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Electrochemiluminescence immunosorbent assay of ricin in ground beef: biotinylated capture antibodies and matrix effects

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Electrochemiluminescence immunosorbent assay of ricin in ground beef: biotinylated capture antibodies and matrix effects

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Ricin is a highly toxic protein present in the seeds of castor (*Ricinus communis*), grown principally as a source of high quality industrial lubricant. Because of the past use of ricin for intentional poisoning, there is a need for analytical methodology to detect ricin in food matrices. Ground beef and other fatty, solid matrices present challenges for extraction and detection of protein constituents. This study focused on the use of streptavidin-coated assay plates, with biotinylated monoclonal antibodies (mAbs) immobilised as capture reagents. It explored matrix effects on immunosorbent analyses of ricin in enzyme-linked and electrochemiluminescent detection systems. A variety of mAb pairs enabled assays with predetermined specificity for ricin vs. the related protein, *Ricinus communis* agglutinin-1 (RCA-1). Extraction of samples at low dilution (1:5) and inclusion of 100 mM galactose in the extraction medium produced excellent quantification of ricin in the 1–20 ng/g range in ground beef.

Keywords: ricin; *Ricinus communis* agglutinin; castor; monoclonal antibody; biothreat; electrochemiluminescence; ground beef

Introduction

Ricin is a highly toxic protein found in the seeds (beans) of the castor plant, *Ricinus communis* (Lord & Roberts, 2005; Lord & Spooner, 2011). This toxin is also found in the by-products of industrial castor oil production (Chen, He, Ahn, Vang, & McKeon, 2006) and products being developed for utilisation of this material (Madeira, Macedo, & Macedo, 2011). In mouse toxicity tests, the mean lethal dose (LD₅₀) of pure ricin is about 8 µg/kg by iv administration and 30 mg/kg by oral dosage (He, McMahon, Henderson, Griffey, & Cheng, 2010a). Although ricin would not be expected to contaminate foodstuffs naturally, there has been concern over the potential for foodborne bioterrorism because of the past use of ricin for poisoning and its documented association with criminal activity (CDC, 2003; Audi, Belson, Patel, Schier, & Osterloh, 2005). For these reasons, it is important to have sensitive methods for detecting ricin. Although analytical methods for ricin, including immunoassays, have been reported, their application to complex, solid matrices has been limited. Polymerase chain reaction (PCR) technology was applied to the detection of castor DNA as a marker for crude ricin in the ground beef matrix (He, Brandon, Chen, McKeon, & Carter, 2007). Activity assays have been used for this

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matrix (He, Lu, Cheng, Rasooly, & Carter, 2008), and immunochemical technology was combined with the amplification potential of PCR to develop an exquisitely sensitive immuno-PCR assay for ricin in several food matrices (He, McMahon, McKeon, & Brandon, 2010b). The assay was then applied to the study of ricin toxicokinetics in a mouse model (He *et al.*, 2010a). Application of electrochemiluminescence detection in immunosorbent systems for ricin detection was first reported by Poli, Rivera, Hewetson, and Merrill (1994). Application of the ECL technology to analysis of beverages was described by Garber and O'Brien (2008). Cho, Keener, and Garber (2009) used multi-well ECL technology to develop an assay for ricin's enzymatic activity in a variety of liquid food matrices. Nevertheless, solid, fatty matrices such as ground beef remain challenging for quantitative, high-throughput analyses. The qualitative analysis of ground beef by an electrochemiluminescence immunosorbent assay was used to detect ricin in ground beef, with a lower limit of detection (LOD) of 0.1 ng/g (Brandon, 2011), using antibodies prepared in this laboratory (Brandon & Hernlem, 2009). In this study, we evaluated several monoclonal antibody (mAb) pairs and two immobilisation strategies, and evaluated both qualitative and quantitative detection of ricin in ground beef. In addition to capture mAbs immobilised by adsorption, we used biotinylated mAbs bound to streptavidin-coated wells for immunosorbent assays of ricin that utilise enzyme-linked and ECL detection systems using the familiar 96-well assay plate format.

Materials and methods

Toxins

Ricin and RCA-1 were obtained from Vector Laboratories (Burlingame, CA, USA) and stocks were stored at 4°C. Polypeptide composition of the agglutinins was verified by SDS-polyacrylamide gel electrophoresis.

Antibodies and conjugated antibodies

Monoclonal antibodies were prepared, purified, characterised and biotinylated or conjugated to tris(2,2'-bipyridyl)ruthenium(II) (Ru[bpy]₃)-N-hydroxysuccinimide ester as described previously (Brandon & Hernlem, 2009; Brandon, 2011). MAb are designated by the corresponding hybridoma clone numbers, prefixed by designation of the conjugate, for example, biotinylated mAb (b-mAb) 1795 and Ru(bpy)₃-mAb 1443.

Sample preparation

Ground beef marked '90% lean' was purchased at local supermarkets and used within 24 h of purchase. Samples were kept on ice during all procedures prior to application of sample to assay wells. Four-gram samples were weighed into 50 mL polypropylene conical centrifuge tubes and spiked with a small volume (generally 8 µL) of working dilutions of the stocks of ricin or RCA-1. Each sample was thoroughly mixed using a plastic spatula, and then 8 mL of extraction buffer were added (phosphate-buffered saline [PBS]-100 mM galactose). Samples were

homogenised for 30 sec at 20,000 rpm using Model GLH-01 homogeniser with a 10 mm × 115 mm, saw tooth generator probe and external speed control SC115 (Omni International, Kennesaw, GA, USA). After pieces of beef stuck to the homogeniser probe were scraped back into the homogenate, the sample was homogenised for an additional 30 sec at the same speed. The control homogenate (0 spike level) is referred to as 'ground beef extract' in several experiments described later. Dilutions of homogenates were generally made within the assay wells.

Assay plates

Colorimetric enzyme-linked immunosorbent assays (ELISAs) were performed in Immulon® 4HBX plates (Dynex, Chantilly, VA, USA). For ECL assays, 96-well standard, uncoated plates and streptavidin-coated plates were obtained from Meso Scale Discovery ([MSD], Gaithersburg, MD, USA; Cat. Nos. L15XA-3 and L15SA-2, respectively).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay wells were coated as described previously (Brandon & Hernlem, 2009). Assay wells contained 100 µL of standards, controls or samples, using PBS-0.05% Tween®-20 (PBST) containing 1% bovine serum albumin (BSA) and 100 mM galactose as diluent (BPTG). Samples were generally assayed neat or as dilutions of 1:2, 1:5 or 1:10 by addition of 100, 50, 20 or 10 µL of sample to assay wells containing 0, 50, 80 or 90 µL, respectively, of BPTG. After samples and standards were applied, plates were sealed and incubated 1 h, with shaking. Wells were emptied by manual pipetting of their contents into 0.5% sodium hypochlorite solution for inactivation of toxin, and were rinsed four additional times with water. Biotinylated detection antibody was then added (100 µL at 100 ng/mL in BPTG, to minimise non-specific binding and binding to agglutinins via their carbohydrate-binding sites). After incubation with shaking for 1 h, wells were washed four times with water. Horseradish peroxidase-conjugated streptavidin (HRP-SA, Invitrogen, South San Francisco, CA, USA) was applied (1:5000, 100 µL/well) and incubated 30 min, with shaking. Following water washes, the assay was developed by adding tetramethylbenzidine substrate solution (TMB, K-Blue, Neogen, Lexington, KY, USA), 100 µL/well. The reaction was stopped after 30 min by the addition of 100 µL/well 0.3 N HCl. Absorbance was read at 450 nm, with subtraction of the absorbance at 650 nm, using Model M2 plate reader using SoftMax® Pro 5.3 software (Molecular Devices, Sunnyvale, CA, USA).

ECL assays

Plate coating

Standard plates were coated as ELISA plates, but with 50 µL of mAb at 2 µg/mL in PBS, 4–16 h. They were washed 5 times with PBST, followed by blocking remaining binding sites with 150 µL/well of 30 mg/mL BSA in PBS for 1 h. Plates were washed and used immediately or stabilised by sucrose treatment, drying and storage, as for

ELISA. Streptavidin-coated plates were coated with biotinylated mAb at 1 µg/mL, then blocked as described earlier, on the day of assay.

Sample application and incubation

Procedures were similar to ELISA, but employed 50 µL samples and PBST for washing. Incubations were 60 min for application of samples and standards (to minimise differences in incubation times) and 30 min for other steps.

ECL assay using mAb-coated assay plates

For assays utilising direct detection of analyte, Ru(bpy)₃-conjugated mAbs were used (50 µL/well, 0.2–1 µg/mL). In initial studies, biotinylated antibodies were used for detection with Ru(bpy)₃-streptavidin as secondary detection reagent. This reagent (MSD Sulfo-Tag® – Streptavidin, Cat. No. R32AD-5) was applied 50 µL/well at 0.5 µg/mL diluted in BPTG. After incubation with detection reagent, plates were again washed, as described earlier, tapped to empty, then 150 µL of tripropylamine solution (MSD 4X Read Buffer with Surfactant, Cat. No. R92TC-2, diluted 1:4 with water) were added. After dispersing any bubbles that formed during pipetting, plates were read immediately on a Sector 2400 Imager, with Discovery Workbench v3.0 software (MSD).

ECL assay using biotinylated mAb/streptavidin assay plates

Samples were applied and incubated as for the ELISA other ECL immunosorbent assays. Plates were washed, treated and read, as for the antibody-coated ECL plates.

Data analysis

Data are means ± standard deviation (SD, $n = 3$), unless otherwise indicated. The lower LOD was computed as the analyte concentration at which the lower one-sided 95% confidence interval (CI) equalled the blank (unspiked sample) + 3 SD. Confidence and prediction intervals were computed using SlideWrite® v6 (Advanced Graphics Software, Carlsbad, CA, USA). Standard curves were fitted to a 4-parameter logistic equation or a linear log-log equation using SlideWrite or SoftMax Pro.

Results and discussion

ECL assay performance

Table 1 shows the assay parameters for both of the ECL assay formats used in this study. In general, the streptavidin-coated plates provided a higher signal for the same mAb pair, but the generality cannot be determined from these data because the two formats used different conjugates, albeit of the same mAbs. The directly coated format uses unconjugated mAb, rather than biotinylated mAb, for capture and the Ru(bpy)₃-mAb, rather than biotinylated mAb for detection. Another difference between the formats is that the immobilisation via streptavidin employs a 1 h

Table 1. Positive and negative control data for ECL assays.

Type of ECL plate	Capture reagent	Detection Ru(bpy) ₃ -monoclonal antibody (mAb)	Luminescence of positive and negative controls ^a (units × 10 ⁻³)	
			+	-
Standard (bare)	<i>mAb</i>			
	1443	1795	10	0.05
	1506	2147	18	0.06
	1797	1443	18	0.06
	2147	1443	15	0.07
	2147	1795	27	0.06
Streptavidin	<i>b-mAb</i>			
	1443	1795	18	0.09
	1506	2147	47	0.10
	1655	2147	25	0.11
	1795	1443	36	0.06
	2147	1443	39	0.06
	2147	1795	29	0.13

^aData are means of 2–5 determinations for each mAb pair. The positive control was 10 ng/mL ricin in BPTG; the negative, buffer only.

incubation of the biotinylated mAb, compared to a 4 h or overnight incubation for direct coating, and binding occurs via specific, high-affinity receptors. There was no significant difference between 4 h and overnight coating for the standard ECL assay plates.

Figure 1 shows the results of typical experiments to determine effectiveness of mAb pairs using streptavidin-coated plates. About 10% of mAb pairs tested resulted in effective sandwich assays.

Figure 2 shows how the choice of antibody pairs results in different selectivity for the two castor agglutinins. The sandwich b-mAb 2147/Ru(bpy)₃-1795 an example of a sandwich that does not discriminate between ricin and RCA-1.

Assay performance in ground beef matrix

In prior reports from this laboratory (Brandon & Hernlem, 2009; Brandon, 2011), 100 mM galactose was used in all immunoassay steps, to minimise binding of ricin to antibody or matrix components. In this study, following initial results with poor ricin recovery from ground beef, we tested the effect of galactose in the extraction medium. Table 2 shows the results of ELISA analysis of samples extracted with PBS or 100 mM galactose in PBS. The ELISA used mAb 1443 and biotinylated mAb 1795, and recoveries were increased by about 40% when 100 mM galactose was added. Extraction with higher galactose concentration (500 mM) did not further increase recovery. Subsequent extractions all included 100 mM galactose in the extraction medium.

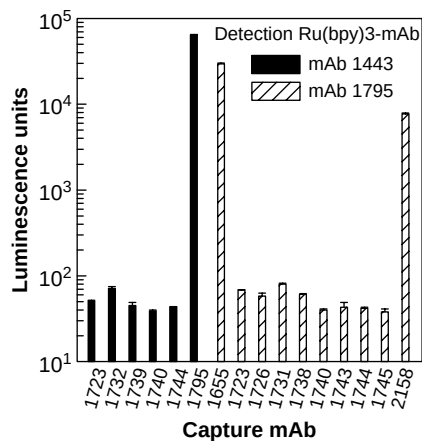


Figure 1. Characterisation of monoclonal antibody (mAb) pairs in ECL assay. Biotinylated capture mAbs were immobilised on streptavidin-coated ECL plates and tested in sandwich immunosorbent assay with Ru(bpy)₃-mAbs 1443 and 1795 for detection. Analyte was 10 ng/mL ricin in BPTG. Blanks (0 ng/mL ricin) ranged from 80 to 120 luminescence units in these assays.

Table 3 shows the result of analysis of ricin spiked in ground beef and analyzed using biotinylated mAb capture on streptavidin-coated ECL plates. The recovery was excellent (83–100%). In comparison, using the same antibodies, recovery measured by ELISA was variable and more subject to matrix effects ($190 \pm 150\%$, $n = 4$), although recovery was improved at higher spiking levels (data not shown).

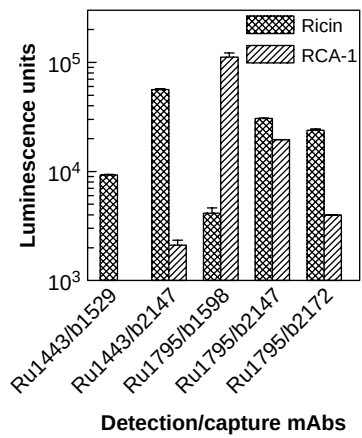


Figure 2. Characterisation of monoclonal antibody (mAb) pairs in ECL assay. Biotinylated mAb/Ru(bpy)₃-mAb sandwich pairs tested for detection of ricin and RCA-1. Samples of negative control (BPTG), and 10 ng/mL of agglutinin in BPTG were applied to wells with immobilised biotinylated mAbs under standard conditions on streptavidin ECL plates. Negative control values ranged from 80 to 120 luminescence units.

Table 2. Effect of extractant on recovery of ricin from ground beef^a.

Homogenate dilution	Recovery (%)			n
	PBS	PBS + 100 mM galactose	Increase (%)	
1:2	59 ± 14	83 ± 7.9	42 ± 13	3
1:4	76 ± 8.3	104 ± 3.0	37 ± 3.9	3

^aMeans ± SD (*n* = 3) of analyses of ground beef spiked with ricin at 0.5, 1 and 2 µg/g and analyzed by sandwich enzyme-linked immunosorbent assay (monoclonal antibody [mAb] 1443/b-mAb 1795). Recovery data are pooled for the 3 spike levels.

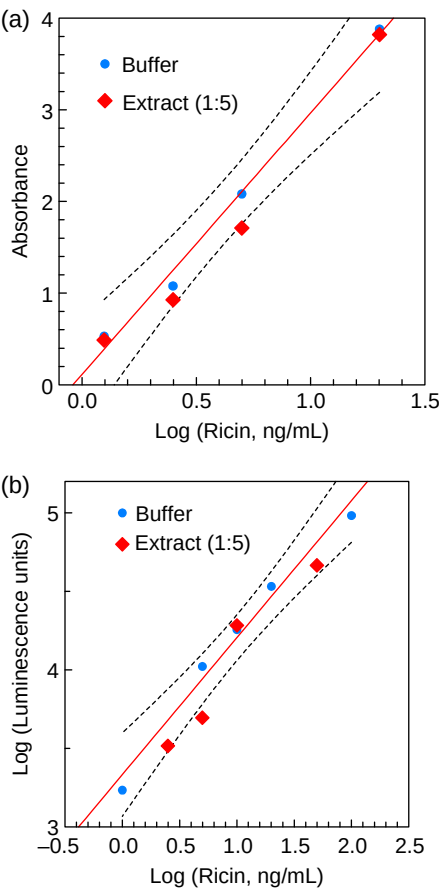


Figure 3. Matrix effects for ground beef extract in enzyme-linked immunosorbent assay (ELISA) and ECL assay. Standard solutions were diluted in BPTG buffer or ground beef extract (control ground beef homogenate at a final dilution 1:5 in buffer) and analyzed by (a) ELISA or (b) ECL, each using monoclonal antibody (mAb) 1443 for capture and mAb 1795 for detection.

Table 3. Recovery data using streptavidin-coated ECL plates^a.

Experiment no.	Detection Ru(bpy) ₃ - monoclonal antibody (mAb)	Recovery (%)		<i>n</i>
		Mean	SD	
1	1443	83	24	4
	1795	98	15	4
2	1443	94	12	6
	1795	110	13	4

^aBoth assay systems employed biotinylated mAb 2147 for capture. Samples were spiked at 1 and 2 ng/g. Recoveries were similar and are pooled.

Previously reported results (Brandon, 2011) showed lower recovery data for ricin in ground beef using ELISA vs ECL immunoassay, based on quantification of assay data determined from the linear portion of a 4-parameter logistic fit of the standard curves. Figure 3 shows that the ground beef matrix at 1:5 dilution produces assay results that fall within the 95% CI for the buffer standards. Thus, this methodology should not require an entire set of standards in matrix, but this should be verified for each experimental sample by inclusion of one or more internal standards (Brandon & Carter, in press).

Conclusions

Enzyme-linked immunosorbent assay and other multiwell immunosorbent assays, such as the electrochemiluminescent detection system employed in this study, are probably most valuable for rapid screening assays that have a high probability of generating negative results because of the nature of the samples. Examples of implementation of such formats are screening for violative levels of residues of agrochemicals or veterinary drugs and testing for the presence of naturally occurring toxins (e.g. aflatoxin) above regulatory action levels. The occurrence of a presumptive positive screening assay alerts the analyst to perform a repeat assay or an alternative 'confirmatory' format (most commonly liquid chromatography/mass spectroscopy). Screening for the presence of intentional contaminants is another example of this analytical purpose. Although the most useful screening assays for biothreat toxins such as ricin are likely to be qualitative, the results presented in this paper demonstrate that assay conditions can be adjusted to provide good quantitative results.

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