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Lactate Dehydrogenase as Safe Endpoint Cooking Indicator in Poultry Breast Rolls: Development of Monoclonal Antibodies and Application to Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

MOHAMED M. ABOUZIED, CHENG HSING WANG, JAMES J. PESTKA, and DENISE M. SMITH*

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

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ABSTRACT

A sandwich enzyme-linked immunosorbent assay (ELISA) was developed to detect lactate dehydrogenase (LDH) as a marker protein for verifying endpoint cooking of uncured poultry products. Monoclonal antibodies were prepared against chicken muscle LDH and used with rabbit polyclonal antibodies developed against turkey or chicken muscle LDH for capture and detection in the assay, respectively. Minimum assay detection limits for turkey and chicken muscle LDH were 1 ng/ml. Turkey and chicken muscle LDH, but not LDH from other species cross reacted in the ELISA. The ELISA was further verified using extracts of turkey breast rolls processed to internal temperatures between 68.3 and 72.1°C. The LDH content of extracts diluted 3- to 6-fold was below 15 ng/ml for turkey rolls processed to 70.9 and 72.1°C. At a 6-fold dilution, LDH content of extracts from rolls processed to 69.7°C was approximately 10 times greater than those processed to 70.9°C. A survey of market precooked poultry products indicated assay validity with precooked turkey roast, but not turkey hams with maximum internal temperature requirements of 68.3°C. Results suggested the sandwich ELISA should be applicable for determining whether turkey breast rolls are processed to the required U.S. Department of Agriculture endpoint temperature of 71.1°C.

The U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) uses several procedures to determine if meat products have been cooked to the proper endpoint temperatures as required in Title 9 of the Code of Federal Regulations. These requirements ensure the destruction of harmful microorganisms and viruses that cause diseases in humans and livestock. Similar requirements exist for processing temperatures in imported products to prevent the spread to the United States of certain exotic diseases in animals (*22*).

Protein solubility of muscle tissue extracts after heating in water or salt solutions has been used as a measure of heat denaturation of protein (*10*). Lee et al. (*13*) and Caldironi and Bäzan (*3*) have demonstrated that water soluble components of beef, pork, and chicken muscles are differentially insolubilized when heated. The degree of insolubilization of water soluble muscle proteins is a function of time of heating, endpoint temperature, and initial quantities of soluble proteins in muscle (*7*). For example, the Coagulation Test (*24*) measures protein solubility loss with temperature in thermally processed meat products. Other approaches include the Bovine Catalase Test which gives a pass/fail indication of proper cooking temperatures for cooked beef (*26*) and the Acid Phosphatase Activity Method which is used to determine if canned hams, picnics and luncheon meats have been properly processed (*25*).

Enzyme assays have also been developed to monitor endpoint cooking temperatures based on residual activity of catalase and peroxidase (*16*), lactate dehydrogenase (LDH) (*4,5,14,18*) and pyruvate kinase (*6*). Townsend and Blankenship (*20*) selected leucine aminopeptidase from among 19 tested enzymes to monitor heat treatment of meat and poultry products. In a related approach, Klinger et al. (*12*) determined that LDH may serve as a test marker because it proportionally decreases in activity in relation to temperature endpoint and duration of heating. Collins et al. (*4,5*) used LDH activity as a marker for endpoint cooking of porcine muscle and bovine muscle.

Recently, we determined that LDH is insolubilized in turkey breast muscle following processing to an endpoint of 71°C (*28*). Polyclonal antibodies were prepared against turkey and chicken LDH and used in a competitive indirect enzyme-linked immunosorbent assay (ELISA) to verify processing temperatures. The LDH content determined by ELISA decreased as endpoint processing temperatures of turkey breast rolls were increased. The aim of the present study was to improve the above approach by preparing monoclonal antibodies to chicken muscle LDH and employ these in an antibody sandwich ELISA for LDH. The assay was used to evaluate processed turkey rolls and commercially available precooked poultry products.

MATERIALS AND METHODS

Materials

BALB\c, 6-8 week-old female mice were purchased from Charles River Laboratories (Wilmington, MA). Polyethylene sorbitan monolaurate (Tween 20), 2,2'-azinobis (3-ethylbenzo-

thiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide, polyethylene glycol (MW 1450), hypoxanthine, aminopterin, thymidine, penicillin/streptomycin solution (pen/strep) (100,000 U/ml), NCTC medium, 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). Bovine serum albumin (BSA) (fraction V) was from Ameresco (Solon, OH). Goat anti-mouse 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 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. (*19*).

Turkey muscle LDH was purified using ammonium sulfate and acetone precipitations and activity determined as described by Wang et al. (*28*). Complete and incomplete Freund adjuvant and Dulbecco's modified medium were from Difco Laboratories (Grand Island, NY). White New Zealand female rabbits were obtained from the Bailey Rabbitry (Alto, MI). Rabbit polyclonal antibodies were prepared against chicken LDH (designated as R-A, R-B, and R-C) or turkey LDH (designated as R-D, R-E, or R-F) as reported previously (*28*). Commercial precooked turkey roasts and hams were purchased from a local retail store.

Monoclonal antibody production

Six groups of female BALB\c mice (6-8 weeks) received 0.2 ml subcutaneous or intraperitoneal injections containing one volume of chicken muscle LDH (10, 25, or 50 μg per mouse) in saline (0.8%) and one volume Freund's complete adjuvant. Two booster injections were given at 2-week intervals in identical fashion, except that incomplete Freund's adjuvant was used. One week after the last injection, serum was obtained from the retrobulbar plexus of each mouse to determine titer and antibody specificity. Three days before removal of the spleen for fusion, an intraperitoneal injection of LDH in saline solution was given to mice whose antisera showed the highest inhibition by competitive ELISA.

Monoclonal antibodies against chicken muscle LDH were produced according to the protocol of Galfre and Milstein (*8*) as modified by Abouzied et al. (*1*). Spleen cells (1×10^8) of immunized mice, that showed the highest serum titer and specificity against LDH, were fused with NS-1 myeloma cells (1×10^7) using 50% polyethylene glycol. Fused cells were suspended in Dulbecco's modified medium containing 20% fetal bovine serum (20% FBS-DMEM) supplemented with 1% NCTC medium, 10 mM sodium pyruvate and penicillin/streptomycin solution (100 U/ml). The cell suspension was seeded into 11 96-well flat bottom tissue culture plates. Plates were incubated at 37°C in a humid atmosphere of 8% CO_2 in air. After 24 h, half of the supernatant fraction from each well was removed and an equal volume of hypoxanthine-aminopterin-thymidine (HAT) selective medium was added. This was repeated every 3 d. Two weeks later, the HAT medium was eliminated by gradual replacement with HT medium (the same composition of HAT medium but without aminopterin). Hybridomas that showed continued production of anti-LDH antibodies were expanded and cloned twice by limiting dilution (*9*) using 15% FBS-15%-macrophage-conditioned-HT medium (*1,19*). Four cell lines {designated as B3C-B8-F3 (B3C), D5E-G8-E4 (D5E), E6B-F8-G4 (E6B), and G4D-D9-G4 (G4D)} that produced high affinity antibodies for chicken muscle LDH were scaled up for further study. Cell lines were grown in 20% FBS-DMEM medium and supernatants collected every 2-3 d. After centrifugation to remove cells, antibodies were purified by precipitation with 50% ammonium sulfate (*11*), dialyzed against three changes of PBS, aliquoted, and lyophilized. Monoclonal antibody isotypes were determined with the ScreenType kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Indirect ELISA

An indirect ELISA (*1*) was used to determine titer, sensitivity, and specificity of anti chicken muscle LDH antibodies in mice sera or in culture following fusion and cloning. For titer determination, microtiter plates were coated overnight (4°C) with 100 μl chicken LDH (3 μg/ml) in 0.1 M carbonate buffer (pH 9.6). Plates were washed four times with 0.01 M PBS, pH 7.2, containing 0.05% Tween 20 (PBS-Tween). Three hundred milliliters 1% BSA (wt/vol) in PBS (PBS-BSA) was added to each well and incubated at 37°C for 30 min to minimize nonspecific binding. After washing four times with PBS-Tween, 50 μl of serially diluted serum were added to each well and incubated for 1 h at 37°C. Unbound antibody was removed by washing four times with PBS-Tween, and 100 μl of goat anti-mouse IgG peroxidase conjugate (1:500 in 1% BSA-PBS) was added to each well. Plates were incubated for 30 min at 37°C, washed eight times with PBS-Tween, and bound peroxidase was determined with ABTS substrate as described by Pestka et al. (*17*). Absorbance was read at 405 nm using a Minireader II (Dynatech), and the titer of each serum was arbitrarily designated as the maximum dilution that yielded at least twice the absorbance of the same dilution of nonimmune control serum.

The competitive indirect ELISA was essentially identical with titer determination, except that, after BSA blocking and washing, 50 μl of standard LDH or meat extract was added to each well simultaneously with 50 μl of the appropriate dilution of LDH antisera or 50 μl of cell culture supernatant.

Sandwich ELISA

The sandwich ELISA was performed by coating microtiter wells with 125 μl of monoclonal or polyclonal LDH antibody diluted in 0.1 M carbonate buffer (pH 9.6) and drying overnight at 40°C in a forced air oven. Wells were washed four times with PBS-Tween and remaining protein binding sites were blocked with PBS-BSA (300 μl per well) for 30 min at 37°C. Muscle extracts or LDH diluted in PBS-BSA (100 μl) were added to each well and incubated for 1 h at 37°C. Plates were washed four more times with PBS-Tween and 100 μl of monoclonal or polyclonal LDH antibody diluted in PBS-BSA was added. After incubation for 1 h at 37°C and washing four times with PBS-Tween to remove unbound second antibody, 100 μl of either goat anti-rabbit IgG (when polyclonal antibody was used as second antibody) or goat anti-mouse IgG (when monoclonal antibody was used as second antibody) peroxidase conjugate diluted (1:500) in PBS-BSA were added to each well and incubated for 30 min. Plates were then washed eight times with PBS-Tween, and bound peroxidase was determined as described above.

Thermal processing and extraction of turkey rolls

Turkey rolls were commercially prepared and formulated using 45.36 kg turkey breast meat, 8.62 kg water, 1.27 kg modified starch, 0.68 kg salt, 0.27 kg sugar, and 0.23 kg Na tripolyphosphate. Each roll weighed about 3.63 kg and measured 10.16-cm diameter by 35.56-cm length. Turkey rolls were smokehouse processed to final internal target temperatures of 68.9, 70.0, 71.1, and 72.1°C by monitoring product temperature throughout the cooking and cooling cycle (*28*).

Extracts from raw and processed turkey products were prepared by cutting 25 g meat from the geometric center of each product, homogenizing with 75 ml cold 0.15 M NaCl, 0.01 M Na

phosphate buffer, pH 7.2 in a Waring Blender for 90 s, followed by centrifugation at 16,000 x g for 20 min at 4°C. The supernatant was filtered through Whatman No. 1 filter paper and protein concentration determined by Biuret method (*28*). Extracts were diluted in PBS-BSA for ELISA.

Statistics

Turkey rolls were processed in triplicate using three separate smokehouse runs. Five commercial roasts and hams were evaluated in triplicate. Basic statistics and 2-way analysis of variance (treatment x replication) were performed using MSTAT software (version C, 1989, Michigan State University, East Lansing).

RESULTS AND DISCUSSION

Subcutaneous injection of mice with 50 μg chicken muscle LDH resulted in the highest end point titer (409,600) and was more efficient in eliciting sensitive antibodies than that induced by intraperitoneal injection or by subcutaneous injection of lower doses (10 and 25 μg per mouse)(Table 1). Spleens from two of these mice were used for fusion with NS-1 cells. Of 650 wells exhibiting hybridoma growth, 7% produced antibody against LDH. After further subcloning, four stabilized subclones designated E6B-F8-G4 (E6B), D5E-G8-E4 (D5E), B3C-B8-F3 (B3C), and G4D-D9-G4 (G4D) were chosen for further characterization and monoclonal antibodies mass production. All were IgG_1 with kappa light chains.

TABLE 1. *Antibody titers to chicken muscle LDH by indirect ELISA after three immunizations.*

Mouse group	Dose (μg)	Injection mode[a]	No. of mice	Responding mice	Titer (range)
1	10	ip	5	1	3200 (3200)
2	25	ip	5	3	11200 (1600-25600)
3	50	ip	5	5	25600 (1600-102400)
4	10	sc	5	2	7200 (1600-12800)
5	25	sc	5	4	25600 (12800-51200)
6	50	sc	5	5	136200 (3200-409600)

[a]ip = intraperitoneal; sc = subcutaneous.

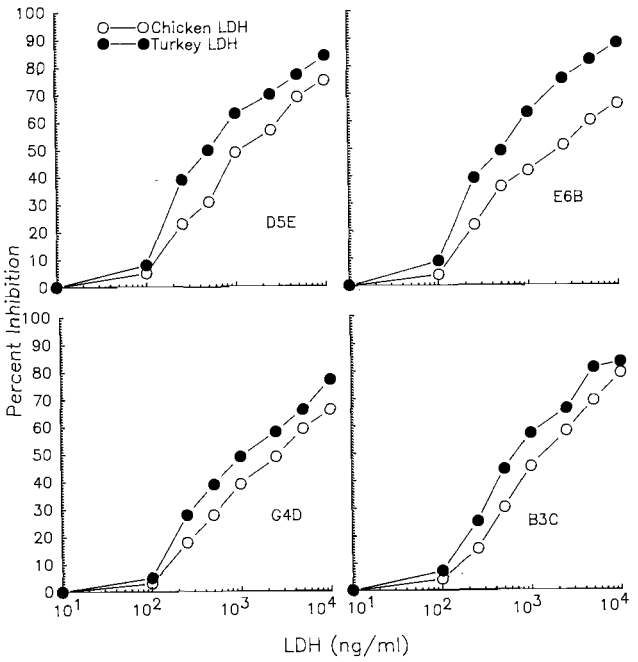

Figure 1. *Detection of chicken and turkey LDH by competitive indirect ELISA using chicken LDH monoclonal antibodies (MAbs). Optimum dilution for MAbs were D5E (1:1000), E6B (1:200), G4D (1:35), and B3C (1:25).*

Typical standard curves for determination of LDH by competitive indirect ELISA using monoclonal antibodies from four cell lines are shown in Fig. 1. The detection limit of LDH by competitive, indirect ELISA using monoclonal antibodies was 100 ng/ml. Although the four cell lines were developed against chicken muscle LDH, they showed significant cross-reactivity with turkey muscle LDH. Cross-reactivity with LDH from chicken heart, porcine heart, porcine muscle, bovine muscle, bovine heart, and rabbit muscle at concentrations up to 50 μg/ml was not detected (data not shown).

To improve sensitivity, a sandwich ELISA was used whereby the microtiter plate was first coated with capture antibody then incubated sequentially with (a) standard and tigen or sample extract, (b) detector antibody that was generated against the same antigen, and (c) antispecies peroxidase conjugate. In this assay, the detector antibody can bind to the antigen either symmetrically, in which it is specific for the same determinant as the capture antibody or asymmetrically whereby detector antibody recognizes a different epitope on the multivalent antigen (*2*). The standard curve obtained when monoclonal (D5E) antibody was used as capture antibody and polyclonal antibody (*RF*) used as detector antibody was more than 100-fold more sensitive (Fig. 2B) than that obtained when polyclonal antibody was used as capture antibody and monoclonal antibody as detector antibody (Fig. 2A). Immunocapture by a polyclonal antibody may have covered most sites leaving few available epitopes for monoclonal antibodies when used as detecting antibodies (*27*). However, when immunocapture was performed with a monoclonal antibody, several epitopes were left free for binding with polyclonal antibodies as the detecting antibody. Of the four cell lines secreting monoclonal antibodies for chicken muscle LDH, D5E was the most effective capture antibody.

The sensitivity and specificity of the sandwich ELISA using detector antibodies prepared against chicken or turkey LDH were compared using LDH from chicken muscle, turkey muscle, rabbit muscle, bovine muscle, bovine heart, porcine muscle, porcine heart, and chicken heart. The standard curve was approximately linear from 1 to 25 ng/ml for chicken or turkey LDH. At concentrations of 1000 ng/ml or higher, chicken heart LDH also showed slight reactivity. Cross-reactivity was not observed for any of the other LDHs tested. In general, turkey LDH bound to capture and detector antibodies more effectively than chicken LDH. Of the six polyclonal antibodies (three prepared against chicken LDH and three prepared against turkey LDH) tested, the most sensitive assay was generated using polyclonal antibodies from rabbit serum injected with turkey LDH (R-F) as the detector antibody (Fig. 3).

Maximum internal processing temperatures of turkey rolls were 68.3, 69.7, 70.9, and 72.1°C which corresponded to target temperatures of 68.9, 70.0, 71.1, and 72.1°C, respectively. To use LDH as an indicator to detect proper

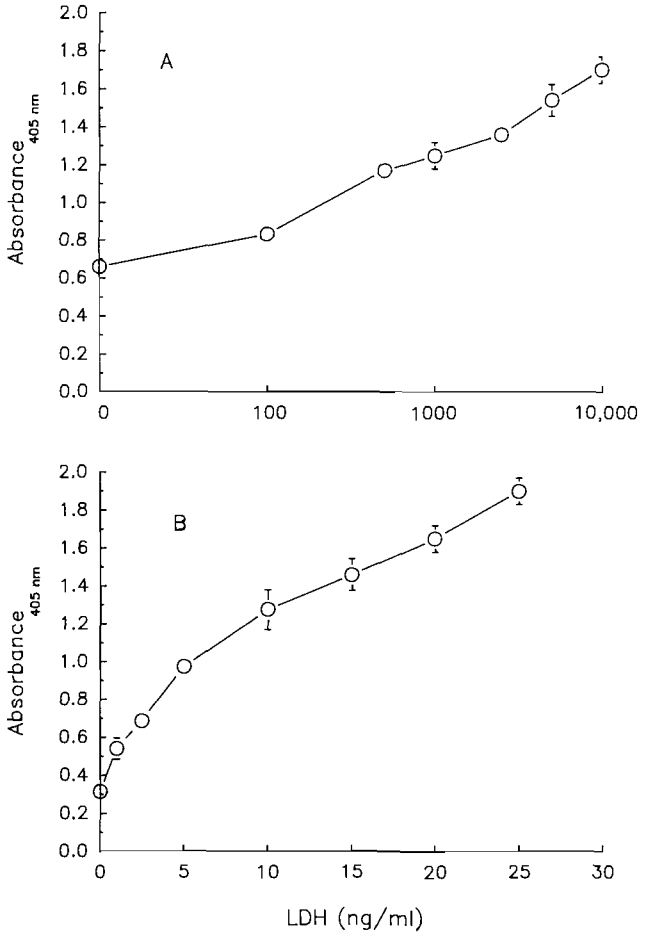

Figure 2. *Detection of chicken muscle LDH by sandwich ELISA. A: Polyclonal antibody prepared against turkey muscle LDH (R-F) was used as capture antibody. Detector antibody was monoclonal antibody D5E prepared against chicken muscle LDH. B: Capture antibody was monoclonal antibody D5E (1:450 dilution) and detector antibody was polyclonal antibody R-F. Bars indicate standard error of the mean.*

endpoint cooking of uncured poultry products, the enzyme must be insoluble in the extraction buffer or undergo a conformational change such that it is not recognized by the antibodies at or near the USDA-FSIS required temperature (71.1°C). The LDH content of extracts decreased as internal processing temperature of turkey rolls was increased (Fig. 4). The LDH content of extracts diluted 3- to 6-fold decreased below 15 ng/ml for turkey rolls processed to 70.9 and 72.1°C. At a 6-fold dilution, LDH content of extracts from turkey rolls processed to 69.7°C was approximately 10 times greater than those processed to 70.9 and 72.1°C.

Stadler et al. (*18*) reported that LDH activity decreased significantly when bovine muscle extracts were heated to 63°C, and only slight activity was detected at 66°C. Marin et al. (*15*) used an indirect ELISA to monitor antigenicity in heated salt soluble beef protein extracts. The authors reported that 50 and 70% of proteins lost antigenicity when heated to 70 and 100°C, respectively, indicating conformational changes in protein structure with heat. Collins et al. (*4*) reported a decrease in LDH activity in extracts of whole muscle ham as heating temperature of 11 to 15-g samples was increased from 65 to 71°C in a water bath. Decreased activity was attributed to heat denaturation of the enzyme and/or from decreased quantities of extractable protein.

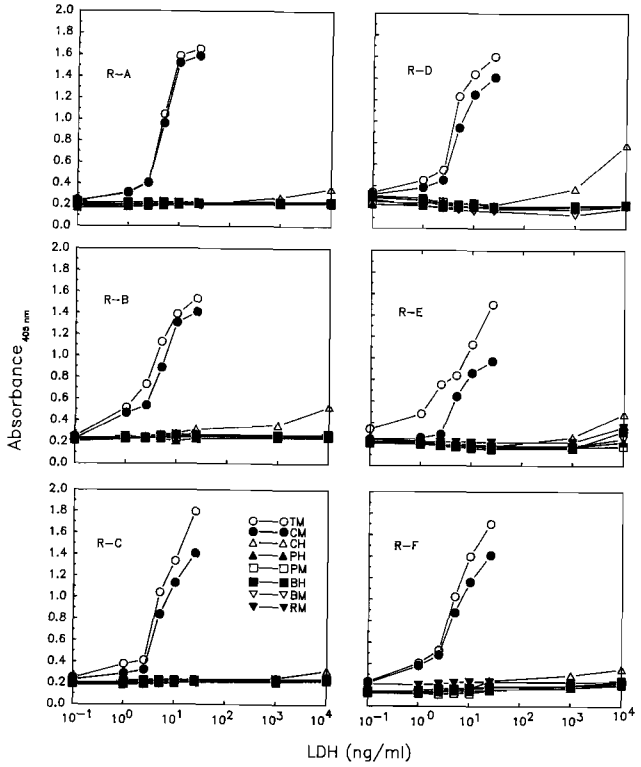

Figure 3. *Specificity of sandwich ELISA for LDH. LDH sources were turkey muscle (TM), chicken muscle (CM), chicken heart (CH), porcine muscle (PM), bovine muscle (BM), bovine heart (BH), and rabbit muscle (RM). Plates were coated with monoclonal antibody D5E for capture. Polyclonal detector antibodies were R-A, R-B, R-C prepared against CM LDH or R-D, R-E, R-F prepared against TM LDH.*

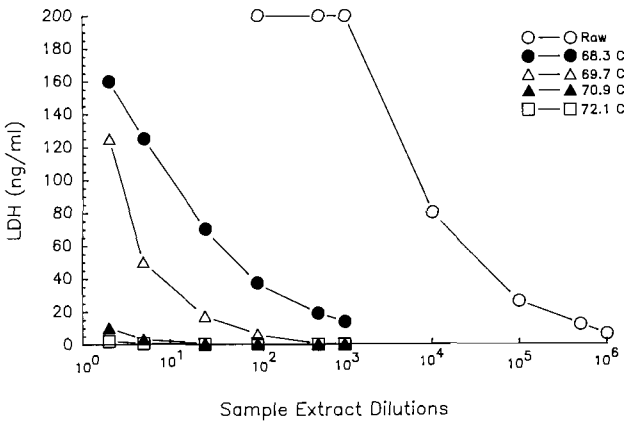

Figure 4. *Effect of processing temperature on LDH concentration of turkey roll extracts as measured by sandwich ELISA. Standard error of the means are ± 2.51, 4.19, 2.29, 0.64, and 0.13 for raw, 68.6, 69.7, 70.9, and 72.1°C endpoint temperature treatments, respectively.*

Similarly, Collins et al. (*5*) observed a decrease in LDH activity in extracts from bovine top round muscle heated in a water bath from 4 to 66°C.

Roast turkey breast and turkey ham obtained from different supermarkets were tested by sandwich ELISA for extract LDH content (Fig. 5). When extracts were diluted 3- to 6-fold, LDH content averaged less than 2 ng/ml in turkey breast as compared to 20-25 ng/ml in turkey ham extracts of the same dilution. The detection of greater quantities of LDH in turkey ham may be due to the lower

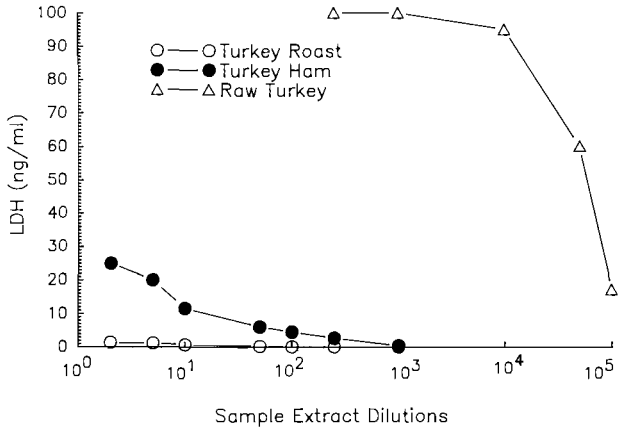

Figure 5. *Detection of LDH in commercially available precooked turkey roasts and hams by sandwich ELISA. Standard error of the means are ± 0.05, 0.99, and 2.56 for roast, ham, and raw turkey breast, respectively.*

USDA-FSIS required minimum internal processing temperature of 68.3°C for cured poultry products (*23*). Also, differences in heat stability of LDH from red and white muscles has been reported (*21*).

The sandwich enzyme immunoassay developed in this study should be suitable for verifying safe processing temperatures of uncured poultry products. The sandwich ELISA showed higher sensitivity than that obtained with competitive indirect ELISAs employing LDH polyclonal antibodies or LDH monoclonal antibodies separately. A further advantage is that the sandwich technique did not require purified LDH to coat microtiter plates as in the indirect ELISA. This immunological approach might be routinely used to detect LDH in uncured poultry products for verification of endpoint cooking temperatures due to its specificity, low cost, and ease of handling. Since this assay is very sensitive for turkey and chicken LDH (detection limit is less than 1 ng/ml) with no cross-reactivity with LDH from other animal sources, it can also be used to detect adulteration of other meats with chicken or turkey.

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