Lactate Dehydrogenase Monoclonal Antibody Sandwich ELISA To Determine Cooking Temperature of Ground Beef

A. Orta-Ramirez, C. H. Wang,[†] M. M. Abouzied,[‡] G. J. Veeramuthu, J. F. Price, J. J. Pestka, and D. M. Smith*

Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824, and Neogen Corporation, Lansing, Michigan 48912

Monoclonal antibodies (MAb) to bovine muscle lactate dehydrogenase (LDH) were prepared and a sandwich enzyme-linked immunosorbent assay (ELISA) was developed, using MAb as capture antibodies and polyclonal antibodies as detector antibodies. The ability of the sandwich ELISA to quantify LDH was tested in a ground beef model system and commercial ground beef products cooked to different endpoint temperatures (EPT). The LDH content of extracts decreased from 805 μg/g of meat in ground beef cooked to 64 °C to 0.24 μg/g of meat cooked to 74 °C. Commercially cooked patties with EPTs between 68.3 and 71.1 °C averaged 3.38 μg of LDH/g of meat. Similar concentrations of LDH (about 3 μg/g of meat) were observed in both the model system and commercially cooked patties at EPT of about 70 °C. Fat content had no effect on the concentration of LDH in ground beef cooked to 69.4 °C. Five freeze−thaw cycles had no effect on the LDH concentration of raw ground beef. Repeated freezing and thawing of raw meat decreased the LDH concentration of cooked ground beef. LDH concentrations in most patties from six fast food restaurants suggested that all were cooked to at least the recommended EPT of 69.4 °C.

Keywords: *Lactate dehydrogenase; endpoint temperature; ground beef; ELISA*

INTRODUCTION

Most outbreaks of *Escherichia coli* O157:H7 have been associated with consumption of undercooked ground beef products. The severity of symptoms, including death in some cases, has made control of this organism a concern for meat processors, government regulatory agencies, food retailers, and consumers. Proper cooking is the simplest method to eliminate the pathogen from food. The meat industry is recommending that hamburger patties be cooked to a minimum temperature of 69.4 °C and held for 10 s to ensure destruction of *E. coli* and other pathogens (Beef Industry Council, 1995).

The U.S. Department of Agriculture−Food Safety Inspection Service (USDA-FSIS) does not have an accurate test to verify minimum internal processing temperatures after beef patties have been cooked (Townsend and Blankenship, 1989). Several researchers have investigated the use of residual enzyme activity to detect the endpoint cooking temperature (EPT) of previously cooked beef products. Residual activity of lactate dehydrogenase (LDH) has been shown to be a potential EPT indicator in turkey products (Wang et al., 1992, 1993; Abouzied et al., 1993), beef model systems (Collins et al., 1991; Stalder et al., 1991), and beef patties (Wang et al., 1996a). The quantification of LDH by a sandwich enzyme-linked immunosorbent assay (ELISA) has been successfully used for the determination of EPT in poultry products (Wang et al., 1992, 1993; Abouzied et al., 1993; Smith et al., 1996). Recently, Wang et al. (1996b) devised a polyclonal antibody (PAb)-based sandwich ELISA (PAb used for both capture and detection) to determine EPT of ground beef.

* Author to whom correspondence should be addressed [telephone (517) 353-9513; fax (517) 353-8963; e-mail 20533dms@msu.edu].

[†] Present address: Department of Food and Nutrition, Providence University, Shalu, Taiwan.

[‡] Present address: Neogen Corp.

Monoclonal antibodies (MAb) have several advantages over PAb. MAb are specific to one epitope and homogeneous and can be produced in unlimited quantities by tissue culture (Harlow and Lane, 1988). Wang et al. (1993) demonstrated that a sandwich ELISA using MAb for antigen capture was at least 100 times more sensitive than an indirect competitive (IC) ELISA using PAb. The goal of this project was to develop a MAb-based immunoassay to determine the EPT of previously cooked ground beef patties. MAb to LDH were prepared, and a sandwich ELISA, using MAb as capture antibodies and PAb as detector antibodies, was developed. The sandwich ELISA was tested in a ground beef model system and commercial ground beef products cooked to different EPTs. The effect of fat content and freeze−thaw cycles on LDH concentrations in ground beef was also determined.

MATERIALS AND METHODS

Materials. Female mice (BALB/c, 6−8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Polyethylene sorbitan monolaurate (Tween 20), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide, poly(ethylene glycol) (MW 1450), hypoxanthine, aminopterin, thymidine, complete and incomplete Freund's adjuvant, Dulbecco's modified medium, ovalbumin, and LDH from chicken muscle (type XXXIV), bovine heart (type III), porcine heart (type XVIII), rabbit muscle (type II), porcine muscle (type XXX-S), bovine muscle (type X), and chicken heart (type VIII) were purchased from Sigma Chemical Co. (St. Louis, MO). Penicillin/streptomycin solution (100 000 units/mL), NCTC-135 medium, fetal bovine serum (FBS), and minimum essential media (MEM) sodium pyruvate were from Gibco Laboratories (Grand Island, NY). Goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase and goat anti-rabbit IgG conjugated to horseradish peroxidase were from Cappel Laboratories (West Chester, PA). Tissue culture plasticware was from Corning Laboratory Science Co. (Corning, NY), and microtiter plates (Immunolon-2 Removawells) from Dynatech Laboratories (Alexandria, VA). The

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myeloma cell line P3/NS 1/1-Ag4-1 (NS-1) (ATCC TIB 18) was from the American Type Culture Collection (Rockville, MD). Macrophage conditioned medium was prepared as described by Sugasawara et al. (1985). Rabbit PAb were prepared against beef muscle LDH as reported previously (Wang et al., 1996b).

Production of MAb. Two groups of mice were injected either subcutaneously or intraperitoneally (five mice each) with 75 μg of bovine muscle LDH in saline (0.8%) mixed (1:1) with Freund's complete adjuvant for a total of 0.2 mL/mouse. Two booster injections were given at 2 week intervals as described above except that incomplete Freund's adjuvant was used. One week after the last injection, serum obtained from the retrobulbar plexus of each mouse was tested by indirect ELISA to determine antibody titer. Three days before removal of the spleen for fusion, an intraperitoneal injection of bovine muscle LDH in saline solution was given to those mice with antisera showing the highest inhibition when tested by IC-ELISA.

MAb were produced following the procedure of Abouzied et al. (1990). Mouse spleen cells (1×10^8) were fused with NS-1 myeloma cells (1×10^7) using 50% poly(ethylene glycol) as the fusion agent. Following fusion, cells were suspended in Dulbecco's modified medium containing 20% FBS, 1% NCTC medium, 10 mM MEM sodium pyruvate solution, and penicillin/streptomycin solution (100 units/mL). The cell suspension was then distributed into six 96-well flat-bottom tissue culture plates. The plates were incubated at 37 °C in a humid atmosphere containing 8% CO_2. After 24 h, half of the supernatant was removed from each well and replaced with an equal volume of hypoxanthine, aminopterin, and thymidine selective medium (HAT medium). Every 3 days the cells were fed exactly as above. After 2 weeks, HAT medium was replaced by HT medium (same as HAT but without aminopterin). Those wells showing cell growth and color change were tested for antibody production by IC-ELISA. Hybridomas showing continued production of anti-LDH antibodies were expanded and cloned twice by limiting dilution (Goding, 1980). Cloned cells were grown in HT medium containing 15% FBS and 15% macrophage-conditioned medium (Abouzied et al., 1990; Sugasawara et al., 1985).

Supernatants were collected following centrifugation at 2500*g* for 30 min. Antibodies were purified by precipitation with 50% ammonium sulfate (Hebert et al., 1973), dialyzed for 3 days against 0.15 M NaCl/0.01 M sodium phosphate, pH 7.2 (PBS), at 4 °C, aliquoted, and frozen. MAb isotypes were determined with the Screen Type Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Cross-reactivity of antibodies with LDH from other species and organs was determined by sandwich ELISA.

Indirect ELISA. An indirect ELISA was used initially to determine antibody titer (Abouzied et al., 1990). The indirect ELISA was performed by coating microtiter plates with 100 μL of bovine LDH (10 μg/mL) in 0.1 M carbonate buffer, pH 9.6, following incubation overnight at 4 °C. Plates were washed four times with PBS containing 0.05% Tween 20 (PBS-T). Next, nonspecific binding sites were blocked by adding 300 μL of 1% ovalbumin in PBS (OA-PBS) to each well and incubating at 37 °C for 30 min. After four washings with PBS-T, 50 μL of serially diluted serum was added to each well and incubated at 37 °C for 1 h. After incubation, excess antibodies were removed by washing four times with PBS-T. To each well was added 100 μL of 1:500 goat anti-mouse IgG peroxidase conjugate in OA-PBS and incubated at 37 °C for 30 min. After eight washings, peroxidase binding was determined by using ABTS substrate (Pestka et al., 1982). Absorbance was read at 405 nm using a Vmax kinetic microplate reader (Model 1234, Molecular Devices Corp., Menlo Park, CA). The titer of each serum was arbitrarily determined as the highest dilution showing an optical density reading that was at least twice the reading of the nonimmune serum at the same dilution.

An IC-ELISA was used to test cells for antibody production and was identical to the indirect ELISA, except that after blocking of nonspecific sites and washing, 50 μL of LDH was added to each well with 50 μL of LDH antisera or 50 μL of cell culture supernatant.

Sandwich ELISA. The sandwich ELISA was performed by coating microtiter plates with 100 μL of MAb LDH antibodies in 0.1 M carbonate buffer, pH 9.6, and drying overnight at 40 °C in a forced air oven. After four washings with PBS-T, nonspecific binding sites were blocked by adding 300 μL of 2% OA-PBS to each well and incubating at 37 °C for 30 min. Plates were washed four times and 50 μL of serially diluted bovine muscle LDH or ground beef patty extracts was added to each well and incubated at 37 °C for 30 min. After four more washings, 50 μL/well of LDH specific PAb in 2% OA-PBS (1:500) was added. Following incubation for 30 min at 37 °C and washing, 100 μL of 1:500 goat anti-rabbit IgG peroxidase conjugate in 2% OA-PBS was added to each well and incubated at 37 °C for 30 min. After eight washings, bound peroxidase was determined by adding 100 μL of K-Blue substrate (ELISA Technologies, Lexington, KY) or ABTS substrate to each well. Color development was stopped by addition of 100 μL/well of stopping reagent and absorbance was read at 650 nm for K-Blue substrate or 405 nm for ABTS substrate. Assay precision was determined as suggested by Deshpande (1996).

Model System. Semitendinosus muscle and beef kidney fat were purchased from a local supplier. Meat and fat were ground twice through the 3 mm plate of a meat grinder (Model K5-A, KitchenAid, Hobart Corp., Troy, OH). Ground beef was adjusted to target concentrations of 10, 15, or 20% fat. Moisture, fat, and protein contents were determined according to AOAC (1990) Methods 950.46B, 991.36, and 981.10, respectively. Ground beef (2.5 g) was extruded into 10 × 75 mm glass thermal death time (TDT) tubes to a constant height of 45 mm using a small syringe stuffer to eliminate air pockets. The tubes were sealed with Teflon tape and heated to internal temperatures between 62 and 74 °C at 2 °C intervals in a circulating water bath (Model 1268-52, Cole-Parmer, Chicago, IL) connected to a temperature programmer (Model 1268-62, Cole-Parmer). The temperature of the water bath was set 0.5 °C above the target temperature. A thermocouple (RTD, 1.6 mm diameter ± 0.5 °C) connected to a Solomat MPM 2000 Modumeter (Stanford, CT) was inserted into the center of a control tube containing 2.5 g of meat and used to monitor temperature. The probe was calibrated using the manufacturer's instructions. Tubes were removed when they reached the target temperature and immediately placed in an ice–water bath. LDH was extracted using 3 volumes (w/v) of PBS as described by Wang et al. (1996b). The LDH concentration of the extracts was determined by sandwich ELISA. Three replicate batches of ground meat were cooked at each temperature.

Freeze–Thaw Cycles. The effect of five freeze–thaw cycles on the concentration of LDH in raw ground beef and ground beef cooked to 69.4 °C was determined. Raw ground meat, containing 10% fat, was placed in TDT tubes as described above, sealed, and frozen at −20 °C. The next day, all TDT tubes were removed from the freezer and meat was thawed in a 25 °C water bath for 45 min. Three tubes were cooked to 69.4 °C, held for 10 s, and then cooled as described previously. Three tubes containing raw meat and three tubes containing cooked meat were extracted and analyzed for LDH content by sandwich ELISA. The remaining tubes containing raw meat were refrozen, and this freeze–thaw cycle was repeated four more times at 24 h intervals.

Commercial Patties. Three groups of ground beef patties (113 g) were cooked in a clam-shell grill by a commercial processor to center temperatures of 54.6–65.6, 68.7–71.1, or 73.9–83.2 °C as determined by a thermocouple inserted into the side of each patty. Patties were cooked, frozen, and sent to Michigan State University in dry ice. Four patties were analyzed from each temperature range within 48 h of cooking. In another experiment, three small (56.8–79.5 g raw weight) and three large cooked patties (113.6 g raw weight) were purchased from each of six fast food restaurants. Ten grams of meat from the center of each patty was extracted and analyzed as described above by sandwich ELISA.

Electrophoresis and Western Blotting. Purified bovine muscle LDH and protein extracts from the model system cooking trials were separated by native polyacrylamide gel

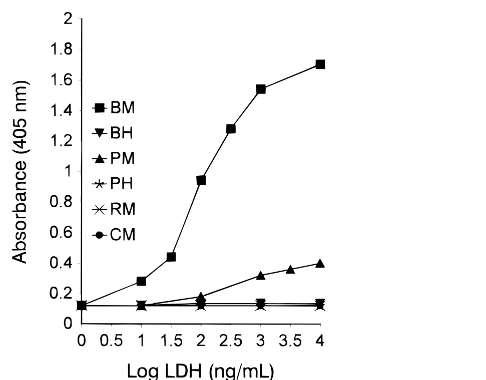

Figure 1. Specificity of bovine muscle LDH antibodies in a sandwich ELISA. LDH sources were bovine muscle (BM), bovine heart (BH), porcine muscle (PM), porcine heart (PH), rabbit muscle (RM), and chicken muscle (CM). Data are the average of three replicates.

electrophoresis (PAGE) and sodium dodecyl sulfate (SDS) PAGE and transferred electrophoretically to a nitrocellulose membrane by Western blotting (Wang et al., 1996b). After transferring, the membrane was washed with PBS-T, blocked with 10 mL of 3% OA-PBS for 30 min at room temperature, and rinsed with PBS-T, and then 10 mL of diluted MAb in 3% OA-PBS was incubated with the membrane for 30 min. Unbound antibody was removed by washing with PBS-T and 10 mL of goat anti-mouse IgG peroxidase conjugate was added to the membrane and incubated for 10 min. The membrane was washed and bound peroxidase determined as described by Wang et al. (1996b).

Statistical Analysis. Each experiment was performed in triplicate. Basic statistics and one-way analysis of variance were performed using MSTAT software (MSTAT software, version C, 1989, Michigan State University, East Lansing, MI). Mean separations were performed using Tukey's test with the mean square error term at the 5% level of probability.

RESULTS AND DISCUSSION

Production of MAb and ELISA Development. Mice injected subcutaneously with bovine muscle LDH had higher titers than those injected intraperitoneally. In addition, when tested by IC-ELISA, sera of mice injected subcutaneously showed higher inhibition than those injected intraperitoneally. A total of 360 wells were seeded with fused spleen cells using the mouse with the highest titer (1.3×10^4) and NS-1 myeloma cells, but only one produced antibodies against LDH. A second fusion was performed using two additional mice that yielded two more positive hybridomas. The three antibody-producing hybridomas were further expanded and cloned. Eight stabilized lines were obtained that showed inhibition above 60% at 10 000 ng of LDH/mL when tested by IC-ELISA. All subclones had similar cross-reactivity with LDH from other species. Subclone A731-12C4 exhibited the highest inhibition (79.5%) and was used in all subsequent experiments.

A sandwich ELISA was developed with MAb as the capture antibodies and PAb as the detector antibodies (MAb−PAb ELISA). The coefficients of variation for the sandwich ELISA at 100, 350, and 500 ng of LDH/mL were 7.9, 7.7, and 4.4%, respectively, indicating acceptable interassay precision (Deshpande, 1996). The minimum detection limit was 10 ng of LDH/mL. This assay was 10 times more sensitive than a PAb−PAb sandwich ELISA for bovine LDH developed by Wang et al. (1996b). No cross-reactivity was observed with the antibodies used in the sandwich ELISA and LDH from bovine heart, porcine heart, rabbit muscle, or chicken muscle (Figure 1). Slight cross-reactivity was observed with porcine muscle at concentrations of 100 ng of LDH/mL and above. The MAb were more specific than the PAb, as Wang et al. (1996b) reported that the PAb cross-reacted with both porcine muscle LDH and bovine heart LDH.

Table 1. Influence of Temperature on the LDH Content of Ground Beef as Measured by Sandwich ELISA

temp (°C)	LDH[a] (μg/g of meat)	temp (°C)	LDH[a] (μg/g of meat)
62	979.82 ± 45.57[a]	70	3.03±0.54[d]
64	804.92 ± 17.43[ab]	72	0.43 ± 0.15[e]
66	589.84 ± 62.38[b]	74	0.24 ± 0.01[f]
68	122.03 ± 18.42[c]		

[a] Means with unlike superscripts differ ($P < 0.05$).

Table 2. Effect of Repeated Freeze−Thaw Cycles of Raw Ground Beef (15% Fat) on the LDH Concentration of Ground Beef Cooked to 69.4 °C As Determined by Sandwich ELISA[a]

cycle	LDH[b] (%)	cycle	LDH[b] (%)
0	100.0 ± 8.5[b]	3	53.3 ± 3.8[bc]
1	79.0 ± 22.3[b]	4	29.6 ± 2.2[c]
2	58.7 ± 3.6[bc]	5	22.5 ± 1.8[c]

[a] Results expressed as a percentage of initial concentration prior to freezing. [b] Means with unlike superscripts differ ($P < 0.05$).

Results of Western blotting experiments showed that the MAb reacted with LDH separated on native PAGE, but not with LDH separated by SDS−PAGE, suggesting that the MAb recognized an epitope of LDH found only in the native protein (data not shown). In contrast, Wang et al. (1996b) reported that the PAb reacted with both native and denatured forms of LDH.

Model System Studies. The LDH concentration decreased when ground beef containing 19.6 ± 0.94% fat was cooked to internal temperatures between 64 and 74 °C in the model system. No differences in LDH content were noted in beef cooked to 62 and 64 °C. The LDH content of extracts decreased from 804.9 μg/g of meat in ground beef cooked to 64 °C to 0.24 μg/g of meat in beef cooked to 74 °C (Table 1). The meat industry is recommending that beef patties be cooked to 69.4 °C. Ground beef in the model system contained 3.03 μg of LDH/g when cooked to 70 °C. Wang et al. (1996b) reported similar residual concentrations of LDH (3.3 μg/g of meat) in ground beef cooked to 70 °C when measured by PAb−PAb sandwich ELISA. Fat contents of 10.3, 15.0, and 18.9% had no effect on the residual concentrations of LDH in ground beef cooked to 70 °C in the model system.

Five freeze−thaw cycles had no effect ($P > 0.05$) on the LDH concentration of raw ground beef. Wang et al. (1993) reported that frozen storage for 16 weeks had no effect on LDH content of turkey breast muscle. Freezing and thawing of raw ground beef had a larger effect on the residual concentration of LDH in ground beef cooked to 69.4 °C (Table 2). One freeze−thaw cycle of the raw meat prior to cooking had no effect on the LDH concentration of cooked meat. However, additional freeze−thaw cycles decreased the residual LDH concentration of the cooked meat. Four freeze−thaw cycles decreased the LDH concentration by 70.4% in the cooked beef. Collins et al. (1991) also reported that the residual activity of LDH after cooking was the same in fresh beef muscle and in muscle subjected to one cycle of freezing and thawing. It is possible that ice crystal damage during freezing and thawing of ground beef allowed LDH to migrate from the muscle cells into the

Table 3. Influence of Temperature on the LDH Content of Commercially Cooked Beef Patties As Measured by Sandwich ELISA

temp (°C)	LDH[a] (μg/g of meat)	temp (°C)	LDH[a] (μg/g of meat)
54.4−65.6	362.44 ± 179.88[a]	73.9−82.2	0.30 ± 0.15[b]
68.3−71.1	3.38 ± 2.78[b]		

[a] Means with unlike superscripts differ ($P < 0.05$).

Table 4. LDH Content (Micrograms per Gram of Meat) of Beef Patties from Fast Food Restaurants[a]

restaurant	small patties	large patties
A	0.03 ± 0.03	0.04 ± 0.02
B	0.10 ± 0.03	0.08 ± 0.07
C	0.01 ± 0.00	ND
D	ND[b]	ND
E	0.99 ± 0.89	3.53 ± 2.29
F	ND	ND

[a] Means ± standard deviation, $n = 3$ patties. [b] ND, not detected. Limit of detection was 0.01 μg of LDH/g of meat.

drip or exudate where it is more easily denatured by heat (Wang et al., 1993).

Commercial Patties. The LDH content of commercially cooked patties also decreased as the processing temperature was increased (Table 3). The LDH concentration of patties cooked between 68.3 and 71.1 °C averaged 3.38 μg/g of meat. Similar concentrations of LDH (about 3 μg/g of meat) were observed in both the model system and commercially cooked patties at a cooking temperature of about 70 °C. Although more research is needed to establish a minimum residual concentration, results in both the model system and commercial patties suggest that a maximum concentration of about 3 μg of LDH/g meat might indicate that ground beef was processed to 70 °C or above.

Ground beef patties from only one restaurant contained LDH at concentrations above 3 μg/g of meat, suggesting that these patties may have been undercooked (Table 4). Ground beef in the model system tests cooked to 74 °C contained 0.24 μg of LDH/g of meat. Most patties obtained from fast food restaurants contained <0.24 μg LDH/g of meat, suggesting they were cooked above the recommended EPT of 69.4 °C.

The MAb−PAb sandwich ELISA described herein could detect differences in LDH concentration in ground beef and patties resulting from different internal cooking temperatures between 66 and 74 °C. More research is needed to determine if a minimum residual concentration of LDH can be established to indicate processing to a desired EPT. Studies are needed to determine the effects of patty size, cooking rate, and other formulation and processing variables on the residual LDH concentration.

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