Effect of Food Matrices on the Biological Activity of Ricin

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ABSTRACT

A cell-free translation assay was applied for the quick detection of ricin in food samples. Three economically important foods—ground beef, low-fat milk, and liquid chicken egg—were tested. The results indicated that ground beef had a very little matrix effect on the assay, whereas low-fat milk and liquid chicken egg showed clear interference on the protein translation. A simple dilution in phosphate-buffered saline (PBS) effectively eliminated the translational inhibition from these foods. The concentrations inhibiting 50% of luciferase translation derived from the current study were 0.01 nM for the pure ricin A chain, 0.02 nM for pure ricin, and 0.087 nM for crude ricin in PBS. In most cases, the half inhibitory concentration values for ricin in food matrices were significantly lower than for those in PBS buffer, suggesting that some components in these food matrices might potentiate the activity of ricin. Thermal stability tests indicated that the ricin A chain was the least stable among the three forms of ricin in all matrices measured. The thermal stability of pure and crude ricins varied depending on the matrices. The specific activities of ricin in PBS buffer were confirmed by a neutralization test with ricin-specific and nonspecific antibodies. This study demonstrates that the cell-free translation assay is a rapid and sensitive method for detection of biologically active ricin toxin in ground beef, low-fat milk, and liquid chicken egg and that food matrices can greatly affect the thermal stability of ricin.

The events of 11 September 2001 have changed the way we live and have given the government and the food industry a new challenge. A terrorist attack on the food supply could have catastrophic public health and economic consequences. Existing surveillance systems are designed to detect and determine the effect of occurrences of infections and intoxications resulting from typical foodborne pathogens or the toxins produced by these organisms. However, our capabilities for detecting atypical threat agents and understanding their behavior in foods are currently very limited (9).

Ricin, one of the most potent toxins known, is produced in the seeds of the castor plant (Ricinus communis). Although its lethal toxicity is about 1,000-fold less than that of botulinum toxin, its heat stability, accessibility, and ease of production in massive quantities make it a potential weapon for terrorism. The Centers for Disease Control and Prevention has listed ricin as a category B agent (4). Ricin exerts toxicity through inhibition of protein synthesis, which results in cell death. It belongs to the large family of ribosome-inactivating proteins and contains two disulfide-linked heterodimeric chains (A and B) with similar molecular mass (32 and 34 kDa). The A chain is an N-glycosidase that can irreversibly inactivate ribosomes. It cleaves a specific adenine at a highly conserved site in the 28S ribosomal RNA in the cytoplasm of eukaryotic cells (6), preventing binding of elongation factor 2, and thereby blocking protein synthesis (19). The B chain is a lectin that binds to galactosyl residues of cell membranes and helps the ricin heterodimer enter target cells. Ricin can enter human bodies through various routes, including inhalation, injection, and intragastric administration. Poisoning could thus occur through ingestion of ricin-contaminated food. The median lethal oral dose for ricin in humans has been estimated to be 1 to 20 mg/kg of body weight on the basis of previous reports of castor bean ingestion (2).

Bioterrorist attacks via contaminating foods have already occurred (17). To better prevent or prepare for the intentional adulteration of food with ricin, sensitive, accurate, and robust assay formats that provide critical information in a timely manner are needed. The most common methods used to detect ricin are antibody-based assays, the mouse bioassay, and cell culture assays. The antibody-based immunoassays can detect ricin specifically and sensitively depending on the quality of antibody used, but they are unable to distinguish active ricins from inactive ricins (8, 14). The mouse bioassay used to be the “gold standard” for the detection of toxins (20); however, it is not practical to use for most laboratories because it is expensive and time-consuming, requires special animal care facilities, and is unrealistic for high-throughput applications. In vitro cell culture assays are sensitive and can detect biologically active ricin in food samples, but they usually require lengthy incubation times (3). Methods based on real-time PCR, such as the detection system we developed for crude ricin (12, 13), are highly specific, sensitive, and scalable to high-throughput applications. However, real-time PCR methods rely on the presence of DNA from the source organism and thus are limited to specific types of ricins, such as crude ricin extracts.

Recently, a cell-free translation (CFT) assay for measuring the biological activities of ribosome-inactivating proteins was developed (10). This method uses luciferase ac-
tivity as a reporter for protein translation. Adding enzymatically active ricin disrupts ribosomal activity, leading to lower luciferase accumulation and decreased luminescence. The toxicity of ricin, therefore, could be measured from the difference in luminescence readings (counts per second [cps]) between the control (lysat without adding ricin) and treated (with ricin) samples.

Ricin, one of the best characterized ribosome-inactivating proteins, could be used as a bioterrorist agent in different forms. The ricin A chain lacks the cellular toxicity of native ricin because it cannot get into the cells without the B-chain binding component, but it possesses the catalytic activity and therefore has the full depurination function in a cell-free system. The difference between pure and crude ricin in terms of stability and bioavailability could be because of the presence of components associated with crude ricin. Although some assays are available for the detection of highly pure toxins or toxins in nonviscous liquids, very few assays have been validated for the detection of toxins in food matrices. Here, we present our research results on the validation of this method for detection of all three forms of ricin in different food matrices and our observations on the thermal stability of ricin when present in food samples.

MATERIALS AND METHODS

Materials. Purified ricin A chain (1 mg/ml), ricin (5 mg/ml), and affinity column–purified polyclonal goat antibody against ricin A chain were purchased from Vector Laboratories (Burlingame, Calif.). A non–ricin-specific affinity-purified goat anti-rabbit immunoglobulin G antibody was purchased from Promega (Madison, Wis.). Crude ricin was prepared as described previously (12). Briefly, dry castor seeds with shells removed were ground in liquid nitrogen to a fine powder with a mortar and pestle, and cold acetone was added to form a uniform homogenate. The resulting mixture was filtered to remove the liquid solvent and then cold acetone was added to form a uniform homogenate. The freshly prepared powder was dried overnight in a vacuum desiccator over P₂O₅ and stored at −20°C for future use.

Nuclease-treated rabbit reticulocyte lysate, complete amino acid mixture (1 mM), luciferase mRNA (1 mg/ml), ribonuclease inhibitor (RNAsin, 40 U/µl), recombinant luciferase, and Bright-Glo Luciferase Assay system were purchased from Promega (Madison, Wis.). Ground beef labeled as containing 20% fat, low-fat milk (with 2% fat), and white chicken eggs were purchased from the local supermarket and stored at 20 or 4°C. Liquid egg was prepared from whole eggs before use by blending the egg white and yolk into a uniform homogenate using a whisk.

Protein assay. The total protein concentration was determined by a microtiter BCA (Pierce, Rockford, Ill.) method. The estimated ricin concentration for the crude preparation was 0.7 mg/ml on the basis of an immunoassay using a commercial lateral flow device (RAMP Ricin Test, Response Biomedical Corp., Burnaby, British Columbia, Canada) and Bis-Tris NuPAGE gel (4 to −12%) electrophoresis (Invitrogen, Carlsbad, Calif.).

CFT assay. The translation lysate mixture consisting of nuclease-treated rabbit reticulocyte lysate, amino acids, RNAsin, nuclease-free water, and luciferase mRNA was prepared in a ratio (vol/vol) of 35:1:1:33:2. After mixing, the lysate was distributed (15 µl per tube) to 1.5-ml microtubes containing 3 µl of phosphate-buffered saline (PBS; as a control) or ricin. The CFT reaction was incubated at 30°C for 90 min with gentle shaking (60 rpm). Aliquots of the reaction were then transferred to a black microtiter plate (5 µl per well). The Bright-Glo Luciferase Assay buffer (100 µl) containing the Bright-Glo Luciferase Assay substrate was added to each well, and luminescence was measured as counts per second in a Victor 2 plate reader (Perkin Elmer, Shelton, Conn.). The linear range of light detection for the luminometer was determined with serial dilutions of recombinant luciferase and Bright-Glo reagent by plotting counts per second versus known luciferase concentrations.

Neutralization test. Goat antibodies against ricin or rabbit immunoglobulin G (1 mg/ml) were diluted at 1:50 in phosphate-buffered saline, pH 7.4, and then mixed with an equal volume of ricin (ricin A chain at 0.1 nM; pure or crude ricin at 2 nM in PBS) for 1 h at 37°C before testing in the translation assay.

Detection of ricin toxicity. To measure ricin toxicity, serial dilutions of ricin were added to the translation lysate at a 1:5 ratio (vol/vol) in microcentrifuge tubes. Translation lysate with PBS in lieu of ricin was used as a control. Translation lysate with water in lieu of luciferase mRNA was used as a blank. Ricin activity was measured as the percentage of translational inhibition by [(cps from PBS control − cps from ricin-treated sample)/(cps from PBS control)] × 100. Data are mean ± standard deviation (SD) of three replicates from a representative experiment. Three individual experiments were performed. Small variations were observed among the individual experiments because of slight changes of ricin activity during storage. Standard curves were plotted with values of percent inhibition versus the log concentration of ricin (pM).

Thermal inactivation of ricin in different food matrices. Aliquots of beef samples (0.1 g) were spiked with ricin (prediluted in 100 µl of PBS) and incubated together for 5 min at room temperature so that the ricin could be absorbed into the food. Samples were then heated in a Thermomixer R (Eppendorf, Westbury, N.Y.) at 63 or 72°C for 3 min and cooled in an ice bath. After adding 900 µl of PBS, the tubes were vortexed for 1 min and then centrifuged at 10,000 × g for 5 min. The supernatant was collected, diluted with PBS, and analyzed for ricin toxicity by the CFT assay. For milk and liquid egg samples, 10 µl of PBS containing varying amounts of ricin was spiked into 90 µl of liquid food in microcentrifuge tubes. After heating for 3 min at 63 or 72°C, samples were cooled on ice. Aliquots of the samples were diluted and analyzed for ricin toxicity by the CFT assay. The effect of food matrix (without the presence of ricin) on CFT assay was presented as the percentage of control according to [(cps from food matrix)/(cps from PBS buffer)] × 100.

Data and statistical analyses. The half maximal inhibitory concentrations (IC₅₀) of ricin, representing the concentration that is required for 50% inhibition of protein translation, were determined by linear regression analysis (InStat 3, GraphPad Software Inc., San Diego, Calif.). Statistical differences between IC₅₀ values were tested by the Tukey-Kramer multiple comparisons test. The differences were considered significant at P < 0.05.

Loss of ricin activity at IC₅₀ after heat treatment was calculated by the formula [(ricin activity in untreated samples − ricin activity in heat-treated samples)/(ricin activity in untreated samples)] × 100.
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DETECTION OF RICIN IN FOODS

Safety considerations. Ricin is extremely toxic! It is a Select Agent and is covered by the requirements of 42 CFR part 73 (7). Specific provisions for handling toxins in any amount can be found in Appendix I at the Centers for Disease Control and Prevention Web site (5). Contaminated glassware or disposable containers were soaked in 3% hypochlorite solution for 24 h.

RESULTS

Detection of ricin activity with the CFT assay. The CFT assay was evaluated for the measurement and detection of ricin in biological samples. First, the linear range of light detection for the luminometer was determined to avoid signal saturation at high light intensities. Figure 1 shows a standard curve of light units versus relative enzyme concentration. Serial dilutions of purified luciferase were made in PBS containing 1 mg/ml bovine serum albumin, which prevents the loss of luciferase through adsorption to the walls of assay tubes. To determine whether the CFT assay can quantitatively detect ricin, serial dilutions of ricin in PBS were added into the lysate mixture. As shown on the semilogarithmic plot in Figure 2, the translational inhibition by ricin was dose dependent. The inhibition curve for the ricin A chain was linear between 5.2 and 52 pM ($R^2 = 0.98$). The curves for pure and crude ricins were linear between 10 and 800 pM (both $R^2 = 0.99$). All three forms of ricin were able to inhibit more than 95% of protein synthesis at the highest concentration of their linear ranges. At the lowest concentration of their linear ranges, the inhibition was less than 25% for the ricin A chain and 0 to 2% for crude and pure ricin. The $IC_{50}$ values determined by the linear regression analysis for the ricin A chain and the pure and crude ricin were 0.01, 0.12, and 0.087 nM, respectively. These results suggest that the ricin A chain was the most active, followed by crude ricin, and the pure ricin was the least active on an equimolar basis (Table 1). Here the ricin concentration for the crude preparation was estimated by protein gel electrophoresis and immunooassay as indicated in “Materials and Methods.” The differences among the three forms of ricin were statistically significant when tested by Tukey-Kramer multiple comparisons (data not shown).

To confirm that the inhibition of translation observed in these assays was due to the presence of active ricin, ricin A chain (0.1 nM) and pure and crude ricin (2 nM) were mixed with an equal volume of affinity-purified polyclonal antibody (0.02 mg/ml) against ricin and incubated at 37°C for 1 h before adding to the translation mixture. Most enzymatic activities of ricin A chain and pure ricin were abolished as indicated in Figure 3. The activity of crude ricin was significantly reduced after pretreatment with anti-ricin antibody, but 20% activity still remained. This is likely because some of the catalytic sites of the ricin were protected by other components in the crude ricin extract, or other ribosomal inactivating proteins were present in the ricin preparation. Preincubation of PBS in lieu of ricin with ricin antibody did not affect luciferase expression. Also, preincubation of ricin with a nonspecific goat antibody did not interfere with ricin activity (data not shown). These results suggest that the observed inhibition of luciferase translation is ricin specific.

Food matrix inhibition of CFT assay. Food samples are highly heterogeneous and contain many different bio-

![FIGURE 1. Linear range of light detection for luminometer. Serial dilutions of purified recombinant luciferase were made in PBS supplemented with 1 mg/ml bovine serum albumin and added to a 96-well plate at 5 µl per well. Bright-Glo Reagent was then added at 100 µl per well; luminescence measurements were integrated over 0.1 s and presented as counts per second (cps). The linear regression was calculated by plotting cps versus luciferase concentration.](image)

![FIGURE 2. Ricin activity (% translational inhibition) versus its log concentration. Ricin activities at varying concentrations were measured with a cell-free translation assay. Results are mean ± SD of three replicates from one representative experiment. Three individual experiments were performed. Equations were obtained by linear regression analysis.](image)

### TABLE 1. $IC_{50}$ values of different forms of ricin in food matrices

<table>
<thead>
<tr>
<th>Food matrix</th>
<th>Ricin A chain (nM)</th>
<th>Pure ricin (nM)</th>
<th>Crude ricin (nM)</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.010 A</td>
<td>0.120 A</td>
<td>0.087 A</td>
<td>3</td>
</tr>
<tr>
<td>Beef</td>
<td>0.015 B</td>
<td>0.074 B</td>
<td>0.054 B</td>
<td>3</td>
</tr>
<tr>
<td>Milk</td>
<td>0.006 C</td>
<td>0.065 B</td>
<td>0.035 C</td>
<td>3</td>
</tr>
<tr>
<td>Egg</td>
<td>0.005 C</td>
<td>0.034 C</td>
<td>0.016 D</td>
<td>3</td>
</tr>
</tbody>
</table>

* $IC_{50}$ values determined by the linear regression analysis for the ricin A chain and the pure and crude ricin were 0.01, 0.12, and 0.087 nM, respectively. These results suggest that the ricin A chain was the most active, followed by crude ricin, and the pure ricin was the least active on an equimolar basis (Table 1). Here the ricin concentration for the crude preparation was estimated by protein gel electrophoresis and immunooassay as indicated in “Materials and Methods.” The differences among the three forms of ricin were statistically significant when tested by Tukey-Kramer multiple comparisons (data not shown).

* Differences between numbers with the same letter were not statistically significant and between numbers with different letters were statistically significant ($P < 0.05$).

* Beef extract was diluted 1:10 in PBS, and milk and egg samples were diluted 1:160 in PBS to avoid inhibition by the food matrices.
logically active proteins that could complicate the result of an in vitro translation assay. To determine the food matrix effects on the performance of CFT assays in the absence of ricin, three economically important food products, ground beef, low-fat milk, and liquid chicken egg, were analyzed. Under our experimental conditions, the beef matrix showed a very small negative effect on luciferase translation at the undiluted concentration of the extract (data not shown) and no effect at a 1:10 dilution of the original extract in PBS, whereas milk and egg samples exhibited clear inhibition in protein translation (Fig. 4) and these inhibitions remained even after pretreating samples with anti-ricin antibody (data not shown). Dilution of these food matrices to 1:160 in PBS completely removed the inhibition from these samples. These results demonstrated that undiluted milk and egg contain components that can inhibit the CFT assay in the absence of ricin. However, the CFT assay could be used for the detection of ricin toxicity in beef and diluted milk and egg matrices.

Change of ricin activities in different food matrices.
To further investigate the effect of food matrices on the biological activities of ricin, ricin A chain and pure and crude ricin were spiked into ground beef, low-fat milk, and liquid chicken egg; ricin activities were then evaluated. To avoid food matrix effects on the assay performance, the beef extract was diluted to 1:10 and the milk and egg samples were diluted to 1:160 in PBS; no inhibition of translation was observed in any nonspiked food samples at these dilutions. Table 1 shows the IC₅₀ values for three forms of ricin in different food matrices. As in PBS, the IC₅₀ values among the three forms of ricin were statistically different in all food matrices tested. From low to high, the IC₅₀ values were ricin A chain, crude ricin, and pure ricin (statistical tests are not shown here). This result indicated that in a cell-free system, the ricin A chain was more active than ricin with both A and B chains, and the crude ricin was more toxic than its pure form. As shown in Table 1, the IC₅₀ values of ricin were generally lower in food matrices than in PBS (with the exception of ricin A chain in the beef matrix). Among three food matrices, the IC₅₀ values of ricin A chain in egg and milk matrices were close to each other but significantly lower than that in the beef matrix. For pure ricin, the IC₅₀ value in egg matrix was lower than that in beef and milk matrices, whereas the IC₅₀ values for crude ricin were significantly different in all three matrices, with the lowest in the egg and the highest in the beef matrix (Table 1).

Thermal inactivation of ricin in three food matrices.
To study the thermal stability of ricin in foods, food samples were spiked with ricin at the concentrations of the respective IC₅₀ and heated at 63 or 72°C for 3 min. These temperatures were chosen because they were recommended by the U.S. Food and Drug Administration for the cooking of raw eggs and meats and the pasteurizing of fluid milk. The loss of ricin activity after heating was calculated on the basis of the equation [(activity before heating – activity after heating)/(activity before heating)] × 100 and was used as a measurement for the thermal stability of ricin. As indicated in Table 2, the loss of ricin biological activity was positively correlated with temperature. Among the three forms of ricin, loss of toxicity for the ricin A chain was the most dramatic in all tested matrices when heated to 63 and 72°C. The thermal stability of pure and crude ricin varied with the food matrix used. In PBS, pure ricin was more resistant to heat treatment than crude ricin, whereas the opposite result was obtained in the beef matrix. In milk, no significant difference was found between the thermal stabilities of pure and crude ricin. Unlike in other matrices, the thermal stability of pure and crude ricin in egg behaved...
differently at 63 and 72°C. At 63°C, the pure ricin was more stable than the crude ricin; however, the reverse was found at 72°C. We also noticed major activity losses for all forms of ricin in the beef and egg matrices heated at 72°C and minor losses in the milk matrix heated at both 63 and 72°C.

**DISCUSSION**

Detection of biothreat agents in the food supply is a relatively new aspect of food safety. Many atypical foodborne agents such as toxins are deadlier than live bacteria on a weight basis, yet they are not detected by standard microbiological culture or sophisticated assays that are based on amplification of nucleic acids. The goals of this study were to determine whether the toxicity of ricin in a buffer solution corresponded to the activity of the ricin in foods and whether the food matrices affected the thermal stability of ricin. Three different forms of ricin were tested, and the interactions between each ricin and food matrix were determined. Compared with other methods, the CFT assay is much faster; the entire assay can be finished within 90 min. Earlier work by Hale (10) showed that, at a high concentration of ricin (≥1 nM), reliable results could even be obtained within 45 min.

Detection of ricin activity in PBS demonstrated that all three forms of ricin exhibited log-linear inhibition of protein translation within the ranges indicated. As reported by others (10, 18), we found that the IC₅₀ value of the ricin A chain was much lower than that of whole ricin molecules, indicating that the ricin A chain had greater inhibition capacity in the cell-free system. This supports the hypothesis (15) that for in vivo toxicity, the ricin A chain is first reductively cleaved from the B chain to release its active site. Unlike intact ricin, the ricin A chain can immediately inhibit protein synthesis in the rabbit reticulocyte lysate mixture. Therefore, it is expected that the ricin A chain is more enzymatically efficient than whole ricin in a cell-free lysate. As to the stability of the ricin A chain without the protection of the B chain, Hazes and Read (11) proposed that the ricin A chain in the cytosol could avoid being degraded and remain toxic partly because the ricin A chain contains very few lysine residues, which are required for attachment of polyubiquitin chains to trigger degradation by the proteasomes. Furthermore, Argent et al. (1) reported that the ricin A chain can refold in the presence of ribosomes, which allows the maintenance of its activity.

Foods are highly heterogeneous and can contain compounds that are highly inhibitory to specific assays. We demonstrated that the CFT assay can detect biologically active ricin in beef extracts. For contaminated milk and liquid egg samples, a dilution of 1:160 in PBS is required to reduce inhibition from these food matrices. The most notable result found in this study was the increased toxicity of three forms of ricin in all food matrices tested, with the exception of the ricin A chain in the beef matrix. This was concluded on the basis of the IC₅₀ values. As indicated in Tables 1 and 2, the IC₅₀ values of three ricin forms were significantly higher in PBS than in ground beef, milk, and liquid egg samples (except for the ricin A chain in beef). The results suggest that these foods might contain components that could potentiate the toxicity of ricin, even when the matrix effects were mitigated by dilution. By comparison, when we performed experiments with CFT using a Shiga-like toxin, another A/B di-chain toxin that inactivates ribosomes, no enhancement of toxicity was observed in any of these food matrices compared with that in PBS (unpublished data). Further work is required to characterize the ricin enhancers present in ground beef, milk, and eggs and to elucidate the roles of the enhancers in ricin cytotoxicity.

Thermal inactivation of ricin experiments demonstrated that the ricin A chain was least stable among the three forms of ricin tested. It is expected because the ricin A chain was shown to be more sensitive to heat treatment in the absence of the B chain. Argent et al. (1) found that, with the use of circular dichroism measurement, the ricin A chain was native at 30°C and was catalytically active but became partially unfolded at 45°C yet was still catalytically active. When the temperature was increased to 55°C and above, it became substantially denatured.

In this study, the heating of all three forms of ricin at 72°C in beef and egg matrices resulted in a more pronounced loss of toxicity. This could be because of the high fat content in these foods or the formation of ricin complexes at high temperatures. The complex could be formed by ricin self-aggregation, binding with other proteins, or inorganic ions in these matrices. Satterlee and Kraft (16) reported that the thermal loss of enterotoxin B molecules was more severe in the presence of a meat protein, myosin.
Our results demonstrated that the current temperatures recommended by the U.S. Food and Drug Administration for the cooking of meats (72°C) and eggs (63°C) were effective for the inactivation of ricin A chain in these matrices but would not ensure complete inactivation of the intact ricin molecules. Similarly, the temperature used to pasteurize fluid milk (72°C) was partially effective in inactivating (by 60%) the ricin A chain, but had no observable effect on the pure and crude ricins.

We have validated the CFT assay for the detection of ricin toxicity in ground beef, low-fat milk, and liquid egg. It was shown that the matrix effect from milk and egg could be easily removed by a simple dilution. Depending on the food matrix, the IC50 values obtained from this assay ranged from 5 to 15 pM for the ricin A chain, 34 to 120 pM for pure ricin, and 16 to 87 pM for crude ricin. With proper controls, the assay could be used to determine the stability of toxins. The sensitive detection of ricin in these food matrices suggests that the CFT assay could be useful for the detection and quantification of other ribosome-inactivating proteins in foods. In addition, this assay is an ideal tool to use in high-throughput screening for potential enhancers or inhibitors of cytotoxicity because it is fast, sensitive, and capable of measuring the biological activity of toxins.

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