Thermal Inactivation of Ricin Using Infant Formula as a Food Matrix

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Ricin is a potent protein toxin found in the seeds of the castor bean plant, *Ricinus communis*. Ricin specifically and irreversibly inactivates ribosomes, promoting cell death by inhibiting protein synthesis. It is composed of a ribosome-inactivating enzyme (A-chain) linked to a lectin (B-chain) by a single disulfide bond. Several reports indicate that ricin can be detoxified by thermal treatment; however, the conditions required for inactivation are not well characterized. In addition, little information exists on the thermal stability of ricin added to foods. The objective of this work was to determine the effects of heat treatments on the detection and toxicity of ricin added to milk- and soy-based infant formulas. Reconstituted infant formula powders containing 100 µg of ricin/mL were heated at 60–90 °C for up to 5 h. The heat-treated formulas were analyzed by ELISA to determine levels of ricin. The residual cytotoxicity of ricin-containing infant formula after heat treatments was determined using RAW264.7 mouse macrophage cells. The ELISA and the cytotoxicity assay indicated that ricin detection and toxicity decreased with increasing heating times and temperatures. Minimal losses in detection and toxicity were found for ricin heated at 60 °C for 2 h. The half-lives of ricin cytotoxic activity in a milk-based infant formula at 60, 70, 75, 80, 85, and 90 °C were >100, 9.8 ± 0.5, 5.8 ± 0.9, 5.1 ± 0.7, 3.1 ± 0.4, and 1.8 ± 0.2 min, respectively; the comparable values for a soy-based infant formula were >100, 16 ± 1.6, 8.7 ± 1.2, 6.9 ± 1.1, 3.0 ± 0.4, and 2.0 ± 0.3 min. ELISA detection was a good indicator of the cytotoxicity of heat-treated ricin. The results indicate that ricin is a relatively heat stable protein and may remain toxic under some food processing conditions.

KEYWORDS: Ricin; stability; thermal processing; infant formula; heat; cytotoxicity

INTRODUCTION

Ricin is a potent cytotoxin found in the seeds of the castor bean plant, *Ricinus communis*, a perennial plant native to tropical and subtropical regions of the world. The plant is cultivated in temperate regions of the world for the oil present in its seeds. Castor bean oil is rich in the hydroxy fatty acid, ricinoleate, which imparts its unique properties that enable it to be manufactured into lubricants, varnishes, paints, fungicides, and cosmetics (1). Castor bean oil is produced by hot or cold pressing of the seeds with or without solvents (3). The material that remains after pressing and solvent extraction, “waste mash” or castor bean meal, contains virtually all of the ricin present in the seeds (1–5% ricin, w/w). In addition to ricin, castor beans contain another toxic component, *R. communis* agglutinin (RCA), a potent lectin (4). Unlike ricin, a cytotoxin, RCA does not have direct cytotoxic activity, but has affinity for red blood cells causing their agglutination and subsequent hemolysis (5).

Ricin is a globular protein composed of two subunits, the cytotoxic A-chain (32 kDa) and the receptor-binding (lectin) B-chain (32 kDa), covalently linked by a single disulfide bond. The toxic effect of ricin comes from its ability to inactivate eukaryotic ribosomes specifically and irreversibly, promoting cell death by inhibiting protein synthesis. The A-chain de-purinates a specific adenine residue of 28S ribosomal RNA, while the B-chain, which contains two galactose binding sites, binds specifically to cell surface glycoproteins and glycolipids facilitating the movement of the A-chain into the cell (5). Extremely low levels of ricin are able to inhibit protein synthesis. Olsnes et al. (6) reported that only one A-chain molecule of ricin is able to inactivate 2000 ribosomes/min. Eiklid et al. (7) found that penetration of a single molecule of ricin into the cytosol is enough to kill a cell.

The lethal toxicity of ricin varies 100-fold among nonhuman species and is affected by the route of exposure. Of the species tested, horses were the most sensitive, while chickens and frogs...
were the least (8). In general, exposure by inhalation is the most lethal route of exposure (9). In mice, the LD₉₅ for ricin is 5–10 g/kg by parenteral routes, 3–5 μg/kg by inhalation, and 20 mg/kg by oral route (5, 8). There are reports in the literature of poisonings in humans due to consumption of castor bean seeds (5, 9). From the poisoning cases, the oral lethal dose of ricin in humans has been estimated at between 1 and 20 mg/kg of body weight (3, 9–12). Symptoms typically occur between 4 and 10 h after exposure and include severe abdominal pain, vomiting, diarrhea, and oropharyngeal irritation (3, 5). In lethal cases, gastrointestinal symptoms are followed by vascular collapse and multiorgan failure.

Ricin has potential for being used as a biological weapon since the castor bean plant is found worldwide and the toxin is relatively easy to isolate and purify. Ricin can be disseminated as an aerosol, by injection, or as a water or food contaminant. Limited information exists on the stability and detection of ricin added to foods before or after processing. Several reports indicate that ricin can be detoxified by thermal treatment (13, 14); however, the conditions required for inactivation are not well characterized. The objective of this work was to study the effects of heat treatments on the detection (by ELISA) and biological activity (cytotoxicity) of ricin in a model food matrix: reconstituted powder infant formulas (milk- and soy-based). The cytotoxicity assay developed here utilized a RAW264.7 murine macrophage cell line since it and other macrophage cell lines exhibit enhanced sensitivity to ricin, presumably due to the presence of high levels of mannose receptors on the cell membrane that facilitate uptake of the toxin (15). The assay, which measures the effects of ricin on cell viability, differs from other tissue culture assays (15–18), which measure the ability of ricin to inhibit protein synthesis, deadenylate ribosomal RNA, or induce apoptosis.

MATERIALS AND METHODS

Materials. Ricin standard (R. communis agglutinin II, 5 mg/mL in phosphate buffered saline (PBS)) was purchased from Vector Laboratories (Burlingame, CA). The ELISA assay for the detection of ricin was obtained from Tetracore Inc. (Rockville, MD). Similac Advance with iron powder milk-based formula (Ross Products Division of Abbott Laboratories, Columbus, OH), Isomil with iron powdered soy-based formula (Ross Products Division of Abbott Laboratories), and Good Start Supreme with Iron powdered milk-based formula (Ross Products Division of Abbott Laboratories (Burlingame, CA). The ELISA assay for the detection of ricin (BioTek model ELx808; Winooski, VT) and software (KC4, BioTek), The Tetracore ELISA kit uses mono- and polyclonal antibodies to detect ricin in samples.

Cytotoxicity Assays. Cell toxicity (cytotoxicity) assays were used to estimate the biological potency of heat-treated Similac Advance and Isomil formulas spiked with ricin. RAW264.7 mouse macrophage cultures (Sigma-Aldrich, St. Louis, MO) were maintained in MegaCell MEM medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich), 2 μM 1-glutamine (Invitrogen, Carlsbad, CA), 100 units/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen) in a 5% CO₂ incubator set at 37 °C in T-75 tissue culture flasks. The cells were used within three passages from frozen stocks. Attached cells were dislodged using a cell scraper and suspended in culture medium, plated (2 × 10⁵ cells/well in 0.1 mL) in black, polystyrene, flat-bottomed tissue culture treated 96-well assay plates (Corning, Corning, NY), and then allowed to attach to the plate surface overnight in a 37 °C incubator with 5% CO₂ atmosphere. Ricin-containing samples in PBS or infant formula were serially diluted in separate 96-well U-bottomed plates with ricin-free PBS or infant formula (11 concentrations per plate), and then each sample was further diluted using cell culture medium in a second 96-well plate. Attached cells in the assay plate were fed with 0.1 mL of medium/well containing ricin dilutions or ricin standards (0.2 mL/well), and incubated at 37 °C for 48 h. CellTiter-Blue viability reagent (20 μL/well; Promega, Madison, WI) was added during the final 4 h of incubation. Cell-dependent generation of fluorescent resorufin product was detected (EX, 550 nm; EM, 590 nm) using a FluoroSkran Ascent 96-well fluorescence plate reader (Thermo Labsystems, Milford, MA).

Data and Statistical Analyses. Ricin inhibitory concentration 50% (IC₅₀) values from cytotoxicity assays were determined by nonlinear regression analysis (SigmaPlot, SyStat Software, Inc., Richmond, CA). The IC₅₀ values for heat-treated infant formula samples were divided by the apparent IC₅₀ values for untreated ricin added to the same infant formula matrix. This ratio reflects the relative residual potency (% activity) of each treated sample compared to untreated ricin. For ELISA assays, % ricin detected was calculated by dividing the level of ricin detected in the heat-treated formula by the amount of ricin detected in the formula at time 0, or when the formula reached processing temperature (2 min).

The % ricin remaining in samples detected by ELISA or cytotoxicity assays at each time point was fitted to eq 1 using nonlinear regression with the Prism software package (GraphPad, San Diego, CA), where A is an empirically determined constant, k is the apparent first-order rate constant, and t is the thermal treatment duration. The apparent half-life (t½) was calculated from first-order rate constants using eq 2:

\[
\% \text{ricin} = A e^{kt}
\]  
(1)

\[
t_{1/2} = \ln(2)/k
\]  
(2)

\[
k = B e^{-E_{act}/RT}
\]  
(3)

First-order rate constants for ricin inactivation in the different infant formula samples detected using ELISA or cytotoxicity assays at each temperature were fitted to the Arrhenius relationship between temperature and reaction rate (eq 3) using nonlinear regression, where B is an empirically derived constant, R is the gas constant, and T is the
temperature in kelvins. The \( z \) values for ricin inactivation were calculated from \( E_a \) using eq 4. The temperature (\( T \), \( K \)) used to calculate the \( z \) values was the midpoint (348 K) in the range of temperatures used in the experiments.

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z \text{ value} = 2.303RT^2/E_a
\] (4)

An F-test statistic was used to compare \( k \) and \( E_a \) values for ricin inactivation determined using nonlinear regression with different infant formula samples and by different methods of analysis. Differences were considered significant when \( p < 0.05 \).

The bias or difference between methods used to determine loss of ricin during processing (ELISA vs cytotoxicity assay) was assessed for each infant formula. For each time/temperature point, % ricin activity (cytotoxicity assay) was subtracted from % ricin detected (ELISA test). Where replicate measurements were made at a common formula/method combination, the mean was taken of the value. After checking for normality of the values by the Wilk–Shapiro test, a \( t \) test that the difference between % ricin activity and % ricin detection was equal to zero was performed using SAS Proc Univariate (SAS Institute, Inc., Cary, NC). \( t \) tests were performed using all temperatures pooled, and separately by temperature.

A comparison of the precision or variance of the two methods (ELISA vs cytotoxicity assay) was made. Analysis of residuals from the analysis of covariance model was used to determine the method error. An analysis was performed for each formula/method where temperature was treated as a class variable and time was a continuous linear covariate. An F test was performed comparing the variances of the residuals from the two methods. This was done separately for Similac and Isomil formulas.

SAFETY. Ricin is a potent protein toxin and must be handled with care. All work with the toxin was done in a laminar flow hood or biological safety cabinet and personal protective gear (lab coats, safety glasses, gloves, etc.) worn. Glassware and materials exposed to the toxin were detoxified by soaking in 5% hypochlorite solution for at least 1 h. All ricin standards and solutions containing the toxin were mixed with an equal volume of >10% hypochlorite solution to prepare them for disposal. These safety precautions should always be followed when handling ricin.

RESULTS AND DISCUSSION

The effects of processing time and temperature on the residual cytotoxicity of ricin in an intact milk protein based infant formula (Similac Advance) and a soy protein based infant formula (Isomil) are shown in log-transformed form in Figure 1. Figure 2 shows the effects of thermal processing on ELISA detection of ricin in Similac Advance, Isomil, and Good Start Supreme, a hydrolyzed whey protein formula. Figures 1 and 2 indicate that rate of loss in ricin cytotoxicity or ELISA detection is highly dependent on temperature and that the extent of loss of ricin increases with processing temperature and time. Minor losses of cytotoxicity and detection were found in the infant formulas processed at 60 °C for <2 h. At 90 °C, over 90% loss in ricin cytotoxicity and ELISA detection was found only after >4 min processing.

Figures 1 and 2 show that ricin inactivation at 60–90 °C follows first-order kinetics. The half-lives and rate constants at each processing temperature and for each infant formula were calculated from cytotoxicity and ELISA detection data (Tables 1 and 2). The \( t_{1/2} \) values for ricin in Similac Advance milk-based infant formula at 60, 70, 75, 80, 85, and 90 °C were >100, 13 ± 0.9, 5.8 ± 0.7, 5.6 ± 0.9, 3.0 ± 0.4, and 2.0 ± 0.2 min, respectively (mean SEM calculated from combined ELISA and cytotoxicity assay results). The comparable values for Isomil soy-based infant formula were >100, 17 ± 4, 8.6 ± 1.1, 7.1 ± 1.3, 2.7 ± 0.4, and 2.1 ± 0.2 min.

The activation energies (\( E_a \)) are given in Table 1 and 2 for ricin inactivation in Similac Advance and Isomil formulas determined from derived first-order rate constants for each temperature treatment using nonlinear regression. Figure 3 reveals linear Arrhenius relationships for ricin inactivation plotted as \( \ln(k) \) versus \( 1/T \) and with indistinguishable slopes for each regression line. Differences between activation energies and \( z \) values calculated for thermal inactivation of ricin in
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Similac Advance, Isomil, and Good Start Supreme infant formulas were insignificant. No significant differences in $E_a$ or $z$ values were apparent whether determined using ELISA or cytotoxicity assay results.

$s$ values for ricin inactivation, as calculated from the cytotoxicity activation energies, were $25 \pm 3 \, ^\circ\text{C}$ in Similac Advance and Isomil formulas. When calculated from the ELISA detection data, the $s$ values for ricin inactivation in Similac Advance, Isomil, and Good Start formulas were $26 \pm 3 \, ^\circ\text{C}$, $25 \pm 5 \, ^\circ\text{C}$, and $28 \pm 6 \, ^\circ\text{C}$.

Results of a 2-way ANOVA indicate that there were statistically significant ($p = 0.0019$) differences in the stability of ricin in Similac Advance vs Isomil formulas as measured by ELISA and the cytotoxicity test. The results indicate slightly greater stability of ricin in the soy-based Isomil than the milk-based Similac Advance formula. A possible explanation for this observation is that the soy proteins or other components of the soy-based formula were better at stabilizing ricin during heating than milk proteins or other components of the milk-based formula.

A $t$-test was used to determine if statistical differences existed between ricin activity measurements as determined by ELISA and cytotoxicity assays. Statistical analysis of the data (Table 3) indicates there was no significant difference ($p = 0.2353$) between ELISA and cytotoxicity assays in their determination of % ricin remaining in heated Similac Advance samples. However, for Isomil formula samples, the ELISA results were, on average, 9.6% greater ($p = 0.0014$) than results generated by the cytotoxicity assay. When differences between results were separated by processing temperature (Table 4), there was only a statistical difference between ELISA and cytotoxicity results generated at 70 $^\circ\text{C}$. The statistical comparison of the precision (variability) of the ELISA vs the cytotoxicity assay indicates no significant ($p > 0.05$) difference in the precision of the two assays. The high level of agreement between the two assays suggests that the epitope(s) responsible for detecting ricin by ELISA were inactivated or denatured at the same rate as the portion(s) of the protein responsible for the toxicity of ricin.

To the authors’ knowledge, these are the first studies that determined the half-life, activation energy, and $z$ value of ricin in a food-based model system during heating. However, the literature contains many references to inactivation of ricin and RCA during thermal treatment of whole or flaked castor seeds or castor bean meal. Jenkins (13) found that autoclaving castor seeds at 15 psi (presumably at 121 $^\circ\text{C}$) for 1 h or boiling solutions of ricin for 2 min fully detoxified ricin as measured in a rat feeding study. Layton (19) reported that catfish fed castor bean meal obtained by steam extraction of the castor seeds (conditions not specified) did not exhibit signs of toxicity while Gardner et al. (20) found that heating flaked castor bean at 100–102 $^\circ\text{C}$ for 12–15 min was sufficient to detoxify the toxic components of castor beans. Okorie and Anugwa (14) found that steaming castor beans at 80 $^\circ\text{C}$ for up to 40 min did not destroy ricin, while dry heating the beans at 140 $^\circ\text{C}$ for 20–30 min was sufficient to inactivate the toxin. Overall, these studies corroborate our finding that found ricin is a fairly heat stable protein toxin. The $s$ values for ricin inactivation in the matrices studied here ($25–28 \, ^\circ\text{C}$) were greater than the $s$ value...
reported for *Clostridium botulinum* 62A toxin (z value = 5.4 °C), similar to that reported for *Staphylococcus aureus* enterotoxin A (z value = 27.8 °C), and less than the z value for *Staphylococcus aureus* enterotoxin B (z value = 32.4 °C) (21–24).

Our results indicate that the processing conditions used to pasteurize fluid whole milk (63 °C, 30 min; 72 °C, 15 s; 89 °C, 1.0 s; 90 °C, 0.5 s, etc.) would not ensure complete inactivation of ricin present in liquid infant formula. Infant formula mixes that are used in the production of powdered infant formulas are typically pasteurized under more extreme thermal conditions (higher temperatures and/or longer hold times) than fluid milk due to their higher solids and fat content. However, it is unlikely that even these conditions would fully inactivate ricin. Based on the inactivation rate constants obtained in this study, the conditions used to manufacture canned liquid formulas (retorted) would be sufficient to fully inactivate ricin. It is important to emphasize that the information presented here applies only to the heat stability of ricin in infant formula. Additional research is needed to determine the thermal stability of ricin in other matrices, in particular acidic foods such as fruit juices.

The ELISA method used here to measure the effects of thermal processing on ricin stability was able to give a good estimate of the amount of residual toxicity as compared with the cytotoxicity test. Advantages of the ELISA over the cytotoxicity test and whole animal assays include decreased cost and more rapid estimation of ricin inactivation. Results from the ELISA can be obtained in <5 h as compared to >2 days for the biological assays. More research is needed to determine if the ELISA method is able to predict ricin cytotoxicity in food matrices other than infant formula.

ACKNOWLEDGMENT

We thank Dr. Eric Garber (FDA/CFSAN), who provided the technical assistance and advice needed to begin this project.

LITERATURE CITED