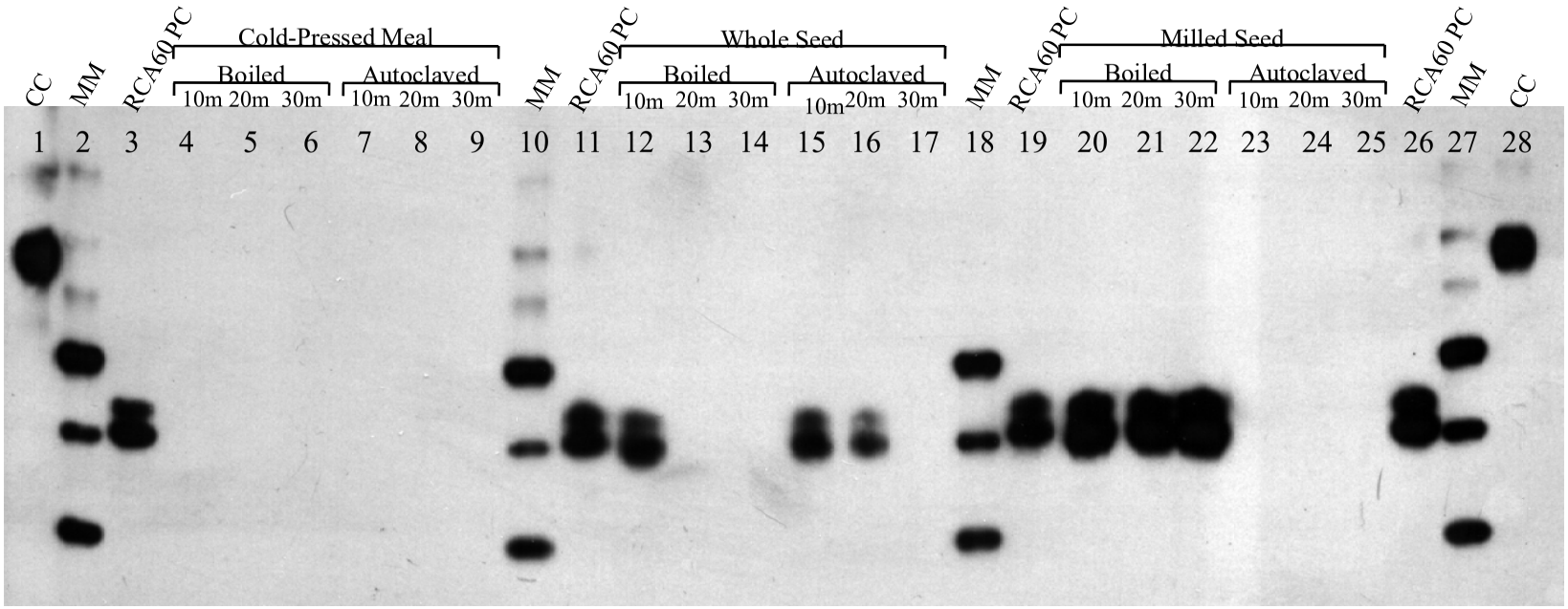

Figure 3.2 Heat treatments of castor seed samples.

All experimental samples were loaded at a total protein concentration of 250ng per well. RCA60 positive control lanes were loaded at a concentration of 31.25ng per well. The bands in lanes 2, 10, 18, and 27 are 220kDa, 120kDa, 60kDa, 40kDa, 30kDa, and 20kDa from top to bottom. CC – Chemichrome™ protein standard (Sigma-Aldrich; St. Louis, MO); MM – MagicMark™ XP Western standard (Invitrogen, Carlsbad, CA); RCA60 PC – ricin A-chain positive control (Vector Labs; Burlingame, CA)

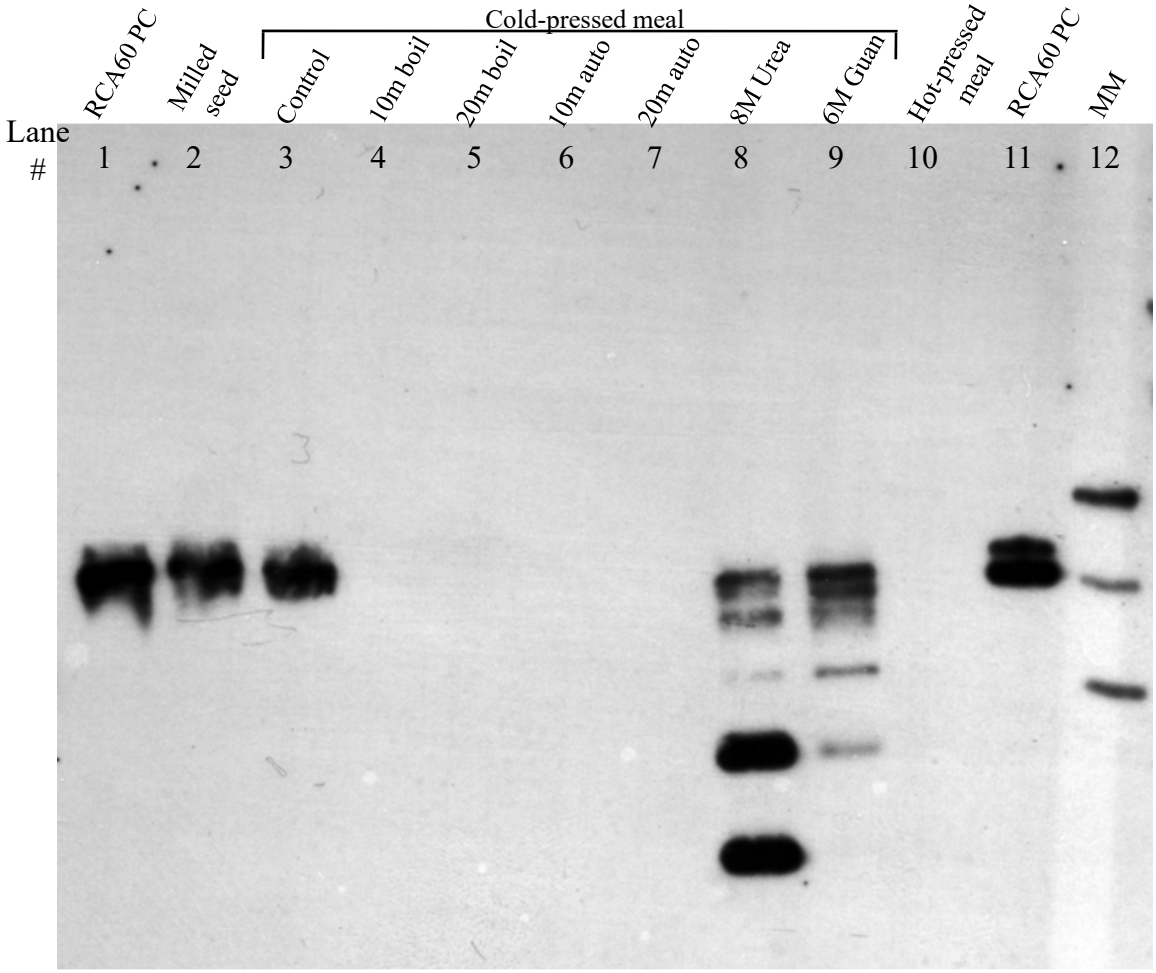

Figure 3.3 Thermal and chemical treatments of pressed seed meal.

All experimental samples were loaded at a total protein concentration of 2.5µg per well. RCA60 positive control lanes were loaded at a concentration of 31.25ng per well. The three bands in lane 12 are 40kDa, 30kDa, and 20kDa. MM – MagicMark™ XP Western standard (Invitrogen, Carlsbad, CA); RCA60 PC – ricin A-chain positive control (Vector Labs; Burlingame, CA); auto – autoclaved; Guan – guanidine-HCl