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Influence of yogurt fermentation and refrigerated storage on the stability of protein toxin contaminants

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ABSTRACT

Dairy products sold in a ready-to-eat form present the risk that adulterants persisting through manufacturing, storage, and distribution would reach consumers. Pathogenic microbes, including shigatoxigenic strains of *Escherichia coli* and the toxins they produce, are common food safety hazards associated with dairy products. Ricin and abrin are plant-derived ribosome-inactivating protein toxins related to the shiga-like toxins produced by *E. coli*. Limited information exists on the effects of manufacturing processes on the stabilities of these heat-resistant ribosome-inactivating proteins in the presence of foods. The goal of this study was to determine how typical yogurt manufacturing and storage processes influence ribosome-inactivating protein toxins. Ricin and abrin were added to skim or whole milk and batch pasteurized. Complete inactivation of both toxins was observed after 30 minutes at 85 °C. If the toxins were added after pasteurization, the levels of ricin and abrin in yogurt and their cytotoxic activities did not change significantly during fermentation or refrigerated storage for 4 weeks. The activities of ricin and abrin were inhibited by skim milk, nonfat yogurt, whole milk, and whole milk yogurt. The results showed minimal effects of the toxins on yogurt pH and %titratable acidity but inhibitory effects of yogurt on toxin activity.
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1. Introduction

Many dairy products, such as yogurt and ice cream, are meant to be consumed with no additional heat treatments. As such, adulterants persisting through manufacturing, distribution, and storage could reach consumers unabated. Typically, pathogenic microbes and the toxins they produce are the major food safety hazards associated with dairy products.

Although enterohemorrhagic and shigatoxigenic strains of *Escherichia coli* (EHEC and STEC) may be tolerated without significant harm in cattle, these microbes are pathogenic in humans (Caprioli et al., 2005) and have caused food poisoning outbreaks involving dairy products (De Schrijver et al., 2008; Guh et al., 2010; Morgan et al., 1993). The shiga-like toxins (Stx1 and Stx2) are the primary virulence factors responsible for EHEC and STEC pathogenicity to humans.

Stx1, Stx2, and shiga toxin, produced by *Shigella* species, are homologous proteins comprised of a single catalytic A subunit and five identical cell-binding B subunits. These three AB_5 microbial toxins belong to the ribosome-inactivating protein (RIP) toxin family. Following B subunit-dependent target cell binding, endocytic uptake, and retrograde delivery through the endoplasmic reticulum into the cytoplasm, the adenine *N*-glycosidase catalytic activity of RIP toxin A subunits attack the so-called α-sarcin/ricin loop of 28S rRNA, a structure required for the elongation of nascent polypeptides. The enzymatic action of RIP toxin A subunits thus prevents protein synthesis and activates cell death pathways (Gray et al., 2008; Olsnes and Kozlov, 2001; Stirpe, 2004).

The most toxic members of the RIP toxin family include the AB heterodimeric plant proteins ricin and abrin that are produced by the castor bean plant (*Ricinus communis*) and the rosary pea plant (*Abrus precatorius*), respectively. Ricin and abrin are designated Category B bioterrorism select agents by the US Department of Health and Human Services (HHS) according to the Public Health Security and Bioterrorism Preparedness and Response Act of 2002.[1] The USDA and US FDA devote considerable resources to enhance the safety of the food supply from acts of bioterrorism involving these and other select agents.

Interest in the use of atypical plant products to supplement yogurt, such as flaxseed lignans (Hyvarinen et al., 2006) and medicinal herbs (Jager et al., 2010), could provide additional opportunities for the introduction of pathogenic EHEC or STEC microbes or perhaps plant-derived additives contaminated with RIP toxins. Careful selection of suppliers and the application of well-designed HACCP and food defense plans (Sandrou and

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[1] 42 CFR § 73. HHS Select Agents and Toxins. Revised December 4, 2012.

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Arvanitoyannis, 2000; Vanschothorst and Kleiss, 1994; Yadav and Sharma, 2011) that include appropriate heat treatments should minimize or eliminate the risk to consumers from EHEC or STEC pathogens or RIP toxins introduced via yogurt additives.

Only limited information exists on the effects of food manufacturing on the stability of protein toxin contaminants. For example, little is known about the long term stability of RIP toxins in fermented milk products such as yogurt. In addition, the potential reciprocal effects of protein toxins on foods have not been studied, such as simple indicators of fermented milk product quality like color, aroma, pH, etc. Furthermore, it has been shown that the biological activities of Stx2 and ricin are inhibited in the presence of certain foods (Friedman and Rasooly, 2013; He et al., 2008; Rasooly et al., 2010, 2012). The thermal stabilities of ricin, abrin, and Stx2 have been reported previously (Krupakar et al., 1999; Levy and Benaglia, 1950; Rasooly et al., 2010; Zhang et al., 2013). Our prior work showed that the thermal stability of ricin is enhanced in yogurt-containing products when compared to other foods consumed by small children (Zhang et al., 2013). For the present investigation, ricin and abrin were studied because of their higher toxicities and because we and others have shown that these two plant-derived RIP toxins are resistant to thermal inactivation. The objectives of this study were to study the effects of the yogurt manufacturing process and refrigerated storage on the stability of ricin and abrin, and to determine if these toxins interfere with the yogurt fermentation process. In addition, we investigated the influence of the dairy product matrix on toxin activity.

2. Materials and methods

2.1. Materials

Whole milk and skim milk were purchased from a local supermarket. Freeze-dried, direct vat set starter cultures used to produce yogurt included a thermophilic culture mix (FDA-DVS-YC-380, Chr. Hansen, Hørsholm, Denmark) and a culture mix containing thermophilic and probiotic microbes (FD-DVS Yo-Fast-88, Chr. Hansen). Purified ricin [*Ricinus communis* agglutinin II, 5 mg/mL in phosphate buffered saline (PBS)] and abrin (1 mg/mL in PBS) were purchased from Vector Laboratories (Burlingame, CA) and Toxin Technology, Inc. (Sarasota, FL), respectively and used as standards.

2.2. Preparation of crude ricin and abrin extracts for stability studies

Crude ricin extracts used for stability tests were obtained from coarsely ground castor beans (Johnny's Selected Seeds, Winslow, ME). The ground beans were mixed with chilled (4 °C) 0.01 M PBST (pH 7.4) for approximately 24 h. Clear ricin extracts were obtained by centrifuging the castor bean/PBST mixture at 4000 rpm for 10 min then filtering the supernatant fluids through Whatman #2 filters into glass flasks. Ricin levels of 5–10 mg/mL extract were obtained by dialyzing (MW cutoff of 6000–8000; Spectrapor, Spectrum Labs, Rancho Dominguez, CA) the extracts against solid polyethylene glycol (MW 20,000, Alfa Aesar, Ward Hill, MA).

Crude abrin extracts for stability experiments were obtained by soaking rosary peas (Toxin Technology, Inc.) in chilled PBST for 24 h at 4 °C. The softened peas were ground in a blender and then centrifuged at 4000 rpm for 10 min. The supernatant fluids were filtered through Whatman #2 filters and then dialyzed against solid polyethylene glycol until abrin levels of approximately 10 mg/mL extract were obtained.

2.3. Yogurt preparation and storage

Direct (cup) set yogurt was produced using methods that mimic conditions used for full-scale production of yogurt (Hyvarinen et al., 2006; Jablonski and Jackson, 2008). Milk (whole or skim) was supplemented with nonfat dry milk powder (2% w/w of milk). The supplemented milk was pasteurized at 85 °C for 30 min and then cooled to 42–45 °C before adding yogurt starter culture (FD-DVS-YC-380 or FD-DVS-Yo-Fast-88). Crude ricin or abrin extracts were added to achieve final toxin levels of 100 μg/mL either prior to (*vide infra*) or following milk pasteurization. No toxin was added to the control yogurt cultures. Inoculated milk with or without toxin was dispensed into sterile, 50 mL polypropylene tubes and the tubes were incubated at 42–45 °C. Tubes (controls and those containing toxins) were removed after 0, 1, 2, 3, 4, 5, 6, and 24 h fermentation and analyzed for pH and titratable acidity. When the inoculated milk reached a pH of 4.4–4.5 and/or titratable acidity of 0.9%, the tubes were placed in refrigerated storage (4–6 °C) for up to 4 weeks. Tubes were also assessed visually for the extent of milk coagulation and degree of whey syneresis. Samples were collected after 1, 2, 3, and 4 weeks of refrigerated storage. Yogurt samples collected during fermentation and refrigerated storage were stored at −20 °C prior to analysis for ricin and abrin levels using ELISA and cytotoxicity assays. In separate experiments we found that repeated freeze/thaw cycles did not affect the cytotoxicity of either RIP toxin in the presence of yogurt (data not shown). Whole milk yogurt experiments were performed in triplicate while skim milk yogurt experiments were performed in duplicate.

2.4. Percent titratable acidity and pH measurements

Percent titratable acidity (expressed as %lactic acid) was determined according to the procedure described by Wehr and Frank (2004). Yogurt and milk samples (9 g or 9 mL) were transferred into white 50 mL beakers or cups, a few drops of phenolphthalein solution (1% w/v in ethanol) were added, and the samples were titrated with a 0.1 M NaOH solution until a faint pink endpoint was achieved. Percent titratable acidity was calculated according to Eq. (1), where V_{NaOH} and C_{NaOH} are the volume (L) and molarity of NaOH titrant used, respectively, and m_{yogurt} is the mass (g) of yogurt culture.

$$\%\text{titratable acidity} = \frac{V_{NaOH}C_{NaOH}\left(\frac{mole\ lactic\ acid}{mole\ NaOH}\right)\left(\frac{90.08\ g}{mole\ lactic\ acid}\right)(100\%)}{m_{yogurt}} \quad (1)$$

A calibrated model 420 Orion pH meter (Thermo Fisher, Waltham, MA) was used for pH measurements.

2.5. Effect of pasteurization on toxin stability

A separate study determined the stability of ricin and abrin under batch pasteurization conditions. Whole and skim milk were fortified with nonfat dry milk (2%, w/w) and the concentration of ricin or abrin was adjusted to 100 μg/mL using crude toxin extracts. One milliliter aliquots of toxin-spiked milk were pipetted into glass test tubes. The tubes were capped and then heated in a block heater (Boekel Industries, Model #11002, Feasterville, PA) at 85 °C for up to 30 min. Triplicate sample tubes were removed from the block heater at the desired time points and cooled in an ice bath. Aliquots of cooled milk were analyzed for residual toxin levels using ELISA.

2.6. Detection of ricin and abrin in yogurt using ELISA and cytotoxicity assays

Yogurt samples were diluted with PBS and residual ricin and abrin levels quantified with commercial ELISA kits (Tetracore, Rockville, MD) according to the method provided by the supplier. The only modification was the incorporation of a seven-point calibration curve with each ELISA plate. Absorbance values at 405 nm were measured using a plate reader (BioTek Model #ELx808; Winooski, VT) and software (KC4, BioTek).

Cytotoxicity assays with RAW264.7 murine macrophage cells were used to determine the biological potency of yogurt samples containing ricin or abrin (Jackson et al., 2006). RAW264.7 macrophage cells provide two advantages for this work; they tolerate exposures to diverse food matrices very well and they are very sensitive to ricin and abrin (Battelli et al., 2001; Jackson et al., 2006, 2010; Tolleson et al., 2012; Zhang et al., 2013). Cells were cultured in MegaCell MEM media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 2 μM L-glutamine (Sigma-Aldrich), 100 units/mL penicillin (Sigma-Aldrich), and 100 μg/mL streptomycin (Sigma-Aldrich) in a 5% CO_2 incubator at 37 °C. RAW264.7 cells were plated (2.0×10^4 cells per well in 0.1 mL) in black, polystyrene, clear-bottomed tissue culture-treated 96-well assay plates (Corning, Corning, NY) and incubated overnight. Preliminary studies indicated that the acidity and the turbidity of yogurt samples could interfere with cytotoxicity assays. To compensate, thawed control and toxin-containing yogurt samples were diluted 1000-fold with media containing 2.5 mM sodium bicarbonate prior to analysis. Triplicate sets of two-fold serial dilutions were prepared for each toxin-containing sample using 0.1% control yogurt in media with 2.5 mM sodium bicarbonate as the diluent and then 0.1 mL aliquots were added to each well (0.977–1000 pM RIP toxins). Two-fold serial dilutions of cadmium chloride (0.195–100 μM) and ricin or abrin standards in yogurt-free bicarbonate-buffered media were included on each plate as positive controls. After two days of incubation at 37 °C, the toxin-containing media in each well was replaced with CellTiter Blue viability reagent (Promega, Madison, WI) diluted 1:5 with media and incubation was continued for 3 h. The amount of resorufin produced by viable cells in each well was measured (EX550, EM590) using a Synergy 4 plate reader with Gen 5 software (BioTek). Data for each replicate dilution series were normalized to 100% and the logarithm of the 50% inhibitory concentration ($log(IC_{50})$) values were determined by fitting log-transformed ricin concentrations ($log(C)$) and normalized fluorescence intensities (F) to equation (2), where m is the Hill slope factor and F_{min} and F_{max} are the minimum and maximum normalized fluorescence intensities, respectively (Prism 6.0 software, GraphPad, San Diego, CA).

$$F = F_{min} + \frac{F_{max} - F_{min}}{1 + 10^{m(\log(IC_{50}) - \log(C))}} \quad (2)$$

2.7. Inhibition of toxin activity by yogurt and milk

RAW264.7 cells were plated (20,000 cells in 0.1 mL media per well) in 96-well assay plates and incubated overnight. Nonfat yogurt, normal yogurt, skim milk, and