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ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETERMINATION OF ENDPOINT PROCESSING TEMPERATURES IN UNCURED POULTRY PRODUCTS

presented by

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By

Cheng-Hsin Wang

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ABSTRACT

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETERMINATION OF ENDPOINT PROCESSING TEMPERATURES IN UNCURED POULTRY PRODUCTS

BY

Cheng-Hsin Wang

Enzyme-linked immunosorbent assay (ELISA) was developed for determining if precooked poultry products have been processed to the required endpoint heating temperature of 71.1 °C. Electrophoresis of extracts from cooked turkey breast rolls revealed a 35,000-Da band which disappeared at 70.9 °C. This band was identified as lactate dehydrogenase (LDH). Polyclonal antibodies (PAbs) against turkey muscle LDH and chicken muscle LDH, and four monoclonal antibodies (MAbs) against chicken muscle LDH were produced. Turkey muscle LDH PAbs cross-reacted with chicken muscle LDH and vice versa, but not with LDH from bovine, porcine or rabbit. Chicken muscle LDH MAbs cross-reacted with turkey muscle LDH but not with LDH from other species.

The LDH content in turkey breast roll extracts as determined by ELISA decreased as the cooking temperature

increased. ELISA accurately differentiated the endpoint cooking temperature within ±1.1-1.2 °C in the temperature range 68.3-72.1 °C. Salt concentration, cooking schedule and product casing diameter of turkey breast rolls did not have a marked influence on ELISA in determining endpoint cooking temperature. A maximum concentration of 0.31 μg LDH/g meat was found to indicate proper processing of turkey breast rolls.

LDH ELISA could not differentiate endpoint temperature of turkey thigh rolls processed between 68.9 and 71.1 °C due to the presence of heat stable LDH isozymes. The intensity of a 66,000 Da-band, identified as turkey serum albumin, decreased in turkey thigh roll extracts as processing temperature increased. Serum protein ELISA differentiated between products cooked to the minimum endpoint temperature of 71.1 °C and those processed 2.2 °C below the minimum.

Relative epitope binding positions on turkey muscle LDH for four MAbs was determined. Epitopes for MAbs B3C and G4D were separated from D5E by a considerable distance. The LDH MAb sandwich ELISA detected 1% adulteration of turkey breast or thigh muscle in raw beef and pork using D5E as capture MAb and biotin-labeled B3C as detector MAb.

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CHAPTER ONE : INTRODUCTION

Precooked meat products are processed to minimum endpoint temperatures as required by federal regulations to ensure the destruction of pathogens that cause diseases in humans and livestock. Besides possible mortaility, food borne illness can have many undesirable economic implications, mainly increased health care costs, lost work time and reputation. Todd (1989) estimated that food borne illness caused by *Salmonella* and *Staphylococcus* costs approximately $4 billion per year in the U.S.

Current methods used by the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) to verify endpoint cooking temperatures of beef and pork products are slow, empirical and need improvement (Townsend and Blankenship, 1989). Precooked uncured poultry products are required to be cooked to an internal temperature of 71.1 °C (USDA-FSIS, 1992); however, the USDA does not have a standard assay for verifying processing temperatures in poultry.

Assays have been developed to assess the endpoint cooking temperatures for meat products and include protein solubility (Davis and Anderson, 1983), color (Favetto et al., 1988; Ang and Huaung, 1992), electrophoresis of

protein extracts (Steele and Lambe, 1982), residual enzyme activity (Bogin et al., 1992), chromatography (McCormick et al., 1987), differential scanning calorimetry of muscle protein (Ellekjaer, 1992) and near infrared spectroscopy (Isaksson et al., 1989). These assays are very time-consuming, require a large number of reagents and/or sophisticated scientific equipment or not accurate and thus have not been adopted for widespread use.

Development of a rapid and accurate assay for endpoint cooking temperatures is highly desirable. Government regulatory agencies, and poultry processors can use such assay to check endpoint cooking temperatures of products and assure safety of their products and those purchased from suppliers.

Enzyme-linked immunosorbent assays (ELISAs) are now being used to detect adulteration, additives, micro-organisms, mycotoxins, pesticides, drugs, and other constituents in foods (Rittenburg, 1990; Samarajeewa et al., 1991). ELISA is recognized as a specific, sensitive, and simple immunoassay.

The purpose of this research was to develop ELISAs to accurately determine if processed poultry products have been cooked to the proper USDA endpoint heating temperature. Detection of soluble marker or indicator proteins will be the basis of a suitable ELISA that is easy to operate and requires no specific equipment to detect the endpoint cooking temperature of poultry products.

The present research was divided into five studies, and the objectives of each study were:

Study I -- (a) to identify and isolate a marker protein which denatures at or near the minimum heating endpoint of processed poultry products as regulated by the USDA-FSIS, (b) to produce polyclonal antibodies for the marker protein, and (c) to develop an indirect competitive ELISA to detect the marker protein for monitoring endpoint cooking temperatures.

Study II -- (a) to produce monoclonal antibodies for the marker protein, and (b) to develop a sandwich ELISA to detect the marker protein for monitoring endpoint cooking temperatures.

Study III -- to verify the ELISA for uncured turkey breast meat products under a range of commercial product formulations, processing conditions, and storage conditions in pilot studies.

Study IV -- (a) to verify the ELISA for an uncured turkey thigh meat product, and (b) develop and test ELISA using other marker proteins using commercially available antibodies to turkey serum proteins and immunoglobulin G.

Study V -- (a) to determine the relative position of monoclonal antibody epitopes on the marker protein, and (b) to design an effective monoclonal-based sandwich ELISA for detecting poultry in beef and pork products.

CHAPTER TWO : LITERATURE REVIEW

2.1 Regulations for Endpoint Cooking Temperature of Meat Products

Title 9 of the Code of Federal Regulations requires thermal treatment for meat products to ensure the destruction of harmful microorganisms and viruses. The possibility of food borne disease outbreaks from precooked meats contaminated by pathogens such as *Salmonella*, *Escherichia coli* O157:H7, *Staphylococcus,* and *Listeria monocytogenes* is a concern of meat processors, consumers and the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS).

Uncured poultry rolls and cured/smoked poultry products are required to be heated to an internal temperature of 71.1 and 68.3 °C, respectively (USDA-FSIS, 1992). Cooked beef and roast beef are required to be cooked using one of the 16 temperature/time combinations, e.g., heated to 54.4 °C and held for 121 min or to 62.8 °C without holding (USDA-FSIS,1985a). The USDA-FSIS (1987) requires cured/smoked and ready-to-eat products containing pork to be heat processed to an internal temperature of at least 58.3 °C to destroy *Trichinae*. Imported canned pork

products are required to be cooked to an internal temperature of 68.9 °C.

The outbreak of foodborne disease in mid-January 1993 resulted in new cooking standards for hamburger (Mermelstein, 1993). The cause of the outbreak was traced to the consumption of undercooked *E. coli* O157:H7-contaminated hamburgers served at Jack-in-the-Box restaurants in Washington, Idaho, California, and Nevada. The regulatory agencies and industry groups recommended that foodservice operators cook ground meat to an internal temperature of 68.3 °C (155 °F) instead of 62.8 °C (145 °F), and the middle should be light gray or brown and the juices should run clear with no trace of pink (Mermelstein, 1993). Moreover, the USDA-FSIS reached a consensus on cooking temperatures for beef patties in August 1993. The agencies agreed on a cooking temperature of 69.4 °C (157 °F) for federal establishment, food handlers and retailers (Anonymous, 1993a).

2.2 Assays for Assessing Endpoint Cooking Temperature

A. Protein Solubility, Electrophoresis and Chromatography

Thermal processing of meat results in denaturation of protein and loss of protein solubility. Davis and Anderson (1983) and Davis et al. (1987) reported that protein extractability decreased as temperature increased when measured by biuret method. However, there was little

change in protein solubility between 67.5 °C and 75 °C in porcine muscle (Davis and Anderson, 1983) and between 65.6 and 68.8 °C in canned pork picnic shoulder (Davis et al., 1987).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of meat extracts showed that the number or intensity of protein bands decreased as the processing temperature increased (Lee et al., 1974; Crespo and Ockerman, 1977; Caldironi and Bazan, 1980; Steele and Lambe, 1982). For example, Caldironi and Bazan (1980) reported a decrease in the intensity of a 55,000-Da band in beef extracts between 68 and 72 °C. Steele and Lambe (1982) found a 56.6-Da band disappeared between 65 and 70 °C in chicken muscle extracts. King (1978) used isoelectric focusing of myofibrillar proteins from raw and cooked sheep muscle, and reported that measurement of relative staining intensities of the various actin components may provide a suitable method for estimating the severity of heat treatment.

Davis and Anderson (1984) used size exclusion high performance liquid chromatography to study the effect of heating temperature and time on the chromatographic profiles of water soluble proteins extracted from bovine and porcine muscle tissue. They reported that the loss of protein solubility of water soluble or sarcoplasmic proteins was related to heating time, endpoint temperature and initial concentration of soluble proteins in muscle.

McCormick et al. (1987) evaluated the use of reverse phase high performance liquid chromatography to separate and quantify water soluble proteins extracted from heated porcine muscle. Their results showed that the concentration of aldolase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase decreased dramatically between 60 and 65 °C; the lactate dehydrogenase content decreased rapidly between 65 and 70 °C.

B. Residual Enzyme Activity

Townsend and Blankenship (1988, 1989) evaluated a commercially available enzyme detection kit (APIZYM, Analtab Products) on raw and heat processed meat and poultry products. The APIZYM is a semi-quantitative micromethod designed for the detection of 19 individual enzymes. Only a minimum amount of leucine aminopeptidase activity could be detected for samples heated to internal temperatures of 71 °C. They suggested that leucine aminopeptidase could be used as marker to determine the endpoint temperature of meat products.

Lactate dehydrogenase (LDH) activity in bovine muscle could be used as a heating endpoint indicator (Stalder et al., 1991; Collins et al., 1991a). Stalder et al. (1991) reported that LDH activity in bovine tissue slurries decreased dramatically at a temperature close to 63 °C at pH 5.6 and 6.4. They also found that LDH activity decreased as salt and phosphate concentration increased.

Carcasses did not differ in LDH activity by sex, but showed a decrease in activity as maturity increased. Collin et al. (1991a) showed that a major portion of LDH activity in bovine muscle was lost upon heating to 63 °C, and only marginal activity was detectable at 66 °C.

Collins et al. (1991b) measured LDH activity in raw, cured, and heated porcine muscle. Their results showed that cured meat lost almost all LDH activity at an endpoint temperature of 63.8 °C regardless of the holding time. They concluded that since the loss of LDH activity in cured meat was well below 68.9 °C, this enzyme was unsuitable as a marker for cured, canned hams.

Townsend and Davis (1991) reported that LDH activity decreased markedly in ground beef and chicken breast as internal temperature increased from 66.7 to 67.8 °C, and decreased gradually as temperature increased form 67.8 to 71.1 °C. They also found that LDH turkey thigh meat was not greatly affected by endpoint temperature or dwell time.

Bogin et al. (1992) screened 12 enzymes extracted from turkey breast muscle heated to 50-70 °C and found that the activities of creatine kinase, malic dehydrogenase, isocitric dehydrogenase and LDH were good markers for evaluating heat treatment. Their results also showed that protein solubility could serve as a good marker, and suggested using both soluble protein and enzyme activity as verification markers for heating meat.

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Hsu et al. (1993) screened 25 enzymes extracted from turkey *Pectoralis major* and *Sartorius* muscles. They found that LDH and malic dehydrogenase from *Pectoralis major* showed medium activity at 65 °C and no activity at 75 °C, and suggested that these two enzymes might have potential as heating endpoint indicators.

Townsend and Davis (1992) studied glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activity in ground beef. They reported that GOT and GPT activity could not be used for determining the endpoint cooking temperature of domestic meat products, but the GOT assay could possibly be used for determining the adequacy of heat treatment of imported cooked beef which must be heat processed to 79.4 °C. Ang et al. (1993) reported that a catalase test could be used to determine endpoint temperature between 69 and 71 °C for precooked chicken and leg meat.

Townsend et al. (1993) evaluated the "COBAS Ready" (Roche Diagnostics Systems) dry chemistry enzyme system for chicken breast, chicken patties, chicken nuggets and cured turkey products cooked to 66.7-71.1 °C. This enzyme system is used to assay three enzymes (aspartate aminotransferase, creatine kinase, and LDH) simultaneously. Their results indicated that the residual enzyme activity was different among poultry products and concluded that the use of the "COBAS" system was product dependent.

C. Differential Scanning Calorimetry and Near Infrared Spectroscopy

Differential scanning calorimetry (DSC) has been used to assess previous heat treatment of meat products (Parsons and Patterson, 1986; Bowers et al., 1987; Ellekjaer, 1992). Bowers et al. (1987) showed that the patterns in the DSC thermogram of beef muscle previously heated to 55, 65, 75 and 85 °C were different. Parson and Patterson (1986) found a correlation between maximum cooking temperature and the onset of denaturation from DSC analysis of heat treated beef. The onset temperature of denaturation was determined from the first slope of the thermogram and its intersection with the base line. However, it was found that the determination of the onset temperature was very subjective (Ellkejaer, 1992). Thus, Ellkejaer (1992) used multivariate statistics to analyze the full DSC-thermogram and reported that the DSC method was able to determine the previous heat treatment temperature of the beef samples with a prediction error of 0.6 °C in the temperature range 50-72 °C. The author also showed that the thermograms of samples previously heat treated to 75 °C and higher temperatures were featureless, because the meat proteins were irreversibly denatured; no prediction of heating temperature could be made from DSC data for these samples.

Isaksson et al.(1988) and Ellekjaer and Isaksson (1992) used near infrared spectroscopy to determine the

temperature of previous heat treatment in minced beef. They indicated that changes in the spectra upon heat treatment might be related to changes in the local environment of the N-H and C=O groups in meat proteins. This was due to denaturation of proteins and changes of state and binding of water to the proteins. The near infrared spectroscopy methods were able to determine the maximum temperature of previously heat treated beef with a prediction error of 2.0-2.1 °C in the temperature range 50-85 °C.

D. Color

Bowers et al. (1987) reported that significant color changes of beef muscle occurred between 60 and 65 °C, and between 75 and 80 °C. Ang and Huang (1994) studied the effect of heat treatment on color changes of chicken leg patties. They found that lightness ("L") and yellowness ("b") increased whereas redness ("a") decreased as end-point temperatures increased; however, color values of cooked products were influenced by packaging method and storage time.

Favetto et al. (1988) developed a color-changing indicator to monitor the time-temperature history during cooking of meats. This system was based on color development by Maillard reaction between reducing sugars soaked on the indicator and lysine from meat. The indicator was placed on the surface of the meat package,

thus eliminating the need of opening the package for inspection.

Another kind of time-temperature indicator has been developed based on the color change of the polymer band due to the polymerization of acetylenic monomer that gradually blackens during time and temperature changes (Singh and Wells, 1986, Taoukis et al., 1991). This kind of time-temperature indicator is often fixed on isolated individual packages and can be read with a scanner.

E. Formation of Indicator Compounds during Processing

Monitoring compounds intrinsically produced from foods during thermal processing provides an alternative method for assessing the integrated time-temperature exposure of foods. It has been reported that three compounds, M-1, M-2 and M-3, were formed in aseptically processed foods (Kim and Taub, 1993). M-1 was observed in heated meat and vegetable products. M-2 was found primarily in heated meats. M-3 was detected in heated fruits and vegetables. The formation of M-1 was from direct degradation of D-fructose, by a Maillard reaction, or by a combination of both. M-1 was identified as 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pryan-4-one (MW=144). Meat proteins and a water-soluble component might be involved in the formation of M-2. The molecular weight of M-2 is similar to M-1. Kim et al. (1993) studied the kinetic characteristics of M-1 and M-2

formation for 0.5 inch meat cubes heated at 121 °C for up to 15 min. They found that the M-2 concentration increased exponentially to a limit value with a first-order rate constant of approximately 0.7 sec^{-1}. The M-1 concentration increased slowly initially, more rapidly between 2 and 6 min, then almost linearly between 6 and 15 min. A linear relationship was observed as the ratio of the M-1 concentration over the M-2 concentration was plotted against the heating time.

Cambero et al. (1992) found a correlation between cooking temperature of beef heated at 55-95 °C and the concentration of free amino acids and inosine 5'-monophosphate in the beef broth. Townsend et al. (1992) reported that analysis of free amino acids and Maillard reaction products for chicken breast processed to 60-80 °C was not successful due to the presence of unknown compounds in the chicken extracts which were not readily separated from the compounds of interest.

F. Current Methods Used by Regulatory Agencies

Presently, the USDA-FSIS uses a residual phosphatase assay (USDA-FSIS, 1986a), bovine catalase test (USDA-FSIS, 1989), or a coagulation test (USDA-FSIS, 1986b) to verify endpoint cooking temperatures of beef and pork.

The acid phosphatase activity method is based on the residual activity of the enzyme after cooking and is expressed as the μmole of phenol formed per 1000 g sample

after reacting with the substrate, disodium phenylphosphate, for 60 min at 37 °C, pH 6.5. The activity is used in a mathematical formula to estimate internal cooking temperature. Processing method, muscle type and ingredients all affect phosphatase activity (Townsend and Blankenship, 1989).

The bovine catalase test is performed by subjectively observing the appearance of foam when a meat sample is immersed in a solution of hydrogen peroxide and shampoo. Although this assay is fairly rapid, errors can occur if the peroxide reagent is weak or inactive, if the analyst does not carefully observe the appearance of extra foam formed by the catalase reaction or if catalase activity is low (USDA, 1989).

The coagulation test measures the loss in protein solubility with temperature when a meat product is thermally processed. The soluble proteins are extracted in 0.9% NaCl, filtered, heated and the temperature at which the first signs of turbidity appear (54-57 °C) is recorded. This temperature is usually within 2 to 3 °C of the endpoint processing temperature. Because visible observation is involved in this test, this test is considered to be empirical and subjective in nature (Townsend and Blankenship, 1989).

2.3 Immunoassays In Food Science

A. Antibody Production

Immunoassays provide sensitive and specific analytical techniques based on the specific and high affinity antigen-antibody reaction. Production of a suitable antibody is an important step in the development of an immunoassay. This is done by immunizing animals with antigen. A variety of macromolecules (molecular weight >10,000) can be used as antigens, almost all proteins, many polysaccharides, nucleoproteins, lipoproteins, and polypeptides (Roitt et al., 1989; Golub and Green, 1991). Haptens are small molecules that by themselves do not induce the production of antibody but are capable of reacting with antibody; when the hapten is conjugated to a carrier (a large molecule such as a protein), the animal responds by producing antibodies both to the hapten and carrier (Roitt et al., 1989; Golub and Green, 1991). Anti-hapten antibody, induced by hapten-carrier conjugate, can react with free hapten. Mycotoxins, pesticides and drugs are the examples of haptens. Bovine serum albumin is the protein most frequently used as the carrier; gamma globulin and polylysine have also been used (Chu, 1984).

Polyclonal antibodies are usually obtained from the serum of a wide range of vertebrate species after several injections of the antigen. Rabbits, mice, rats, hamsters,

and guinea pigs are the five most commonly used laboratory animals; horses, pigs, sheep and donkeys are used commercially for production of large volume of antisera (Harlow and Lane, 1988). Antiserum is said to be "polyclonal", because its immunoglobulins are derived from multiple clones and vary in affinity and specificity (Pestka, 1988). In contrast to polyclonal antibodies, monoclonal antibodies are produced by cloning a single B-lymphocyte, and therefore have identical specificity (Kohler and Milstein, 1975). Monoclonal antibodies are produced by the fusion of spleen cells from an immunized mouse with a myeloma cell line by the addition of polyethylene glycol which promotes membrane fusion (Fazekas de St Groth and Scheidegger, 1980; Roitt et al., 1989). The fusion mixture is then grown in culture with medium containing "HAT". HAT is a mixture of hypoxanthine, aminopterin and thymidine. Aminopterin is a powerful toxin which blocks a metabolic pathway. This pathway can be bypassed if the cell is provided with the intermediate metabolites hypoxanthine and thymidine. Thus, spleen cells can grow in HAT medium, but the myeloma cells die in HAT medium because they have a metabolic defect and cannot use the bypass pathway. The spleen cells die in culture naturally after 1-2 weeks. Only fused cells can survive because they have the immortality of myeloma cells and the metabolic bypass of the spleen cells. After screening and cloning steps, hybridoma cell

lines secreting monoclonal antibodies are produced.

B. Types of Immunoassays Commonly Used in Food Science

(1) Immunodiffusion

The immunodiffusion test relies on the formation of antigen-antibody precipitates at or near the equivalence zones via diffusion in the agar gels (Roitt et al., 1989). This test has been used in species identification for meat products (Mageau et al., 1984; AOAC, 1990). Immunodiffusion tests are simple to set up and require no expensive equipment. However, immunodiffusion tests have several disadvantages. These tests have low sensitivity and require the use of large quantities of antigens and antibodies, the tests may take several days for the weak precipitin lines to develop and be visualized by special staining techniques, and precipitin lines from immunologically different components may be superimposed upon each other (Kang'ethe, 1990).

(2) Immunoblotting

Immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection (Harlow and Lane, 1988). The immunoblotting procedure involves the separation of protein samples by gel electrophoresis, transfer of the separated polypeptides to a membrane support (such as nitrocellulose or nylon), and detection with antibodies. This technique

has been used to identify specific polypeptides from different oat varieties and to determine the molecular weight of these polypeptides (Zawistowski and Howes, 1990).

(3) Enzyme-linked immunosorbent assay (ELISA)

ELISA is probably the most rapidly growing and widely used immunological technology in food science, and recognized as specific, sensitive, effective and simple immunoassay (Rittenburg, 1990; Fukal, 1991; Samarajeewa et al., 1991; Morgan et al., 1992). ELISA often reduces the use of expensive equipment and analysis time as compared to gas chromatography and high performance liquid chromatography. This technology is based on the binding of antigen or antibody to a solid surface and monitoring the reaction with enzyme labeled antibody or antigen. Enzyme reaction with colorless substrate results in color development, which can be measured spectrophotometrically or by visual comparison with standards.

ELISA is an extension of radio-immunoassay (RIA), which uses radioisotopes such as ^{3}H, ^{14}C and ^{125}I as the antibody or antigen label (Rittenburg, 1990). Although RIA is still commonly used in clinical diagnostics, this technology has several major disadvantages for food and agricultural analysis. Labeling of the antibody or antigen with radioisotopes is difficult and expensive, the shelf life of isotopically labeled antibody or antigen is

generally short, a scintillation counter is expensive, and disposal of radioactive waste is a problem (Pestka, 1988; Rittenburg, 1990).

Polystyrene or polyvinylchloride microtiter plates, nitrocellulose membrane, diazotized paper, and activated beads have been used as solid supports in ELISA (Harlow and Lane, 1988). The use of 96-well microtiter plates enable ELISA to screen a large number of samples at the same time.

Horseradish peroxidase and alkaline phosphatase are most commonly used as enzyme labels; ß-galactosidase, urease and glucose oxidase have also been used (Harlow and Lane, 1988). Glutaraldehyde, periodate, and maleimido-bensoyl-*n*-hydroxy-succinimide ester coupling procedures are some commonly used methods to conjugate enzymes to antibody and antigen (Harlow and Lane, 1988). Substrates for peroxidase include *o*-toluidine, 5-amino-salicylic acid, and 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS); ABTS is the most convenient chromogen because of its high sensitivity and stability (Chu, 1984). Alkaline phosphatase is frequently used with *p*-nitro-phenyl phosphate or the bromochloroindolyl phosphate-nitro blue tetrazolium substrate (Harlow and Lane, 1988).

Direct competitive ELISA, indirect competitive ELISA, and double antibody sandwich ELISA are frequently used in food analysis (Rittenburg, 1990). In the direct competitive ELISA (Figure 2.1), antibody is bound on the

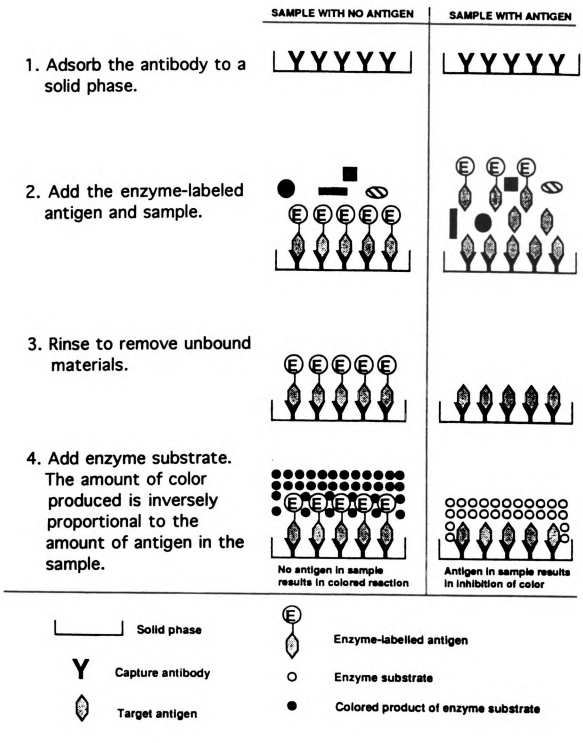


Figure. 2.1. Direct competitive ELISA.
(Adapted from Rittenburg, 1990)

solid surface. Enzyme labeled antigen (or enzyme labeled hapten) is incubated simultaneously with the sample. Any antigen (or hapten) in the sample will compete with enzyme labeled antigen (or enzyme labeled hapten) for the antibody binding sites. Thus, more antigen in the sample, less enzyme-labeled antigen will be bound on the solid phase antibody. The bound enzyme-labeled antigen is determined by the color formation via enzyme and substrate reaction. The amount of color is inversely proportional to the concentration of antigen (or hapten) in the sample. The known antigen standards can be used to prepare a standard curve to determine the content of antigen in the sample. For instance, direct competitive ELISA has been used to detect mycotoxins in foods (Chu, 1984; Pestka, 1988).

In the indirect competitive ELISA (Figure 2.2), antigen or hapten-protein conjugate is coated on the solid phase. Antibody is incubated simultaneously with the sample. Any antigen in the sample will compete with the solid phase antigen for the antibody binding sites; therefore, more antigen in the sample, less antibody will be bound on the solid phase antigen. Anti-immunoglobulin enzyme conjugate is added to determine the total bound antibody. The amount of color formed is inversely proportional to the amount of antigen (or hapten) in the sample or standard. Indirect competitive ELISA has been used in the detection of adulteration of meat products

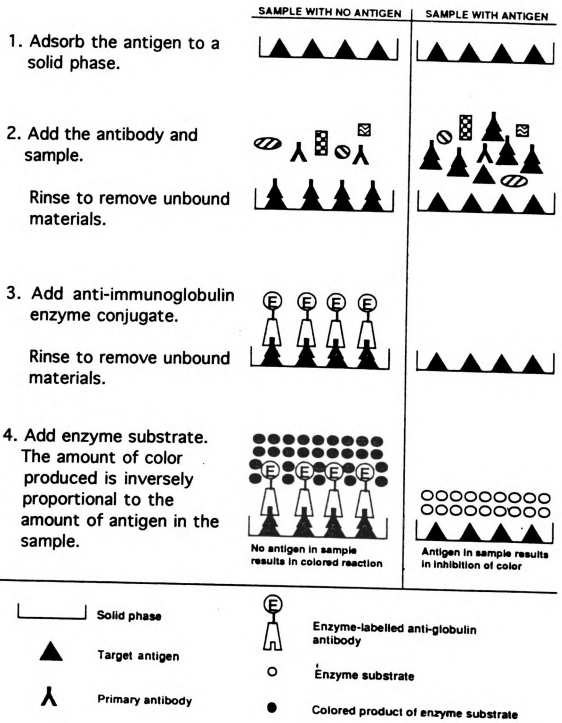


Figure 2.2. Indirect competitive ELISA.
(Adapted from Rittenburg, 1990)

with other meat species and soy protein (Rittenburg et al., 1987; Dincer et al., 1987; Sawaya, 1990).

Double antibody sandwich ELISA (Figure 2.3) is used to measure concentrations of bivalent or polyvalent antigens; since the antigen is sandwiched between the solid phase antibody and the enzyme-labeled antibody, this assay is often referred to as a sandwich assay (Rittenburg, 1990). Excess immobilized antibody is incubated with the sample or standard. The enzyme-labeled antibody is used to detect the bound antigen. The amount of color produced is proportional to the concentration of antigen in the sample or standard. Sandwich ELISA has been used to detect staphylococcal enterotoxins (Saunders and Bartlett, 1977), and determine gluten in foods (Skerritt and Hill, 1990).

C. Applications of ELISA

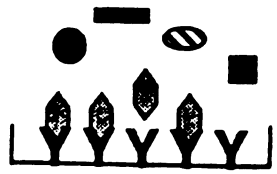
(1) Detection of toxins, microorganisms and residues

ELISA has been developed to detect mycotoxins, such as aflatoxins, T-2 toxin, and zearalenone that are a chemically diverse group of toxic secondary metabolites produced in agricultural commodities by fungi (Pestka, 1988). Conventional analyses of mycotoxins using chromatography are time consuming, tedious and expensive; ELISA can detect mycotoxins within 10-15 min and the cost of using ELISA is only 6 and 2% of gas chromatography and high performance liquid chromatography, respectively

1. Adsorb the antibody to a solid phase.

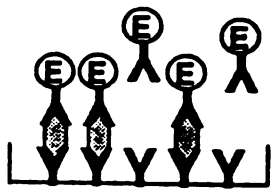
2. Add the sample. The solid phase antibody will capture antigen from the sample.

   Rinse to remove unbound materials.

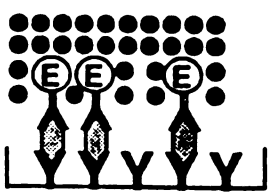


3. Add enzyme-labeled antibody.

   Rinse to remove unbound materials.



4. Add enzyme substrate. The amount of color produced is proportional to the amount of antigen in the sample.



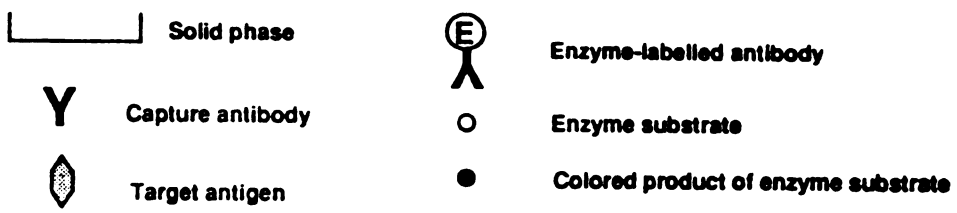


Figure 2.3. Double antibody sandwich ELISA. (Adapted from Rittenburg, 1990)

(Samarajeewa et al., 1991).

In contrast to conventional microbiological or animal tests, ELISA has been demonstrated to be an inexpensive and rapid assay in detecting microbial toxins and microorganisms (Samarajeewa et al., 1991). For example, ELISA has been developed to detect *Staphylococcus aureus* enterotoxins (Saunders and Bartlett, 1977; Fey et al., 1984; Schonwoalder et al., 1988), *Clostridium botulinum* toxins (Lewis et al., 1981; Gibson et al., 1988), and *Clostridium perfringens* enterotoxins (Stelma et al., 1985; Bartholomew et al., 1985). Cell wall polysaccharides and flagella extracts are two types of *Salmonella* antigens used to raise antibodies (Ibrahim, 1986). Monoclonal antibodies have been produced to detect *Salmonella* (Robison et al., 1983; Paterson and Tiffin, 1988). ELISA can detect Salmonella at 10^5 to 10^6 per milliliter of a 24-hr enrichment culture (Samarajeewa et al., 1991).

Consumers are concerned about the health effects of pesticide residues on foods. Several ELISAs have been developed to detect pesticides, such as diflubenzuron (Wie et al., 1982), metalaxyl (Newsome, 1985), chlorsulfuron (Kelley et al., 1985), and paraoxon (Brimfield et al., 1985). Antibiotics and other drugs are used to prevent or treat diseases in animals. The residues of these chemicals in animal foods could induce antibotic resistance or cause harmful effects to human health.

ELISA has been used to detect cephalexin residues in milk, hen tissue and eggs (Kitagawa et al., 1988), gentomycin and sulfamethazine in swine (Singh et al., 1989), and dimetridazole in turkey (Stanker et al., 1993).

(2) Adulterants and food additives

Detecting adulteration of meat products with unlabeled meat species is important for economic, health and religious reasons (Andrews et al., 1992). Substitution of sheep, horse, and kangaroo meats was found in Australian beef exports (Whittaker et al. 1983). Deer meat was used in products labeled as beef and pork sausage (Anonymous, 1993 b). Whittaker et al. (1982, 1983) produced antibodies against whole serum from different species and developed an ELISA to differentiate between unprocessed beef, sheep, horse, kangaroo, pig and camel meat. Dincer et al. (1987) developed an ELISA to detect species-specific serum albumins. Antibodies against muscle proteins also have been used in ELISA to differentiate between raw beef, pig, horse and chicken meat (Martin et al., 1988a,b,c; Martin et al., 1991). Kang'ethe and Gathuma (1987) developed an ELISA using antisera to thermostable muscle antigens, that enabled species identification in cooked and autoclaved meat products. Berger et al. (1988) isolated the heat-resistant antigens from raw skeletal muscle tissue and found that these antigens were immunoreactive after

heating to 120 °C for 15 min; an ELISA was developed to detect poultry and pork in cooked and canned meat foods. The poultry and pork ELISAs could detect chicken/turkey and pork at 126 and 250 ppm level, respectively. Andrews et al. (1992) used a similar approach to detect beef, sheep, deer and horse meat in cooked meat products by ELISA.

It has been reported that addition of ovalbumin in canned mushrooms increases the apparent weight of drained mushrooms by 10 to 20%; coagulated protein bound to mushrooms to block drainage of water and increase water retention (Breton et al., 1988). Since canned mushrooms are purchased by their drained weight and the retained water increase the weight, the amount of added ovalbumin needs to be regulated. Therefore, Breton et al. (1988b) developed an ELISA to identify and quantify ovalbumin in canned mushrooms. Soy protein is often added to meat products as binder to increase water and fat retention and to improve product texture; soy protein is also used to extend or substitute for meat proteins to lower the cost of meat products (Kinsella, 1979). Rittenburg et al. (1987) developed an ELISA to quantify soy protein in meat products.

(3) Food Quality

ELISA has been used to determine food constituents that provide important processing and quality

characteristics. It has been shown that baking characteristics were related to the protein components of wheat (Payne et al., 1987). Valdimarsdottir et al. (1989) developed an ELISA to quantify the water-soluble pentosans that contribute to good dough characteristics of rye. A gluten ELISA has been used to quantify gluten in flours and to predict bread making quality (Mills et al., 1990).

(4) Commercial ELISA Kits

A number of ELISA kits are available on the market for detecting toxins, microorganisms, residues and adulteration (Pestka, 1988; Fukal, 1991; Samarajeewa et al., 1991). These kits are easy to use, convenient, and require fewer technical skills compared with chemical assays. However, in adopting a commercial ELISA kit for the laboratory, both kit manufacturers and potential users should consider the following critical questions: the limits of detection and sensitivity range of the assay, the cross-reactivity of the antibody to analogs present in the sample, the description of realistic sampling protocols, the effectiveness of the recommended extraction procedure, the ability to screen a large numbers of samples, the stability of the kit in the field, and the reproducibility of the kit (Pestka, 1988).

CHAPTER THREE: ANTIBODY DEVELOPMENT AND ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE PROTEIN MARKER LACTATE DEHYDROGENASE TO DETERMINE SAFE COOKING END-POINT TEMPERATURE OF TURKEY ROLLS

(Published in Journal of Agricultural and Food Chemistry, 1992, Vol. 40, No. 9, 1671-1676.)

3.1 Abstract

An indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed for determining the end-cooking temperature of turkey breast rolls. Electrophoresis of extracts from turkey rolls processed to internal temperatures between 68.3 and 72.1 °C revealed a single protein which disappeared from the extract at 70.9 °C. This protein was identified as lactate dehydrogenase (LDH) on the basis of molecular weight determination by sodium dodecyl sulfate electrophoresis, enzyme assay, and LDH-specific stain on native polyacrylamide gels. Polyclonal antisera were raised in rabbits against purified turkey muscle LDH and commercial chicken muscle LDH and yielded titers ranging from 3.6×10^5 to 1×10^6 after 10 weeks of immunization. Turkey muscle LDH antisera cross-reacted with chicken muscle LDH and vice versa but not with other species. The LDH content in precooked turkey breast roll extracts as determined by ELISA

decreased as the end-cooking temperature increased, suggesting that the assay could serve as a simple and rapid method to determine adequacy of processing.

3.2 Introduction

Recent concern by meat processors, consumers and the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) over the possibility of foodborne disease outbreaks from precooked meats contaminated by pathogens such as *Salmonella*, hemorrhagic *Escherichia coli*, *Campylobacter* and *Staphylococcus* have prompted the need for rapid, accurate assays to verify that meat products receive sufficient heating to destroy these microorganisms.

The U.S. Department of Agriculture uses several assays to determine if precooked meat products have been cooked to the proper end-point temperatures as required in Title 9 of the Code of Federal Regulations (CFR). The Food Safety and Inspection Service describes procedures for a coagulation test (USDA, 1986b) for beef and pork products, a bovine catalase test (USDA, 1989), for roast beef and a residual acid phosphatase activity method (USDA, 1986a) for canned hams, picnics, and luncheon meats. Title 9 of the CFR requires precooked uncured poultry products to be cooked to an internal temperature of 71.1 °C; however, the USDA does not have a standard

assay for verifying processing temperatures in poultry.

Several investigators have developed other enzyme assays to monitor end-point cooking temperatures based on the residual activity of catalase, peroxidase (Morozova and Soboleva, 1974), lactate dehydrogenase (LDH) (McCormick et al., 1988; Stadler et al., 1991; Collins et al., 1991a,b), pyruvate kinase (Davis et al., 1988), glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase (Townsend and Davis, 1990). These assays are very time consuming or require a large number of reagents and/or sophisticated scientific equipment and thus have not been adopted for widespread use.

Several sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) techniques have also been evaluated for assessing the end-point cooking temperatures of meat products (Steele and Lambe, 1982; Lee et al., 1974; Alvarez, 1990). The loss of protein solubility has been used to measure the degree of heat denaturation of muscle proteins extracted from heated muscle with water or low ionic strength salt solutions (Lee et al., 1974; Davis et al., 1987; McCormick et al., 1987). These methods are accurate but time consuming and not practical for routine use. A rapid qualitative assay for end-point cooking temperatures based on soluble protein composition is highly desirable.

Immunoassays are now being used to detect undeclared meat species, nonmeat proteins, microorganisms,

mycotoxins, hormones, pesticides, and other contaminants in meat and meat products (Dincer et al., 1987; Sawaya et al., 1990; Fukal, 1991). Relatedly, enzyme-linked immunosorbent assays (ELISAs) have been developed to detect the presence of native and heat-denatured ovalbumin in food products (Breton et al., 1988a; Breton et al., 1989). The goal of this project was to develop an ELISA to accurately determine if poultry breast rolls have been processed to the proper USDA end-point heating temperature. An indicator protein in turkey muscle extracts was identified and isolated. Polyclonal antibodies were raised in rabbits against this indicator protein, and an indirect competitive ELISA was devised to verify the end-cooking temperature of turkey rolls.

3.3 Materials & Methods

A. Materials

Polyethylene sorbitan monolaurate (Tween 20), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide, and LDH from chicken muscle, chicken heart, bovine muscle, bovine heart, porcine muscle, porcine heart, and rabbit muscle were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) (fraction V) was from Ameresco (Solon, OH). Goat anti-rabbit IgG conjugated to horseradish peroxidase was obtained from Cappel Laboratories (West Chester, PA).

Complete and incomplete Freund adjuvant were purchased from Difco Laboratories (Detroit, MI) and polystyrene microtiter ELISA plates (Immunolon-2 Removawells) from Dynatech Laboratories (Alexandria, VA). Rabbits (New Zealand white female) were obtained from the Bailey Rabbitry (Alto, MI). Commercial precooked turkey breast roasts and hams were purchased from a local retail store. All other chemicals were reagent grade or better.

B. Processing of Turkey Rolls

Turkey rolls were commercially prepared and formulated using 45.36 Kg of turkey breast meat, 8.62 Kg of water, 1.27 Kg of modified starch, 0.68 Kg of salt, 0.27 Kg of sugar and 0.23 Kg of Na tripolyphosphate. Product was stuffed into moisture-proof casings. Each roll weighed about 3.63 Kg and measured 10.16 cm in diameter by 35.56 cm in length. Product was transported to Michigan State University and processed the next day.

Turkey rolls were smokehouse processed to target internal temperatures of 68.9, 70.0, 71.1 and 72.2 °C using the smokehouse schedule in Table 3.1. Internal product temperature was monitored by a thermocouple thermometer throughout the cooking and cooling cycle. Smokehouse runs were performed in triplicate. Turkey rolls were removed from the smokehouse approximately 1 °C before the desired internal temperature to allow for post-processing temperature increases, cooled in an

Table 3.1. Processing schedule for turkey rolls

Stage	Time, min	Internal temp, °C	Dry bulb, °C	Wet bulb, °C
1	60		60.0	60.0
2	120		65.6	65.6
3		66.7	71.1	71.1
4		70.0	73.9	73.9
5		72.2	75.6	75.6

ice-water bath for 1.5 hr, then stored overnight at 4 °C before analysis.

C. Extraction of Protein from Turkey Rolls

A 3-cm slice of meat, immediately adjacent to a thermocouple location, was cut from the turkey roll. A 3 cm diameter core was cut from the center of the slice. Twenty-five grams of meat from this core sample was homogenized with 3 volumes (w/v) cold phosphate buffer saline (PBS, 0.15 M NaCl, 0.01 M Na phosphate buffer, pH 7.2) in a Waring blender for 90 sec (3 repetitions of 30 sec on, 10 sec off). The homogenate was centrifuged at 16,000 x g for 20 min at 4 °C, the supernatant was filtered through Whatman No. 1 filter paper, and protein concentration was determined by the biuret method with BSA as the standard (Gornall et al., 1949). Percentage extractable protein was determined by dividing the protein content of the extract by the protein content of the turkey rolls and multiplying by 100.

D. Electrophoresis

To determine protein composition of the meat extracts, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) or native PAGE (Davis, 1964) was performed using either a Mini-Protein II electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) or a Hoeffer vertical electrophoresis unit (Model SE 600)

and a Bio-Rad power supply (Model 1000/500). For SDS-PAGE, stacking and separating gels of 4 and 12% acrylamide, respectively, were used. Molecular weights were determined on SDS-PAGE gels by comparing relative mobilities of protein bands on those of molecular weight standards (SDS-6H and SDS-7, Sigma) (Weber and Osborn, 1969). Protein bands were stained with Coomassie Brilliant Blue R 250 or silver stain (Merril, 1990).

The native PAGE separating gel (pH 8.8) was made up of 4.5% acrylamide and covered with a thin layer (5 mm) of 3% acrylamide gel (pH 8.8). Native PAGE gels were stained for the presence of LDH using an LDH-specific staining solution based on the methods of Fine and Costello (1963) and Fritz et al. (1970). One hundred milliliters of the stain contained 0.1 M Tris, pH 8.5, 0.02 M lithium lactate, 35.3 mg of NAD^+, 17.7 mg of nitro blue tetrazolium chloride, and 0.7 mg of phenazine methosulfate. The gel was stained at 25 °C for 1 h in the dark, and the reaction was stopped by soaking the gels in 7.0% v/v acetic acid.

E. Determination of LDH Activity

Activity was determined using an LDH diagnostic kit (DG 1340-K, Sigma) at 25 °C. One unit of LDH activity was expressed as 1 μmol of NADH oxidized per minute.

F. Purification of LDH from Turkey Breast

Turkey LDH was purified on the basis of the recommendations of Scopes (1970). Turkey breast was ground through the 4-mm plate of a KitchenAid grinder (Hobart Corp., Troy, OH) and then homogenized with 2 volumes (w/v) of cold distilled water in a Waring blender for 30 s. The homogenate was stirred for 30 min at 4 °C and filtered through gauze. The filtrate was adjusted to pH 5.0 with 1 M acetic acid and centrifuged at 16,000 x g for 20 min at 4 °C. The supernatant was heated to 65 °C in an water bath and then cooled immediately in an ice-water bath. The heated solution was centrifuged at 16,000 x g for 45 min at 4 °C. The supernatant was brought to 0.80 saturation with solid ammonium sulfate and then centrifuged at 16,000 x g for 20 min at 4 °C. The precipitate was collected and dissolved in 0.15 M acetate buffer (pH 5.0). This solution was heated to 63 °C in a water bath and then cooled in an ice-water bath. The precipitate formed was discarded, and the ammonium sulfate saturation was raised to 0.45 with saturated ammonium sulfate solution, and left at 4 °C overnight. The solution was centrifuged as described above and the precipitate discarded. The ammonium sulfate saturation was raised to 0.60 with saturated ammonium sulfate solution, and held at 4 °C for crystallization of LDH. This turbid solution was centrifuged at 16,000 x g for 20 min at 4 °C. The precipitate was dissolved in distilled

water and dialyzed against distilled water. The dialysate was cooled to about 0 °C, cold acetone (-10 °C) was added to 40% acetone concentration (v/v), and the mixture was left to stand at -7 °C for 30 min. This solution was centrifuged at 4000 x g for 15 min at -7 °C. The precipitate was dissolved in PBS and dialyzed against PBS.

G. Production of Polyclonal Antibodies

Rabbits (three per group) were injected subcutaneously with 500 μg of purified turkey muscle LDH or commercial chicken muscle LDH mixed with saline-Freund's complete adjuvant (1:1) in a volume of 1.0 mL. Five weeks later these were boosted by subcutaneous injection with 500 μg of enzyme in saline-Freund's incomplete adjuvant (1:1) in a volume of 1.0 mL. At week 8, rabbits were boosted intramuscularly with 200 μg of enzyme in 1:1 saline-Freund's incomplete adjuvant. Rabbits were bled via marginal veins at intervals and sacrificed 10 weeks after initial immunization. Antibodies were purified by ammonium sulfate (33% saturation) (Herbert et al., 1973).

H. Indirect ELISA

For antisera titration, microtiter wells were coated overnight (4 °C) with 100 μL of LDH (5 μg/mL) in 0.1 M carbonate buffer (pH 9.6). Plates were washed four times with PBS containing 0.05% (V/V) Tween 20 (PBS-Tween).

Wells were incubated with 300 μL of 1% BSA (W/V) in PBS (PBS-BSA) at 37 °C for 30 min to minimize nonspecific binding. After washing, 50 μL of serially diluted serum was added to each well and incubated for 1 h at 37 °C. Unbound antibody was removed by washing four times, and 100 μl of goat anti-rabbit IgG peroxidase conjugate (1:500 in 1% BSA-PBS) was added to each well. Plates were incubated for 30 min at 37 °C and washed eight times, and bound peroxidase was determined with ABTS substrate as described by Pestka et al. (1982). 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.

A competitive ELISA was used to test the ability of LDH antibodies for detection of LDH in processed turkey and chicken meat products. The competitive assay 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 followed by 50 μL of the appropriate dilution of LDH antisera. Plates coated with turkey or chicken LDH were stored at 4 °C for at least 8 weeks without loss of activity and were used to determine sera titer and detect and quantify the amount of LDH in turkey or chicken products. Sera of all six rabbits were used throughout this study with a dilution ranging from 1:50,000 to 1:150,000.

I. Western Blot Analysis

Purified turkey LDH and turkey roll extract proteins were transferred electrophoretically (1 hr at 100 V) from the SDS-PAGE gel to nitrocellulose membrane (0.45 μm, Schleicher & Schuell, Keene, NH) in a Mini Trans-Blot unit (Bio-Rad) using 25 mM Tris, 192 mM glycine buffer, and 20% (v/v) methanol buffer (pH 8.3). After transferring, the membrane was washed with PBS-Tween, blocked with 10 mL 3% BSA-PBS for 30 min at room temperature, and rinsed with PBS-Tween and 10 mL of the appropriately diluted antibody in 3% BSA-PBS incubated with the membrane at room temperature for 30 min. Unbound antibody was removed by washing with PBS-Tween, and 10 mL of goat anti-rabbit IgG peroxidase conjugate (1:2000 in 3% BSA-PBS) was added to the membrane and incubated at room temperature for 10 min. The membrane was washed with PBS-Tween, and bound peroxidase was determined with 15 mL of substrate solution (24 mg of 3,3',5,5'-tetramethylbenzidine and 80 mg of dioctyl sulfosuccinate dissolved in 10 mL of ethanol, 30 mL of 0.1 M citrate-phosphate buffer, pH 5.0, and 20 μL of 30% H_2O_2) at room temperature. Staining was stopped by washing with water.

J. Statistics

Experiments with turkey rolls and commercial turkey products were performed in triplicate. Basis statistics and two-way analysis of variance (treatment x replication)

were performed using 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.

3.4 Results and Discussion

Average maximum internal temperatures of the 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 72.2 °C, respectively. Extractable protein decreased from 25.3% in the unheated rolls to an average of 3.6% at internal temperatures between 68.3 and 72.1 °C (Table 3.2). Extractable protein content did not differ ($P > 0.05$) among turkey breast rolls processed between 68.3 and 72.1 °C.

A representative SDS-PAGE electrophoretogram of meat extracts from turkey rolls processed to different endpoint temperatures is shown in Figure 3.1. Extracts from unheated turkey rolls had over 17 bands. Fewer bands were observed in the heated samples. Band patterns of extracts from meat processed to 68.3 °C were not different from those of meat processed at 69.7 °C except for a decrease in the intensity of a 35,000-Da band. The intensity of this band decreased further as processing temperature was increased to 70.9 °C. A protein band with molecular

Table 3.2. Effect of end-point cooking temperature on lactate dehydrogenase (LDH) activity and extractable protein content of turkey roll

Internal temp, °C	Extractable protein,[a] %	LDH activity[a] units/g of sample
unheated	25.3±0.2[b]	734.68±44.04[b]
68.3±0.3	3.8±0.08[c]	22.04±12.16[c]
69.7±0.3	3.6±0.03[cd]	2.28±0.35[d]
70.9±0.3	3.4±0.02[d]	1.35±0.09[d]
72.1±0.3	3.5±0.04[d]	1.18±0.06[d]

[a]Expressed as mean ± standard deviation of three replicate values.
[b-d]Means in the same column followed by the same letter are not different (P>0.05).

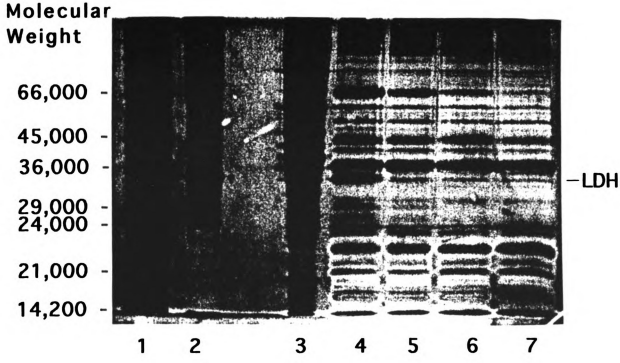


Figure 3.1. Representative sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of muscle extracts from turkey rolls heated to different end-point temperatures. Proteins were visualized with silver stain. (Lane 1) Molecular weight marker; (lane 2) chicken muscle LDH (from Sigma); (lane 3) unheated turkey roll; (lane 4) 68.3 C; (lane 5) 69.7 C; (lane 6) 70.9 C; (lane 7) 72.1 C. Twenty micrograms of protein was loaded on each gel lane.

weight of 66,000 was observed in all extracts but decreased markedly in extracts from turkey rolls processed to 72.1 °C. The 35,000- and 66.000-Da bands were presumptively identified on the basis of molecular weight as a subunit of LDH and monomeric phosphoglucomutase, respectively (Scopes, 1970).

On native PAGE, bands with the same mobility as isolated turkey muscle LDH (Lane 2) were observed in unheated turkey rolls (lane 1) and in turkey tolls processed to 68.3 (lane 3) and 69.7 °C (lane 4) but not in rolls processed to 70.9 (lane 5) or 72.1 °C (lane 6) (Figure 3.2). LDH activity in the extracted fraction decreased form 22.0 units/g at 68.3 °C to 2.3 units/g at 69.7 °C. LDH activity at 70.9 and 72.1 °C was not different ($P > 0.05$) and averaged 1.3 units/g of meat. Using reversed-phase high-performance liquid chromatography, McCormick et al. (1987) also showed decreased LDH content in porcine muscle extracts between 65 and 70 °C.

Since LDH was not detected on native gels using a specific enzyme stain and activity was negligible in extracts of turkey tolls processed to internal temperatures of 70.9 °C, this protein was selected as the indicator protein for immunochemical verification of safe end-point cooking temperatures of turkey breast products. The isolated turkey breast muscle LDH migrated the same distance as a commercial chicken muscle LDH on SDS-PAGE

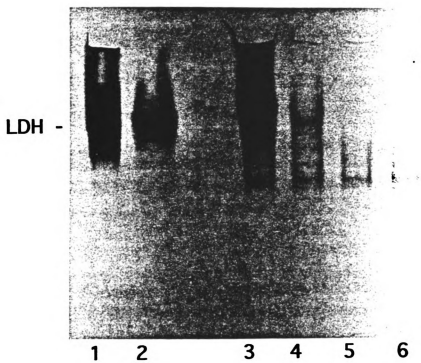


Figure 3.2. Representative native polyacrylamide gel electrophoretogram with LDH-specific stain of muscle extracts from turkey rolls heated to different end-point temperatures. (Lane 1) Unheated turkey roll; (lane 2) isolated turkey muscle LDH; (lane 3) 68.3 °C; (lane 4) 69.7 °C;(lane 5) 70.9 °C; (lane 6) 72.1 °C. Twenty micrograms of protein was loaded on each gel lane.

(Figure 3.3) and had an enzyme activity of 300 units/mg of protein.

For generation of specific antibodies for an LDH assay, rabbits were immunized with commercial chicken LDH assay, or with purified turkey LDH. Antibody titers were detected in the sera of the immunized rabbits as early as 4 weeks after the initial immunization (Table 3.3). The antibody titer rose sharply at week 7 following one booster injection and reached a maximum at week 10 following a second booster injection. Figures 3.4 and 3.5 contain titration curves for rabbits immunized with chicken LDH and turkey LDH, respectively. Generally, higher titers were observed from rabbits injected with turkey LDH compared to those injected with chicken LDH.

A competitive indirect ELISA (CI-ELISA) was devised using turkey or chicken LDH as solid phase/inhibitor pairs (Figure 3.6). It was determined that antibodies prepared against turkey LDH were more effective than antibodies prepared against chicken LDH regardless of the solid phase/inhibitor pair. The specificity of the polyclonal antibodies in CI-ELISA was further examined by evaluating reactivity with LDH from rabbit, bovine, and porcine skeletal muscle and bovine, porcine, and chicken heart (Figures 3.7 and 3.8). None of these enzymes inhibited binding of either turkey or chicken LDH antibodies up to concentrations of 25,000 ng/L, thus verifying assay specificity for turkey and chicken muscle LDH.

Figure 3.3. Representative sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of chicken and turkey breast muscle lactate dehydrogenase (LDH). (Lane 1) Chicken muscle LDH (from Sigma); (lanes 2 and 3) isolated turkey muscle LDH. Gels were stained with Coomassie Blue.

Table 3.3. Production of polyclonal antibodies against lactate dehydrogenase (LDH) of chicken or turkey muscles in rabbits[a]

Weeks after immunization	Antibody titer					
	Chicken LDH			Turkey LDH		
	A	B	C	D	E	F
4	6.0×10^3	1.2×10^4	2.5×10^4	1.0×10^5	2.0×10^5	2.5×10^4
7	2.1×10^5	3.5×10^5	1.8×10^5	3.6×10^5	6.8×10^5	2.1×10^5
10	5.1×10^5	8.1×10^5	3.5×10^5	7.1×10^5	1.0×10^6	5.4×10^5

[a]A,B and C refer to rabbit antisera immunized with chicken LDH. D,E and F refer to rabbit antisera immunized with turkey LDH.
[b]Booster injection were performed at weeks 5 and 8.

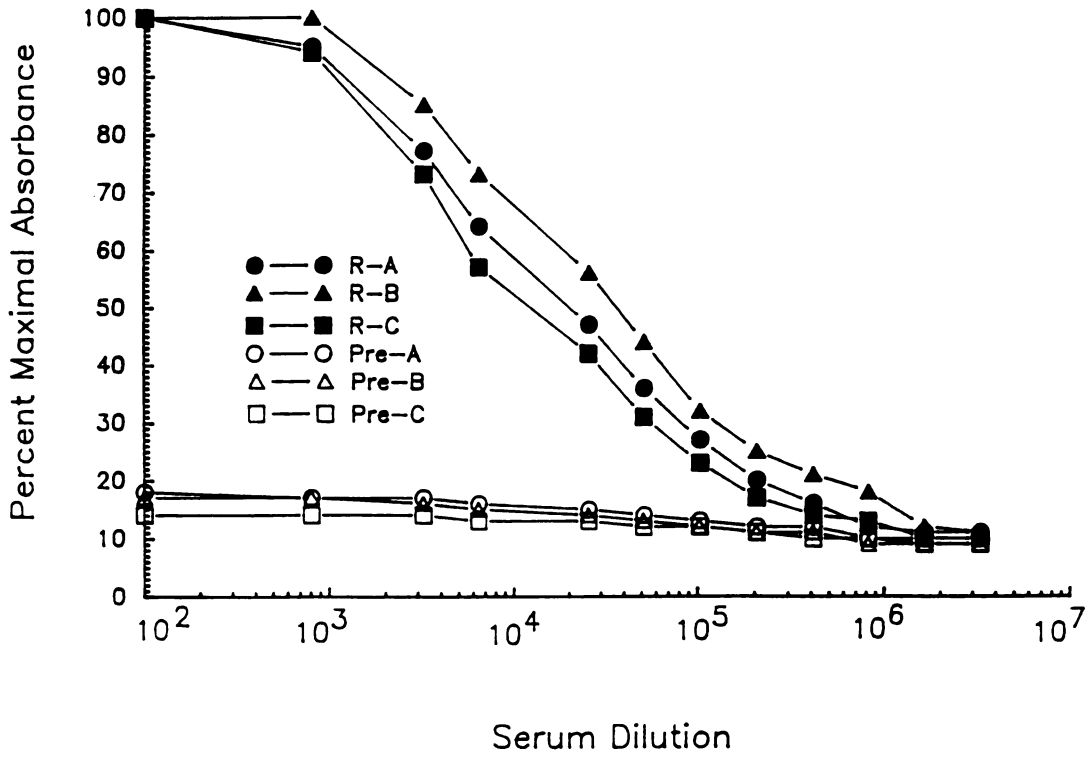


Figure 3.4. ELISA titration of rabbit anti-chicken lactate dehydrogenase antibodies. Rabbit antisera R-A, R-B, and R-C were obtained 10 weeks after initial immunization. PRE indicates preimmune serum.

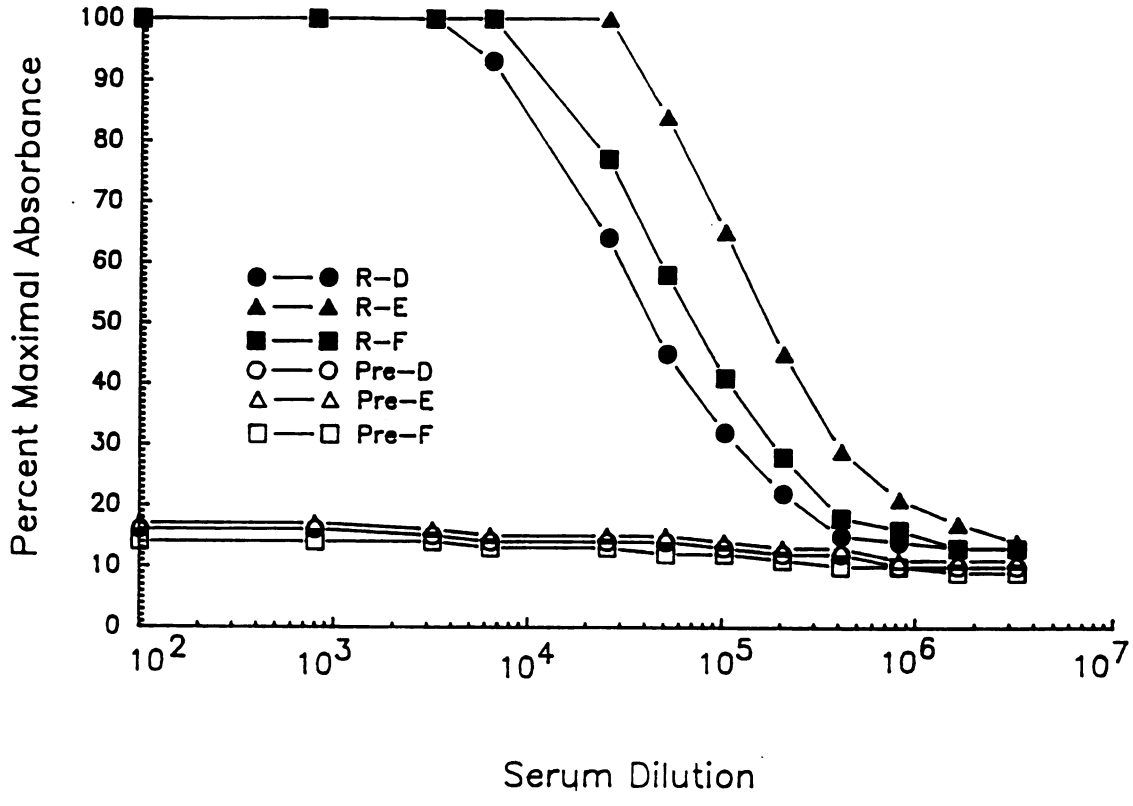


Figure 3.5. ELISA titration of rabbit anti-turkey lactate dehydrogenase antibodies. Rabbit antisera R-D, R-E, and R-F were obtained 10 weeks after initial immunization. PRE indicates preimmune serum.

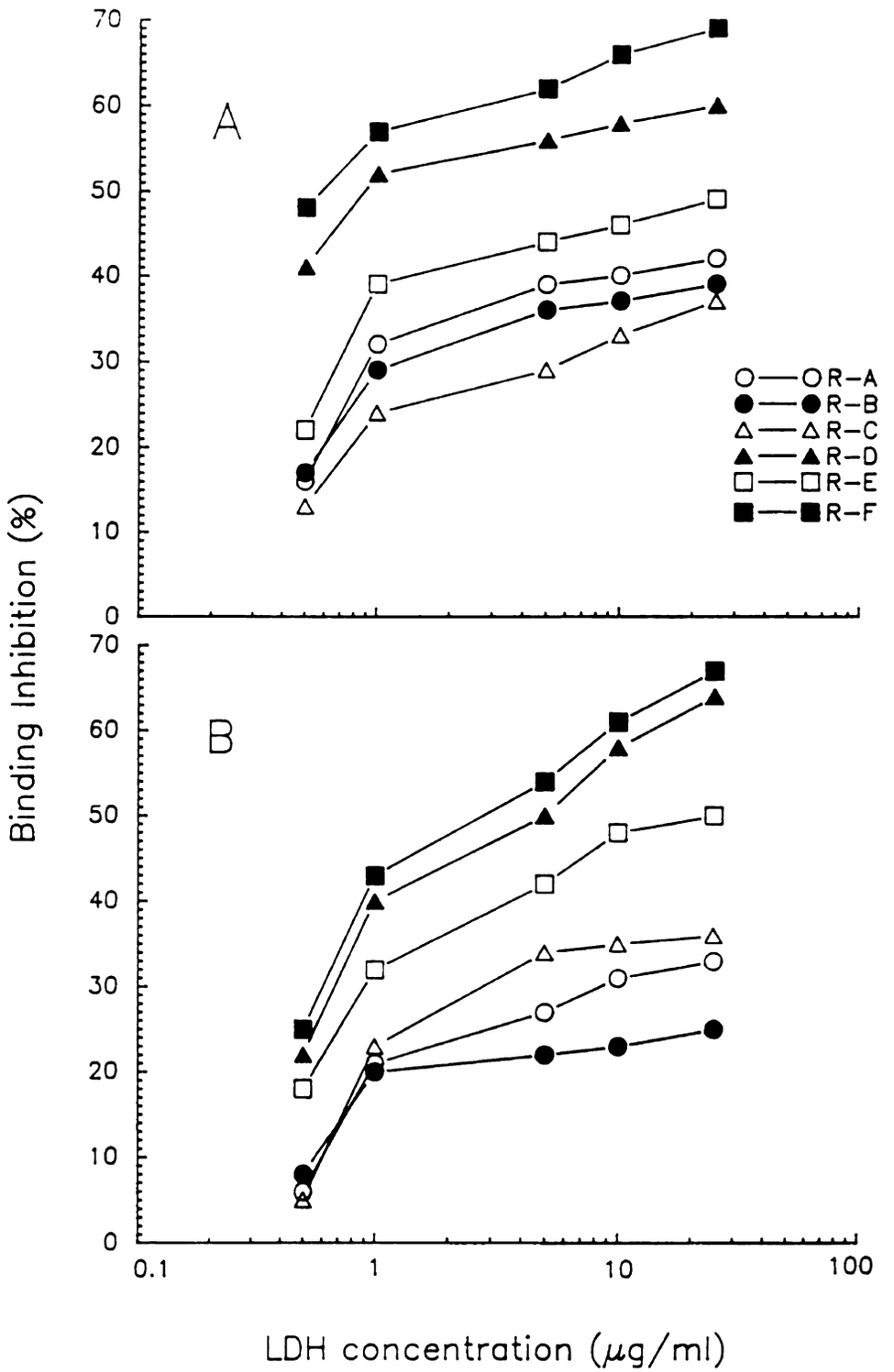


Figure 3.6. ELISA standard curves of rabbit polyclonal antibodies prepared against chicken lactate dehydrogenase (LDH) (R-A, R-B, and R-C) or turkey LDH (R-D, R-E, and R-F). (A) Turkey LDH was used as solid phase and as inhibitor in the competitive ELISA; (B) chicken LDH was used as solid phase and inhibitor.

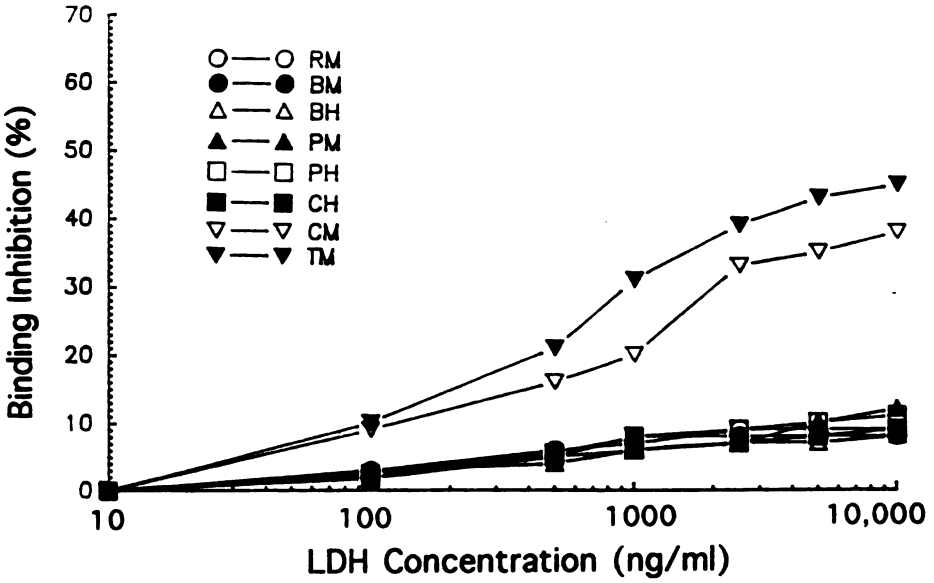


Figure 3.7. Cross-reactivity of chicken lactate dehydrogenase (LDH) polyclonal antibodies with LDH from different animal sources: rabbit muscle (RM), bovine muscle (BM), bovine heart (BH), porcine muscle (PM), porcine heart (PH), chicken heart (CH), chicken muscle (CM) and turkey muscle (TM). Serum of rabbit B was used.

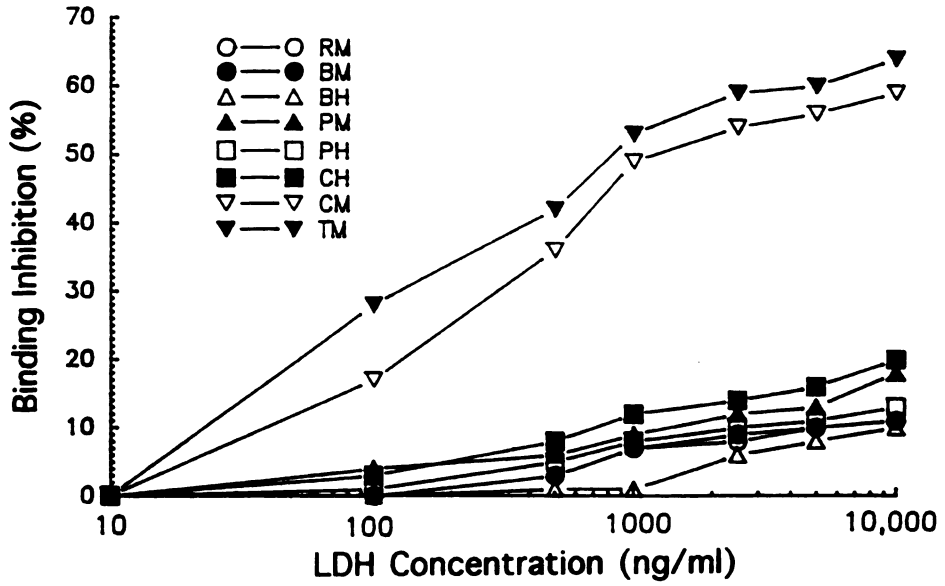


Figure 3.8. Cross-reactivity of turkey lactate dehydrogenase (LDH) polyclonal antibodies with LDH from different animal sources. Abbreviations of LDH sources are the same as in Figure 7. Serum of rabbit F was used.

Western blot analysis revealed that polyclonal antibodies to turkey LDH reacted with purified turkey muscle LDH and with LDH processed turkey roll extracts (Figure 3.9). The intensity of the LDH band identified by Western blot decreased as end-point heating temperature of the turkey rolls increased. Some binding was observed to protein bands other than turkey muscle LDH, which may be attributed to nonspecific protein binding, the presence of other LDH isozymes, or very slight cross-reactivity of the polyclonal antibodies with proteins other than LDH.

The LDH in turkey extracts decreased as the end-cooking temperature increased as determined by CI-ELISA (Figure 3.10). Percent inhibition for extracts diluted 2-5 fold exceeded 10% for raw turkey and samples heated to 68.6 and 69.7 °C but dropped below 10% for samples heated to 70.9 and 72.1 °C. The results indicated that LDH was virtually absent at these latter two temperatures and suggested that a simple ELISA could be used to assess whether the USDA-required safe cooking end-point temperature of 71.1 °C was reached.

Commercially processed turkey breast roast and turkey ham were tested for the presence of LDH by SDS-PAGE, enzyme assay, and CI-ELISA. As shown on electropho-retograms (Figure 3.11), LDH was not observed in turkey breast roast (lane 1) but was observed in turkey ham (lane 2). LDH activities for turkey breast roast and turkey ham were 1.76 and 33.44 units/g of sample, respectively.

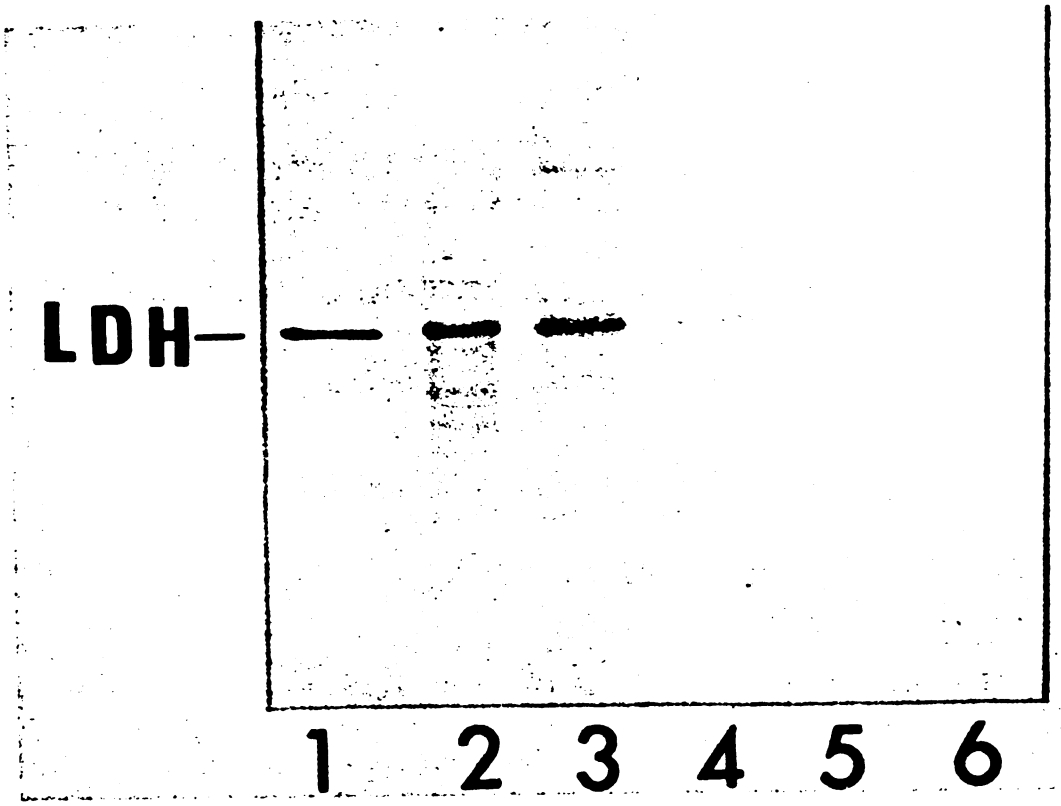


Figure 3.9. Western blot of isolated turkey muscle LDH and muscle extracts from turkey rolls heated to different end-point temperatures. (Lane 1) Isolated turkey muscle LDH; (lane 2) unheated turkey roll; (lane 3) 68.3 °C; (lane 4) 69.7 °C;(lane 5) 70.9 °C; (lane 6) 72.1 °C. The amounts of protein loaded on the gel lane were 0.07, 1.2, and 20 μg for isolated turkey muscle LDH, unheated turkey roll, and heated turkey rolls, respectively.

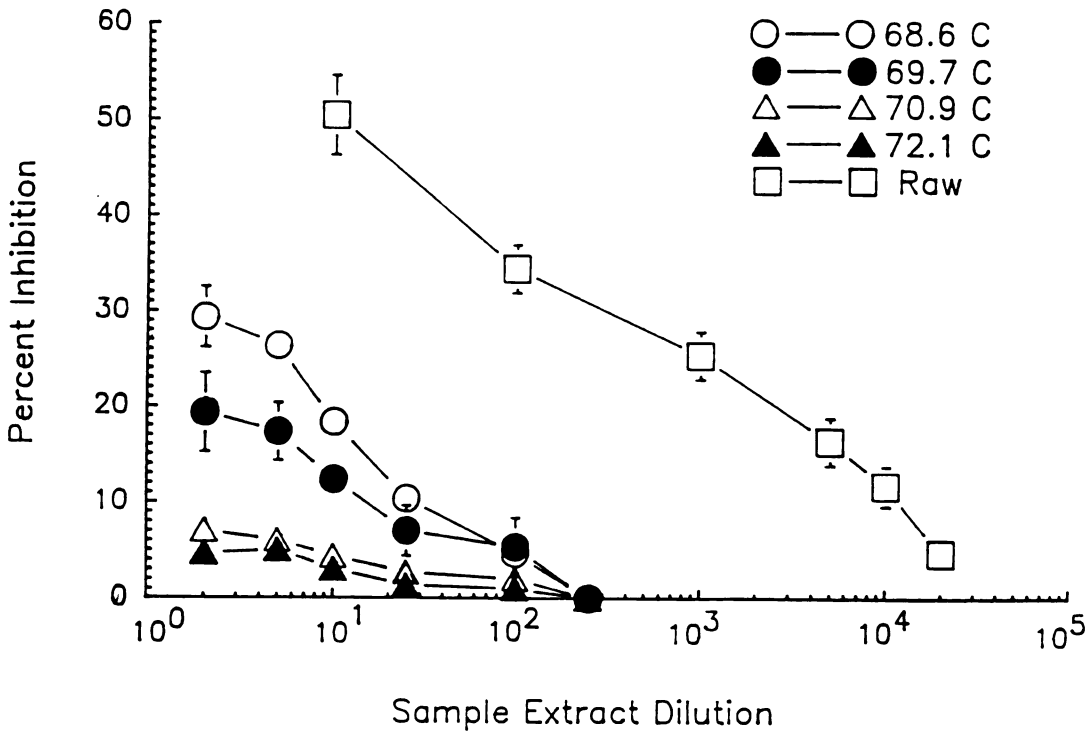


Figure 3.10. Indirect competitive ELISA to determine the cooking end-point by measuring LDH in turkey rolls cooked to different processing temperatures. Data are the average of triplicate samples. Bars indicates standard error of the mean. Data points without bars indicate error bars fit within points.

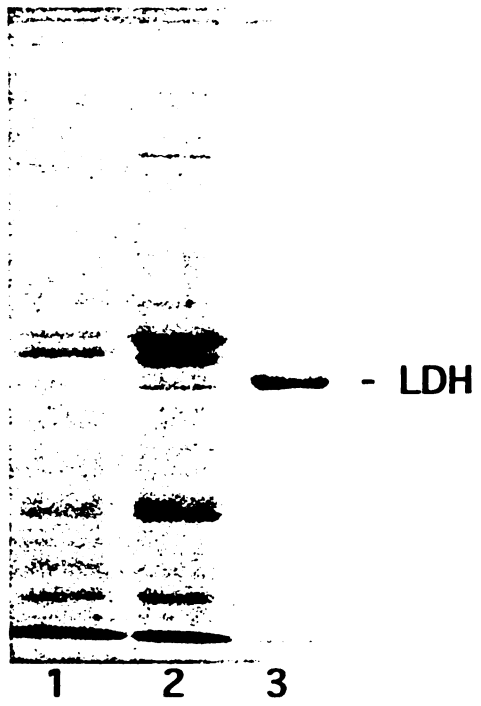


Figure 3.11. Representative sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of muscle extracts of commercial turkey breast roast and turkey ham. (Lane 1) Turkey breast roast; (lane 2) turkey ham; (lane 3) chicken LDH (from Sigma). Twenty micrograms of protein was loaded on each gel lane.

These results agreed with the indirect competitive ELISA (Figure 3.12). Percent inhibition for turkey ham was greater than 10 within the dilution range 2-10-fold, indicating the internal temperature did not reach 71.7 °C. In contrast, inhibition for turkey breast roast was below 10%, indicating the adequacy of heat processing. Since turkey ham is processed to a lower temperature (68.3 °C) than turkey breast products, more LDH was detected by CI-ELISA.

In summary, we have identified LDH as an indicator protein and demonstrated the feasibility of using ELISA to determine the end-cooking temperature of turkey tolls. ELISA accurately differentiated the end-cooking temperature within ±1.1-1.2 °C in the temperature range 68.3-72.1 °C. Although LDH activity was consistent with ELISA, it was very difficult to set a critical value of LDH activity for the adequacy of heat treatment. LDH-specific stain and native PAGE were very sensitive for determining the end-cooking temperature but were more complicated than ELISA and the LDH activity assay. ELISA can screen a large number of samples at the same time by using 96-well microtiter plates, and the resulting color in ELISA can be measured spectrophotometrically or by visual comparison with standards. Thus, ELISA could serve as a simple and rapid method to determine adequacy of processing of turkey rolls. Experiments are in progress to validate the assay under a range of ingredient

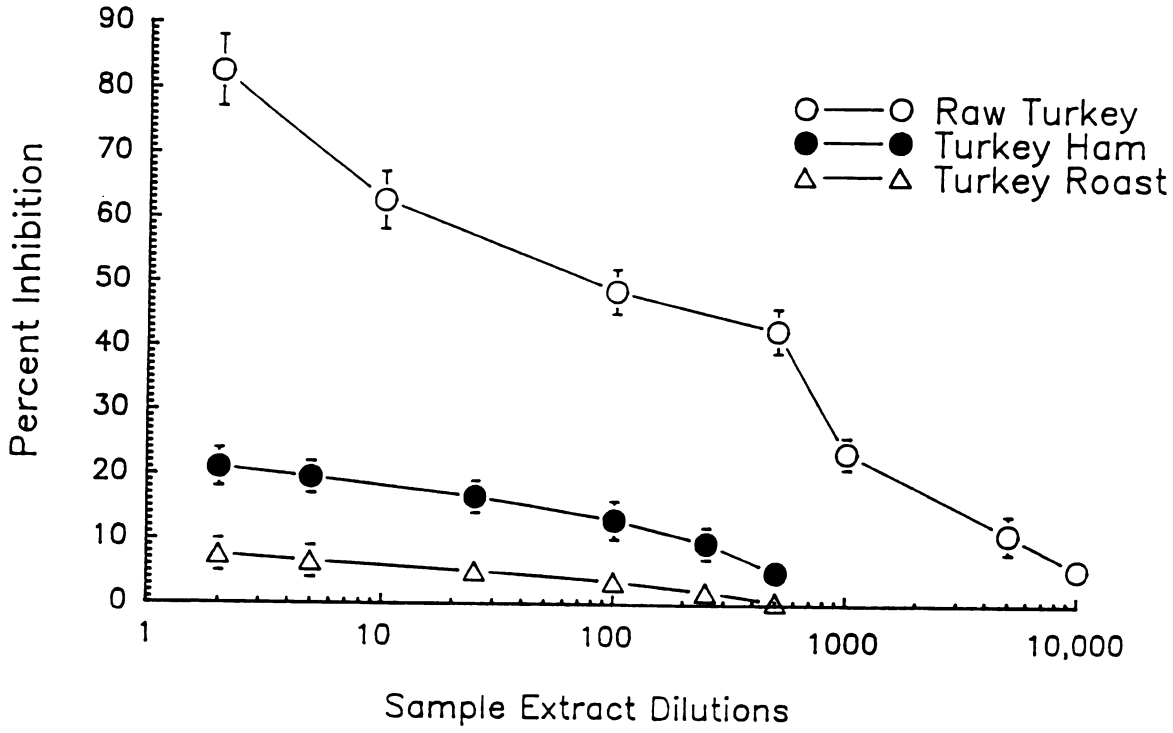


Figure 3.12. Indirect competitive ELISA of commercial turkey breast roast and turkey ham extracts. Extracts were diluted in 1% bovine serum albumin in phosphate buffer saline (PBS-BSA). Turkey was extracted 1:3 (w/v) and then diluted further in 1% PBS-BSA. Bars indicate standard error of the mean. Data points without bars indicate error bars fit within points.

formulations and processing conditions.

CHAPTER FOUR: LACTATE DEHYDROGENASE AS SAFE ENDPOINT COOKING INDICATOR IN POULTRY BREAST ROLLS: DEVELOPMENT OF MONOCLONAL ANTIBODIES AND APPLICATION TO SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)
(Published in Journal of Food Protection, 1993, Vol. 56, No. 2, 120-124, 129)

4.1 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 (MAb) were prepared against chicken muscle LDH and used with rabbit polyclonal antibodies (PAb) 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 USDA endpoint temperature of 71.1 °C.

4.2 Introduction

The United States 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 temperatures attained in imported products in order to prevent the spread to the U.S. of certain exotic diseases in animals (USDA, 1982).

Protein solubility in muscle tissue extracts after heating in water or salt solutions has been used as a measure of heat denaturation of protein (Hamm and Deatherage, 1960). Lee et al. (1974) and Caldironi and Bazan (1980) have demonstrated that water soluble components of beef, pork and chicken muscle 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 (Davis and Anderson, 1984). For example, the Coagulation Test (USDA, 1986b) 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 (USDA, 1989) and the Acid Phosphatase Activity Method which is used to determine if canned hams, picnics and luncheon meats have been properly processed to 68.8 °C (USDA, 1986a).

Enzyme assays have also been developed to monitor end-point cooking temperatures based on the residual activity of catalase and peroxidase (Morozova and Soboleva, 1974), lactate dehydrogenase (LDH) (Collins et al., 1991a,b; McCormick et al., 1987; Stadler et al., 1991) and pyruvate kinase (Davis et al., 1988). Townsend and Blankenship (1989) selected leucine aminopeptidase from among 19 individual enzymes to monitor heat treatment of meat and poultry products. In a related approach, Klinger et al. (1982) 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. (1991 a,b) 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 (Wang et al., 1992). 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 pre-cooked poultry products.

4.3 Materials & Methods

A. Materials

BALB\c, 6-8 weeks 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 (PEG, 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, Ohio). 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. (1985).

Turkey muscle LDH was purified using ammonium sulfate and acetone precipitations and activity determined as described by Wang et al. (1992). 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) LDH as reported previously (Wang et al., 1992). Commercial precooked turkey roasts and hams were purchased from a local retail store.

B. Monoclonal Antibody Production

Six groups of female BALB\c mice (6-8 wk) received subcutaneous or intraperitoneal injections of chicken muscle LDH (10, 25 or 50 µg/mouse), in 0.2 ml emulsified with one volume of 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 the 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 (1981) as modified by Abouzied et al. (1990). Spleen cells ($1x10^8$) of immunized mice that showed the highest serum titer and specificity against LDH, were fused with NS-1 myeloma cells ($1X10^7$) using 50% PEG. The 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 eleven 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 hr, 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 days. 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 (Goding, 1980) using 15% FBS-15%- macrophage-conditioned-HT medium (Abouzied et al., 1990; Sugasawara et al., 1985). 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 grown in 20% FBS-DMEM medium and supernatants collected every 2-3 days. After centrifugation to remove the cells, antibodies were purified by precipitation with 50% ammonium sulfate (Hebert et al., 1973), dialyzed against 3 changes of PBS, aliquoted and lyophilized. Monoclonal antibody isotypes were determined with the ScreenType kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

C. Indirect ELISA

An indirect ELISA (Abouzied et al., 1990) 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). The 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 (w/v) 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 hr 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. (1982). 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 non-immune 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.

D. 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 the remaining protein binding sites were blocked with PBS-BSA (300 µl/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 hr 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 hr 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.

E. 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 (Wang et al., 1992).

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 sec 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 (Wang et al., 1992). Extracts from raw and processed turkey products were diluted in PBS-BSA for ELISA.

F. 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, MI).

4.4 Results & Discussion

Subcutaneous injection of mice with 50μg 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/mouse) (Table 4.1). 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.

Typical standard curves for determination of LDH by competitive indirect ELISA using monoclonal antibodies from four cell lines are shown in Figure 4.1. 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 detectable (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 antigen or sample extract (b) detector antibody that was

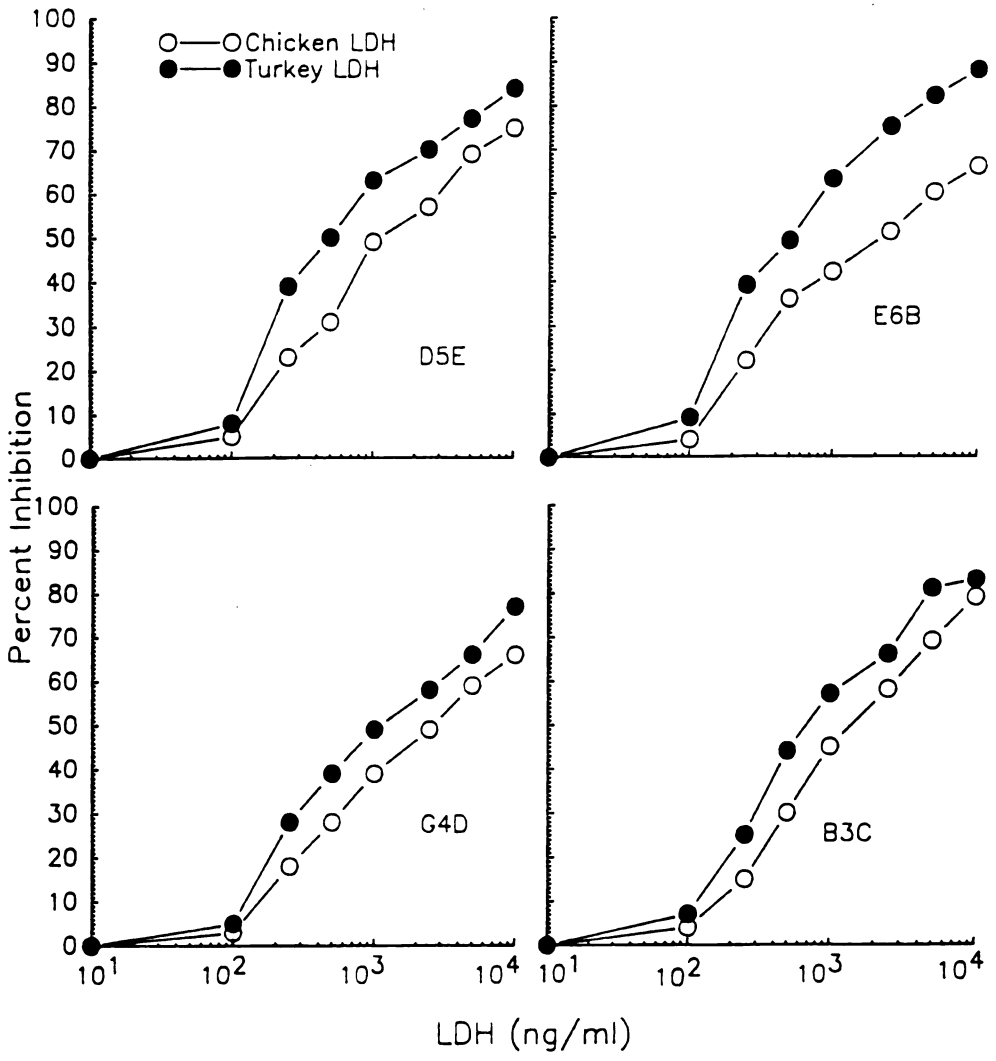


Figure 4.1. Detection of chicken and turkey lactate dehydrogenase (LDH) by competitive indirect enzyme-linked immunosorbent assay (ELISA) using chicken LDH monoclonal antibodies (MAbs). Optimum dilution for MAbs were D5E (1:1000), E6B (1:200), G4D (1:35) and B3C (1:25).

Table 4.1. Antibody titers to chicken muscle LDH by indirect ELISA after three immunizations

Mouse group	Dose (μg)	Injection mode[a]	Number 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.

generated against the same antigen, and (c) anti-species 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 (Butler et al., 1986). 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 (Figure 4.2B) than that obtained when polyclonal antibody was used as capture antibody and monoclonal antibody as detector antibody (Figure 4.2A). Immunocapture by a polyclonal antibody may have covered most sites leaving few available epitopes for monoclonal antibodies when used as detecting antibodies (Varshney et al., 1991). 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 was 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

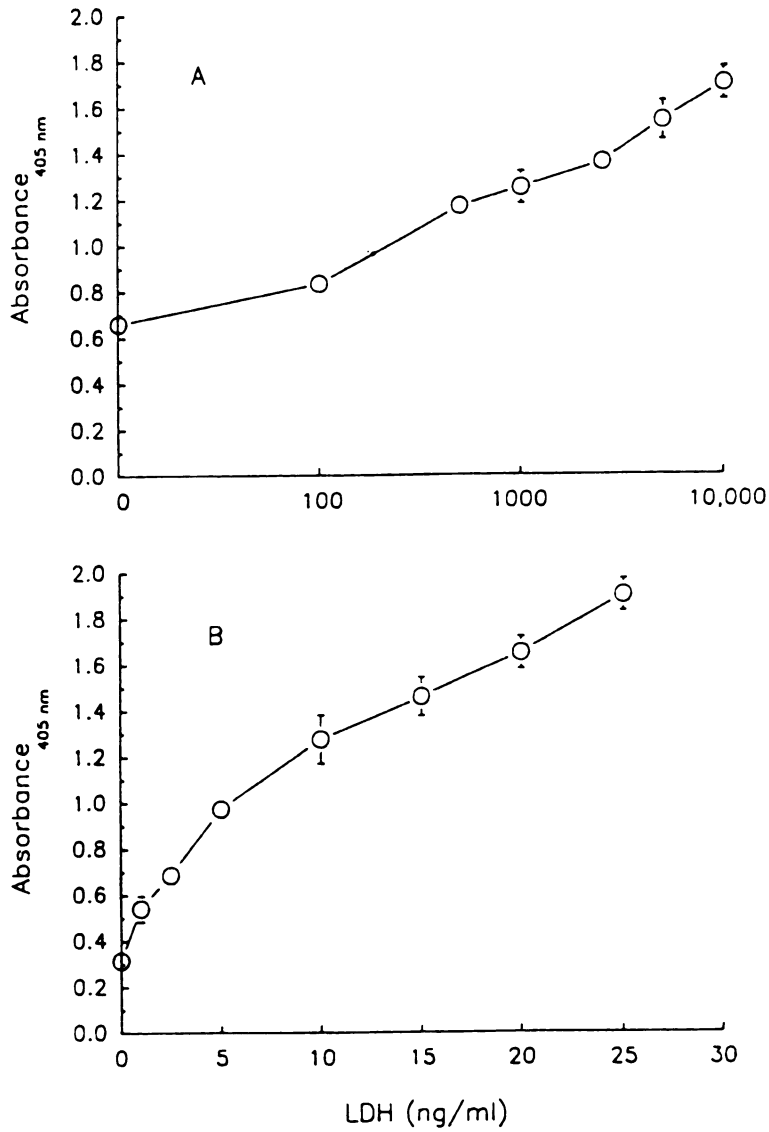


Figure 4.2. Detection of chicken muscle lactate dehydrogenase (LDH) by sandwich enzyme-linked immunosorbent assay (ELISA). A: Polyclonal antibody (PAb) prepared against turkey muscle LDH (R-F) was used as capture antibody. Detector antibody was monoclonal antibody (MAb) D5E prepared against chicken muscle LDH. B: Capture antibody was MAb D5E (1:450 dilution) and detector antibody was PAb R-F. Bars indicate standard error of the mean.

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 (3 prepared against chicken LDH and 3 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 (Figure 4.3).

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 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 (Figure 4.4). 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. (1991) reported that LDH activity

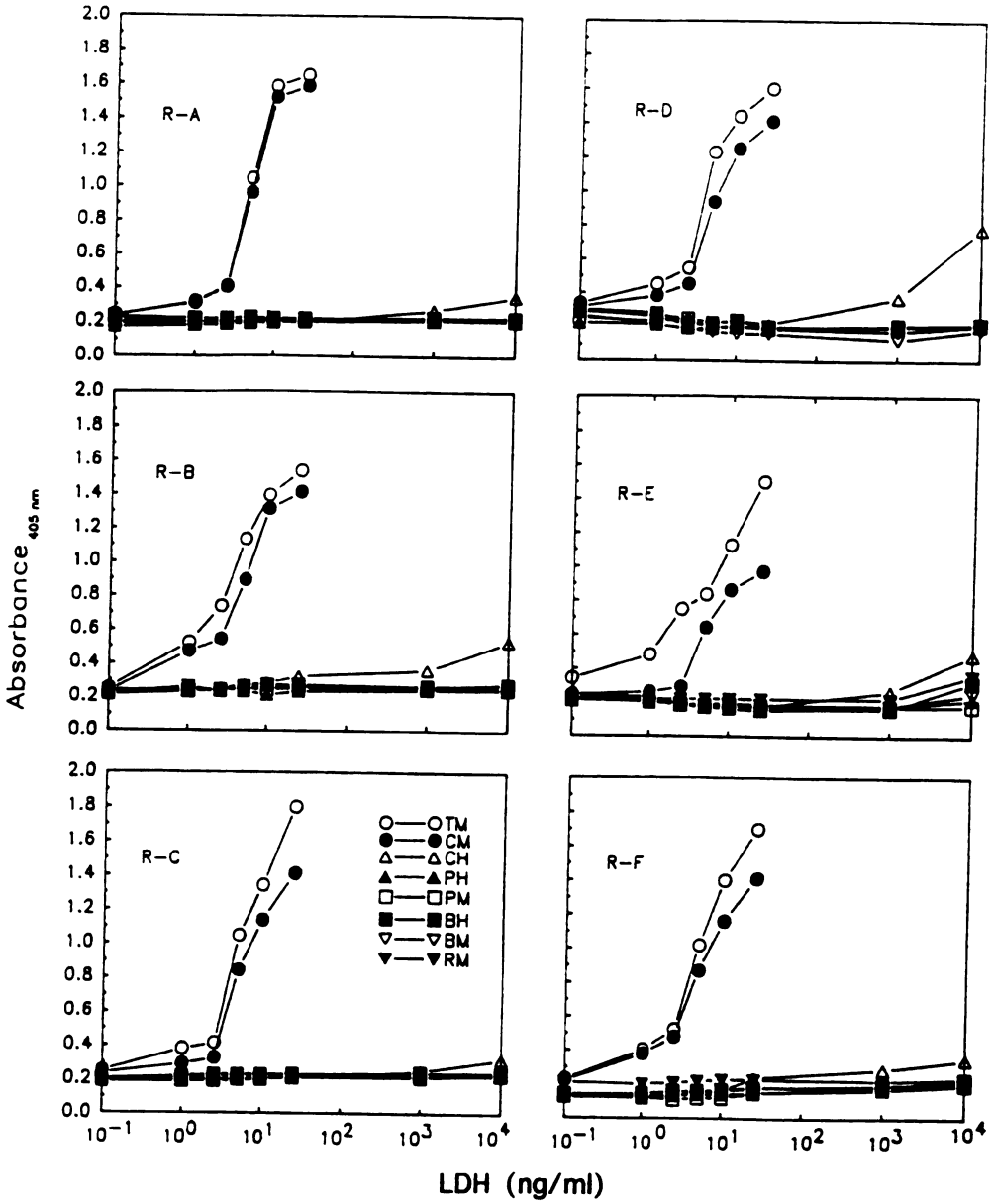


Figure 4.3. Specificity of sandwich enzyme-linked immunosorbent assay (ELISA) for lactate dehydrogenase (LDH). LDH sources were turkey muscle (TM), chicken muscle (CM), chicken heart (CH), porcine muscle (PM), bovine muscle (BM), bovine heart (BH) and rabbit muscle (RM). Plates were coated with monoclonal 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.

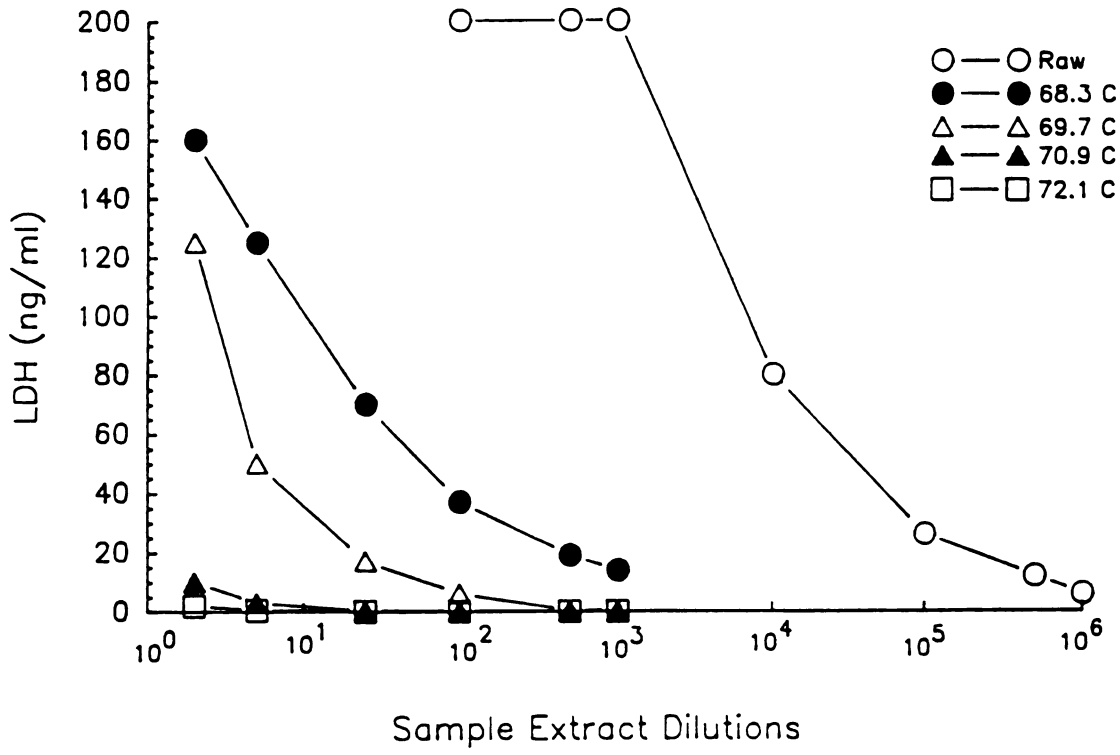


Figure 4.4. Effect of processing temperature on lactate dehydrogenase (LDH) concentration of turkey roll extracts as measured by sandwich enzyme-linked immunosorbent assay. Standard error of the means are ± 2.51, 4.19, 2.29, 0.64 and 0.13 for raw, 68.6, 69.7, 70.9 and 72.1 °C endpoint temperature treatments, respectively.

decreased significantly when bovine muscle extracts were heated to 63 °C and only slight activity was detected at 66 °C. Marin et al. (1992) 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 °C and 100 °C, respectively, indicating conformational changes in protein structure with heat. Collins et al. (1991a) reported a decrease in LDH activity in extracts of whole muscle ham as heating temperature of 11-15g 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. Similarly, Collins et al. (1991b) 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 (Figure 4.5). 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 was expected as USDA-FSIS requires a minimum internal temperature of 68.3 °C for cured poultry products (USDA, 1985). Also, differences in heat stability of LDH from red and white muscles has been reported (Townsend and Davis).

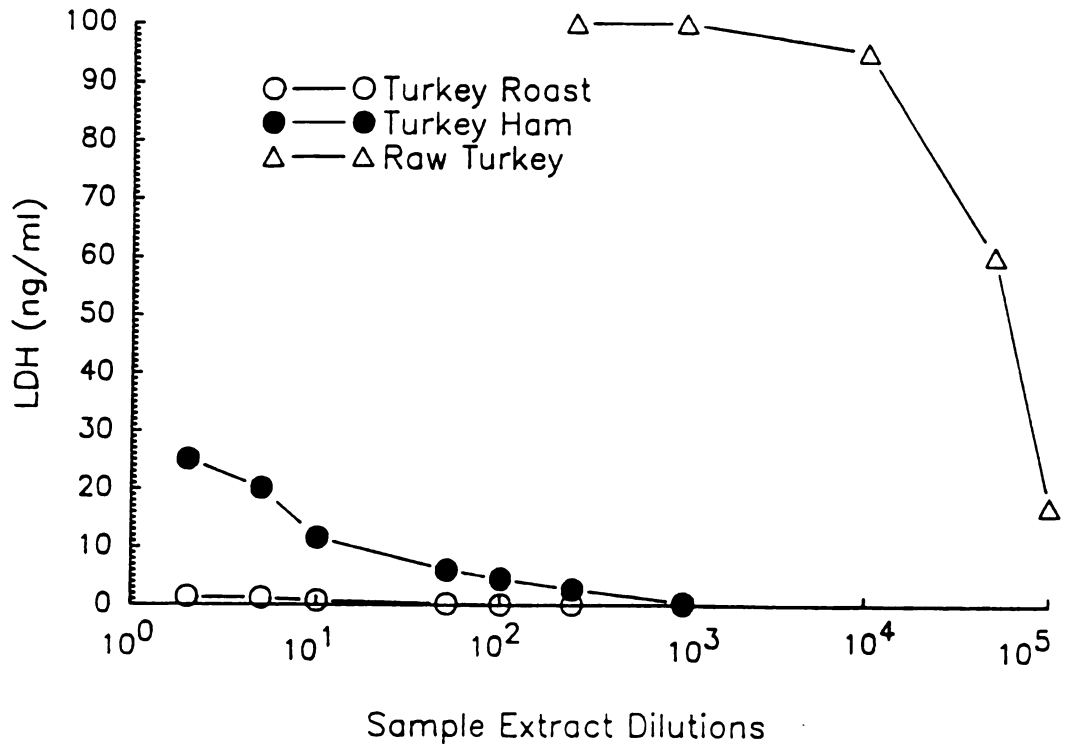


Figure 4.5. Detection of lactate dehydrogenase (LDH) in commercially available precooked turkey roasts and hams by sandwich enzyme-linked immunosorbent assay. Standard error of the means are ± 0.05, 0.99, and 2.56 for roast, ham and raw turkey breast, respectively.

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.

CHAPTER FIVE : ELISA DETERMINATION OF TURKEY ROLL ENDPOINT TEMPERATURE: EFFECTS OF FORMULATION, STORAGE, AND PROCESSING
(Published in Journal of Food Science, 1993, Vol. 58, No. 6, 1258-1261, 1264.)

5.1 Abstract

Effect of refrigerated and frozen storage, salt concentration, cooking schedule and product diameter were compared on determination of minimum endpoint cooking temperature of turkey breast rolls by measuring extractable protein content (EP), lactate dehydrogenase (LDH) activity and LDH concentration by sandwich enzyme-linked immunosorbent assay. LDH concentration differed in rolls processed to 70.0 °C and 71.1 °C, whereas EP and LDH activity did not differ at these temperatures. Salt concentration, cooking schedule and product casing diameter did not markedly influence LDH concentration. LDH content of uncooked rolls decreased during frozen storage. A maximum concentration of 0.31 μg LDH /g meat indicated proper processing.

5.2 Introduction

Precooked meat products are processed to minimum

endpoint temperatures as required by federal regulations to ensure destruction of pathogens that could cause diseases. The U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) uses a residual phosphatase assay (USDA-FSIS, 1986a), catalase test (USDA-FSIS, 1989), or a coagulation test (USDA-FSIS, 1986b) to verify endpoint cooking temperatures of beef and pork. Precooked uncured poultry products are required to be cooked to an internal temperature of 71.1 °C (USDA-FSIS, 1992); however, the USDA does not use a standard assay for verifying processing temperatures in poultry. Assays have been developed to assess the processing temperatures of meat products and include protein solubility (Steele and Lambe, 1982; Davis and Anderson, 1983), color (Favetto et al., 1988; Ang and Huang, 1992), electrophoresis of protein extracts (Steele and Lambe, 1982), differential scanning calorimetry of muscle proteins (Ellekjaer, 1992) and near infrared spectroscopy (Isaksson et al., 1989; Ellekjaer and Isaksson, 1992).

The feasibility of relating residual enzyme activity to internal processing temperature of meat products has been studied extensively (Davis et al., 1988; Townsend and Blankenship, 1989; Collins et al., 1991a, b; Anonymous, 1992; Bogin et al., 1992; Townsend and Davis, 1992; Kormendy et al., 1992). Several researchers have suggested that lactate dehydrogenase (LDH) activity could be used as an endpoint processing indicator in beef, pork

and turkey muscle (Bogin et al., 1992; McCormick et al., 1987; Collins et al., 1991a; 1991b; Stalder et al., 1991). LDH concentration was reported as an endpoint cooking indicator in commercially prepared turkey breast rolls (Wang et al., 1992). An indirect competitive enzyme-linked immunosorbent assay (ELISA) (Wang et al., 1992) and sandwich ELISA (Abouzied et al, 1993) using antibodies against the indicator protein, LDH, have been developed. While both competitive and sandwich ELISA accurately differentiated the endpoint cooking temperature within ±1.1-1.2 °C in the temperature range 68.3-72.1 °C, the sandwich ELISA showed higher sensitivity.

Several formulation and processing variables may alter the LDH content and activity of turkey rolls. Our objective was to compare the effectiveness of LDH activity measurements, LDH concentration by ELISA and extractable protein content to verify compliance to the USDA minimum endpoint temperature of turkey breast rolls. The effect of refrigerated and frozen storage on LDH activity and concentration of raw turkey breast meat and raw and cooked formulated rolls was also investigated.

5.3 Materials & Methods

A. Materials

Polyethylene sorbitan monolaurate (Tween 20), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS),

and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) (Fraction V) was from Ameresco (Solon, OH). Goat anti-rabbit IgG conjugated to horseradish peroxidase was obtained from Cappel Laboratories (West Chester, PA). Microtiter plates (Immunolon-2 Removawells) were from Dynatech Laboratories (Alexandria, VA). Turkey muscle LDH was purified using ammonium sulfate and acetone precipitation as described previously (Wang et al., 1992). All other chemicals were of reagent grade or better.

B. Processing of Turkey Rolls

Breast meat from 18-week-old turkeys (13.62-15.44 kg) was obtained on the day of slaughter from Bil Mar Foods, Inc. (Zeeland, MI). Turkey rolls were formulated using 45.36 kg fresh skinless turkey breast meat, 8.62 kg water, 1.27 kg modified starch (Staley, Decatur, IL), 0.68 or 1.13 kg NaCl, 0.27 kg sugar and 0.23 kg Na tripoly-phosphate (Rhone-Poulenc, Canbury, NJ). Turkey breast meat was ground through a 24x48 mm kidney plate (Model 4146, Hobart Mfg. Co., Troy, OH). Ground meat was placed into a mixer (Butcher Boy, Model 250 F, Lasar Mfg. Co., Los Angeles, CA) and blended with water, salt, sugar and Na tripolyphosphate for 3 min. Modified starch was added and mixed for 6 min. The raw mixture was placed into a Vermag 500 Continuous Vacuum Stuffer (Robert Reiser Co., Canton, MA) and stuffed into moisture-proof casings of

10.16 or 6.35 cm diameter (Teepak, Oak Brook, IL). After stuffing, the casings were clipped. Turkey rolls were processed in a microprocessor (Model PC-5809, Powis Corp., Oak Grove, MO) controlled smokehouse (Drying Systems, Inc., Chicago, IL) to target maximum internal temperatures of 68.9, 70.0, 71.1 and 72.2 °C using three smokehouse schedules (Tables 5.1, 5.2, and 5.3). Each turkey roll was removed from the smokehouse when the internal temperature was about 1 °C below the target temperature, (to allow for post smokehouse temperature rise), and immediately cooled in an ice bath. Internal product temperature was monitored using RTD probes (Omega, Stamford, CT) throughout the cooking and cooling cycle. All recorders and probes were calibrated using manufacturer's instructions. Additional verification of temperatures was done using Type T special limits of error (SLE) thermocouples (Omega, Stamford, CT). Smokehouse runs were performed in triplicate.

C. Processing Conditions

Four different processing/formulation conditions were evaluated. In process A, turkey rolls were formulated with 1.2% salt, stuffed into casings of 101 mm diameter by 35.4 cm length (about 3.6 kg) and processed using the short smokehouse schedule (about 6.5 hr total cook time) (Table 5.1). Process B was the same as process A, except turkey rolls were formulated with 2.0% NaCl. Process C

Table 5.1. Short processing schedule for turkey breast rolls stuffed into 101 mm diameter casings[a]

Stage	Cycle time (min)	Internal temp. (°C)	Dry bulb temp. (°C)	Wet bulb temp. (°C)
1	60	(30.6±2.4)	60.0	60.0
2	120	(57.8±2.1)	65.6	65.6
3	60	(63.5±2.0)	71.1	71.1
4	60	(67.9±1.0)	73.9	73.9
5	(77±6)[b]	71.1	75.6	75.6

[a]Total cook time for turkey rolls processed to an internal temperature of 71.7 °C was about 6.5 hr.
[b]Values in parentheses indicate mean ± standard deviation of internal product temperature at the end of a cycle time to reach internal product temperature of 71.7 °C.

Table 5.2. Long processing schedule for turkey breast rolls stuffed into 101 mm diameter casings[a]

Stage	Cycle Time (min)	Internal temp. (°C)	Dry bulb temp. (°C)	Wet bulb temp. (°C)
1	60	-	48.9	48.9
2	60	(38.7±1.1)	54.4	54.4
3	60	(49.0±0.8)	60.0	60.0
4	60	(55.9±0.6)	65.6	65.6
5	60	(61.8±0.4)	71.1	71.1
6	120	(70.3±0.3)	76.7	76.7
7	(22±5)[b]	71.7	79.4	79.4

[a]Total cook time for turkey rolls processed to an internal temperature of 72.2 °C was about 7.5 hr.
[b]Values in parentheses indicate mean ± standard deviation of internal product temperature at the end of a cycle time to reach internal product temperature of 71.7 °C.

Table 5.3. Processing schedule for turkey breast rolls stuffed into 6.35 cm diameter casings[a]

Stage	Cycle time (min)	Internal temp. (°C)	Dry bulb temp. (°C)	Wet bulb temp. (°C)
1	60	(37.8±0.8)[b]	48.9	47.8
2	60	(52.2±0.5)	60.0	58.9
3	60	(71.9±0.3)	79.4	79.4

[a]Total cook time for turkey rolls processed to an internal temperature of 71.7 °C was about 3 hr.
[b]Values in parentheses indicate mean ± standard deviation of internal product temperature at the end of a cycle.

was the same as condition A, except the turkey rolls were processed using the long smokehouse schedule (about 7.5 hr total cook time) (Table 5.2). Turkey rolls identified under process D were made with 1.2% salt, stuffed into casings of 63 mm diameter by 50.8 cm length (about 1.8 kg) and processed using the smokehouse schedule (about 3 hr total cook time) identified in Table 5.3.

D. Proximate Analysis and pH

Moisture, fat and protein content of the raw formulated rolls were determined using AOAC (1990) 950.46B, 991.36 and 981.10 respectively. For pH determination, 10 g of each turkey formulation were blended with 90 ml of distilled water in a Waring Blender for 1 min and pH of the homogenate was measured. All experiments were performed in triplicate.

E. Extraction of Protein and LDH Activity

Meat (25g) was excised from the geometric center of each turkey roll, adjacent to a thermocouple location, and homogenized and extracted in 0.15 M NaCl, 0.01 M Na phosphate buffer, pH 7.2 (PBS) as described by Wang et al. (1992). Protein concentration of the extract was determined by biuret method with BSA as the standard (Gornall et al., 1949). Percentage extractable protein was determined by dividing the protein content of the extract by that of the turkey rolls and multiplying by

100. The LDH activity in each extract was determined as described by Wang et al. (1992).

F. Sandwich ELISA

Production of antibodies against turkey and chicken muscle LDH was described previously (Wang et al., 1992; Abouzied et al., 1993). The sandwich ELISA was performed by coating microtiter wells with 100 µl of monoclonal LDH antibody D5E (Abouzied et al., 1993) diluted (1:400) 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 containing 0.05% Tween 20 (PBS-Tween), and 300 µL of 1.0% BSA in PBS (PBS-BSA) was added to each well to block the remaining protein binding sites and incubated for 30 min at 37 °C. After washing three times with PBS-Tween, muscle extracts or purified turkey muscle LDH diluted in PBS-BSA (50 µl) were added to each well and incubated for 1 hr at 37 °C. Plates were washed four more times with PBS-Tween, and 50 µl of polyclonal LDH antibody F (Wang et al., 1992) diluted (1:1500) in PBS-BSA was added. After incubation for 1 hr at 37 °C and washing four times with PBS-Tween, 100 µl of goat anti-rabbit IgG peroxidase conjugate diluted (1:500) in PBS-BSA were added to each well and incubated for 30 min. Plates were then washed 8 times with PBS-Tween, and bound peroxidase activity was determined with ABTS substrate as described by Pestka et al. (1982). Absorbance was read at 405 nm

using a Minireader II (Dynatech, Alexandria, VA). Purified turkey LDH was used to prepare a 0 to 200 ng/ml LDH standard curve in each plate and was used to calculate the amount of LDH in meat extracts. Results were expressed as nanograms LDH/g muscle or turkey roll.

G. Refrigerated and Frozen Storage of Raw Muscle and Formulated Rolls

Fresh turkey breast muscle was frozen at -12 °C in the Zipper Seal bags (Gordon Food Service, Inc., Grand Rapids, MI) for 2, 4, 8, 16, 26 and 42 wk. Turkey rolls from process A were sliced (starting from 10.1 cm from the end of the roll) into five pieces (2.8 cm thickness and 10.1 cm diameter) and vacuum packed in polyethylene-laminated nylon pouches (Koch, Kansas City, MO) for storage at 4 °C (1 week) and -12 °C (6, 13, 32 and 60 wk). Frozen meat was thawed overnight at 4 °C before analysis. All experiments were performed in triplicate.

H. Statistics

Basic statistics and one-way analysis of variance were performed using 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.

5.4 Results & Discussion

The proximate composition and pH of raw formulated turkey rolls from different processes were compared (Table 5.4). Proximate composition ranged from 73.0-74.2% moisture, 19.1-20.0% protein, and 1.4-2.0% fat, respectively. The pH of raw turkey formulation was not significantly different between the processes.

A. Effect of Processing

Extractable protein did not differ ($P>0.05$) in raw turkey rolls prepared using the four processes (Table 5.5). Extractable protein decreased from about 25.0% in raw turkey rolls to 3.8% at an internal processing temperature of 68.3 °C in all processes. Within each process, extractable protein content did not differ ($P>0.05$) in turkey breast rolls processed to 68.3 °C and above. A similar pattern of solubility loss was reported during heating of meat by Davis and Anderson (1983) and Davis et al.(1987); protein extractability decreased as temperature increased (by biuret method). However, there was little change in protein solubility between 67.5 and 75 °C in porcine muscle (Davis and Anderson, 1983) or between 65.6 and 68.8 °C in canned pork picnic shoulder (Davis et al., 1987).

There was no difference ($P>0.05$) in LDH activity among raw formulated turkey rolls from the four processes

Table 5.4. Proximate composition and pH of raw formulated turkey rolls from different process conditions[a]

Process[b]	Moisture (%)	Protein (%)	Fat (%)	pH
A	73.7±0.23	19.5±0.12	1.4±0.11	6.2±0.02
B	73.1±0.17	20.0±0.06	1.6±0.10	6.3±0.01
C	73.0±0.17	19.9±0.07	2.0±0.05	6.2±0.01
D	74.2±0.11	19.1±0.13	1.5±0.08	6.3±0.02

[a]Values are the average of triplicate determinations.
[b]Process A:Turkey rolls formulated with 1.2% NaCl, stuffed into 101 mm diameter casings and processed using a short smokehouse schedule. The other processes are the same as A except, B: 2.0% NaCl; C: long smokehouse schedule; D: 63 mm diameter casing.

Table 5.5. Effect of endpoint cooking temperature on extractable protein and lactate dehydrogenase (LDH) activity and content of turkey rolls from different process conditions[a]

Internal temp. (°C)	Extractable protein (%)	LDH activity (U/g of sample)	LDH conc (μg/g sample)
		Process A	
Raw	25.3±0.20[b]	736.2±44.04[b]	2409.9±221.1[b]
68.3±0.3	3.8±0.08[c]	22.0±12.16[c]	5.28±0.50[d]
69.7±0.3	3.6±0.02[cd]	2.3±0.35[d]	0.76±0.12[e]
70.9±0.3	3.4±0.02[ce]	1.4±0.09[d]	0.06±0.02[f]
72.1±0.3	3.5±0.04[ce]	1.3±0.06[d]	0.01±0.01[f]
		Process B	
Raw	26.7±1.98[b]	693.7±67.54[b]	2287.8±453.6[b]
68.6±0.3	3.4±0.18[cf]	2.8±0.36[d]	3.83±0.76[d]
69.7±0.3	3.0±0.12[cf]	1.4±0.12[d]	0.77±0.13[e]
70.9±0.3	3.1±0.10[cf]	1.1±0.02[d]	0.07±0.00[f]
71.9±0.3	2.9±0.22[def]	1.1±0.01[d]	0.03±0.01[f]
		Process C	
Raw	24.1±1.96[b]	633.3±35.83[b]	2391.9±575.1[b]
68.6±0.3	3.1±0.08[ef]	2.5±0.08[d]	3.75±0.78[d]
69.7±0.3	2.9±0.03[def]	1.4±0.31[d]	0.92±0.19[e]
70.9±0.3	2.7±0.08[ef]	1.1±0.12[d]	0.06±0.03[f]
71.9±0.3	2.8±0.22[def]	1.1±0.08[d]	0.03±0.01[f]
		Process D	
Raw	23.9±1.24[b]	755.7±42.22[b]	2885.1±539.4[b]
68.9±0.3	2.9±0.11[def]	27.2±12.73[c]	33.02±7.94[c]
70.0±0.3	2.8±0.09[def]	2.8±0.73[d]	3.81±0.90[d]
71.1±0.3	2.5±0.13[f]	1.4±0.13[d]	0.22±0.09[f]
72.2±0.3	2.6±0.13[f]	1.3±0.01[d]	0.03±0.01[f]

[a]Process A: Turkey rolls formulated with 1.2% NaCl, stuffed into 101 mm diameter casings and processed using a short smokehouse schedule. The other process conditions are the same as A except, B: 2.0% NaCl; C: long smokehouse schedule; D: 63 mm diameter casing. Expressed as mean ± standard deviation of three replicates.

[b-f]Means in the same column followed by the same letter are not different (P>0.05).

(Table 5.5). A decrease ($P<0.05$) in LDH activity was observed in extracts of all turkey rolls processed to 68.3-68.9 °C using all process conditions when compared to raw turkey rolls. Some differences in LDH activity among processes were observed in those processed to endpoints of 68.3-68.9 °C. The LDH activity at 68.3-68.9 and 69.7-70.0 °C in processes A and D were similar. The LDH activity in extracted turkey breast rolls processed to 68.6 °C was 2.8 U/g in process B, lower than 22.0 U/g at 68.3 °C in process A. Stalder et al. (1991) showed that increasing NaCl from 1.5 to 3.0% NaCl in bovine semimembranosus slurries (pH 6.4) slightly decreased residual LDH activity at 63 °C. Thus, a higher salt content (2.0% vs. 1.2% NaCl) and a slightly higher heating temperature (68.6 vs. 68.3 °C) used in process B might be responsible for the decreased LDH activity. In process C, the LDH activity in extracted turkey breast rolls processed to 68.6 °C was 2.5 U/g, also lower than process A. This may be attributed to the longer processing time and slightly higher endpoint temperature in process C.

The LDH activity did not differ ($P>0.05$) in turkey breast rolls processed to 69.7-70 °C and above in any process condition. This suggested that salt content, smokehouse schedule and product diameters did not influence LDH activity at these temperatures. These results also suggested that protein extractability and LDH activity were not suitable methods for verifying endpoint

cooking temperatures of turkey rolls. These tests did not differentiate between products cooked to the required minimum endpoint temperature of 71 °C and those processed 2 °C below that point.

In contrast to those results, as internal processing temperature of turkey rolls increased, LDH content decreased in all process conditions (Table 5.5). This decrease resulted from heat denaturation and insolubilization of the enzyme (Wang et al., 1992; Abouzied et al., 1993). When turkey rolls were processed to 70.9 and 71.9-72.1 °C, the LDH content was 0.07 μg/g or less for turkey rolls processed under conditions A, B, and C. Salt content (1.2 and 2% NaCl) and cooking schedule (short and long smokehouse schedule) has little effect on effectiveness of the ELISA to differentiate between rolls processed to 71 °C and those processed 2 °C below that endpoint. The LDH content was 0.22 μg /g for turkey rolls processed to 71.1 °C and above using process D, higher than those obtained from other process conditions. These differences might be due to different product size and smokehouse schedules used in process D.

Relatively large changes in LDH concentration were observed when turkey rolls were processed to 68.3 °C and above, whereas only small changes in LDH activity were observed under the same conditions. These results suggest that loss of LDH activity did not directly correlate with loss of protein solubility and binding ability of

antibodies to LDH in ELISA. Possibly loss of LDH activity resulted from a conformational change in the region of the active site. However, such change did not influence LDH solubility and binding of antibodies to another portion of the LDH molecule.

A critical concentration of LDH could be estimated for determination of endpoint cooking temperature of turkey breast rolls form the different process conditions. The LDH content of meat was less than 0.22 ± 0.088 μg/g for turkey rolls processed to the USDA endpoint 71.1 °C and above under all conditions. Thus, a maximum critical concentration of 0.31 μg LDH/g (mean + S.D.) of turkey roll might indicate the adequacy of thermal processing of turkey breast rolls.

B. Frozen Storage of Turkey Muscle

The effect of frozen storage was compared on LDH concentration, LDH activity, and extractable protein content of raw turkey breast muscle (Table 5.6). Extractable protein content did not change ($P>0.05$) and averaged 22% in raw turkey muscle during frozen storage at -12 °C for 42 wk. Turkey breast muscle averaged 2450 μg LDH/g throughout 42 wk frozen storage. The LDH activity of raw turkey muscle did not change during 16 wk frozen storage, but increased between 16 and 42 wk. Bogin et al. (1992) reported that storing turkey breast at -12 °C for up to 8 wk decreased protein solubility and LDH activity.

Table 5.6. Effect of frozen storage (-12 °C) on lactate dehydrogenase (LDH) concentration, LDH activity, and extractable protein content of raw turkey breast muscle[a]

storage time (wk)	Extractable protein (%)	LDH activity (U/g of sample)	LDH conc (μg/g of meat)
0	22.4±0.70[b]	620.7±58.05[b]	2778.0±522.9[b]
4	22.3±0.56[b]	615.2±34.13[b]	2579.7±109.9[b]
8	23.6±0.69[b]	639.3±59.73[b]	2178.9±184.5[b]
16	21.1±0.86[b]	696.7±27.87[bc]	2326.5±195.0[b]
26	21.9±0.39[b]	757.5±25.45[cd]	2482.8±254.1[b]
42	22.9±0.24[b]	837.5±35.15[d]	2360.4±216.0[b]

[a]Expressed as mean ± standard deviation of three replicate values.
[b-d]Means in the same column followed by the same letter are not different (P>0.05).

Collins et al. (1991b) showed that frozen storage of ham muscles at -10 °C for 8 days decreased LDH activity. However, Collins et al. (1991a) reported that frozen storage at -24.5 °C for 5 days had little effect on LDH activity in beef top round muscles. Large quantities of sarcoplasmic protein have been observed in the muscle exudate or drip after thawing (Awad et al., 1968). Little drip loss was observed during thawing of turkey muscle in our study, which might explain the difference in LDH activity among our results.

C. Refrigerated and Frozen Storage of Turkey Rolls

Turkey rolls from process A were used to study the effect of refrigerated (4 °C) and frozen storage (-12 °C) on extractable protein, LDH activity and LDH concentration. These values in turkey rolls processed to 68.3, 69.7, 70.9 and 72.1 °C did not differ ($P>0.05$) at 0 and 1 wk of refrigerated storage. Extractable protein content of raw formulated turkey rolls decreased from 25.3% to 15.0% after 60 wk frozen storage at -12 °C (Table 5.7). A decrease ($P<0.05$) in extractable protein, from 3.8% to 3.3%, was found in turkey rolls processed to internal temperature 68.3 °C after 13 wk frozen storage, but no change ($P>0.05$) in extractable protein was measured between 13 and 60 wk frozen storage. Extractable protein did not differ ($P>0.05$) in turkey rolls processed to 69.7 °C and above during frozen storage. The LDH activity of

Table 5.7. Effect of frozen storage (-12 °C) on extractable protein and lactate dehydrogenase (LDH) activity of turkey rolls[a]

	Internal cooking temperature of turkey rolls (°C)				
	Raw	68.3±0.3	69.7±0.3	70.9±0.3	72.1±0.3
Extractable protein (%)					
0 wk	25.3±0.20[b]	3.8±0.08[b]	3.6±0.02[b]	3.4±0.02[b]	3.5±0.04[b]
6 wk	19.8±0.37[c]	3.6±0.08[b]	3.5±0.17[b]	3.5±0.18[b]	3.4±0.09[b]
13 wk	17.9±0.22[d]	3.3±0.06[c]	3.3±0.14[b]	3.3±0.04[b]	3.3±0.11[b]
32 wk	15.5±0.54[e]	3.3±0.06[c]	3.5±0.06[b]	3.5±0.05[b]	3.3±0.14[b]
60 wk	15.0±0.44[e]	3.3±0.14[c]	3.6±0.12[b]	3.6±0.15[b]	3.5±0.17[b]
LDH activity (U/g of sample)					
0 wk	736.5±44.04[b]	22.0±12.16[b]	2.3±0.35[b]	1.4±0.09[b]	1.2±0.06[b]
6 wk	53.1±20.06[c]	11.7±2.91[b]	1.8±0.01[bc]	1.1±0.11[c]	1.1±0.32[b]
13 wk	8.9±2.80[d]	10.6±2.31[b]	1.8±0.04[bc]	1.2±0.05[bc]	1.1±0.10[b]
32 wk	1.9±0.17[e]	8.9±1.86[b]	1.5±0.08[c]	1.1±0.04[c]	1.0±0.05[b]
60 wk	2.1±0.20[e]	10.1±2.14[b]	1.6±0.22[c]	1.2±0.04[bc]	1.2±0.07[b]

[a]Turkey rolls were from process A, formulated with 1.2% salt, stuffed into 101 mm diameter casings and processed using a short smokehouse schedule. Expressed as mean ± standard deviation of three replicates.
[b-e]Means in the same column followed by the same letter are not different (P>0.05).

raw formulated turkey rolls decreased during frozen storage, and only about 0.3% initial activity remained after 32 wk frozen storage. However, LDH activity did not change ($P>0.05$) in turkey rolls processed to 68.3 °C and 72.1 °C and changed slightly in those processed to 69.7 °C and 70.9 °C during frozen storage. Our results suggested that frozen storage for 60 wk has little effect on LDH activity of cooked turkey rolls.

LDH content in raw formulated turkey rolls decreased ($P<0.05$) during frozen storage as measured by ELISA (Table 5.8). Although some variation in LDH content was observed in cooked turkey rolls, it did not differ at 0 and 60 wk storage at -12 °C. Since turkey rolls were sliced into several pieces for the storage study, the variation in LDH content might be due to slight differences in endpoint temperatures among slices.

During preparation of formulated turkey rolls, mixing in the presence of NaCl and Na tripolyphosphate causes cell disruption and fiber swelling with subsequent release of cell contents (Price and Schweigert, 1987). Protein solubility, and LDH content and activity decreased after frozen storage of raw formulated turkey rolls but did not decrease in frozen raw turkey muscle. Thus proteins released from muscle cell during preparation of raw formulated turkey rolls might be more susceptible to proteolysis, denaturation and insolubilization during frozen storage.

Table 5.8. Effect of frozen storage (-12 °C) on lactate dehydrogenase (LDH) concentration of turkey rolls[a]

	Internal cooking temperature of turkey rolls (°C)				
	Raw	68.3±0.3	69.7±0.3	70.9±0.3	72.1±0.3
0 wk	2409.9±221.1[b]	5.28±0.50[b]	0.76±0.10[b]	0.06±0.02[b]	0.01±0.00[b]
6 wk	251.42±48.03[c]	8.28±0.46[c]	1.20±0.14[c]	0.09±0.02[b]	0.03±0.01[c]
13 wk	29.52±8.13[d]	6.90±1.13[bc]	1.16±0.19[c]	0.09±0.03[b]	0.03±0.01[c]
32 wk	2.26±0.36[e]	5.54±0.94[b]	0.85±0.10[bc]	0.06±0.02[b]	0.03±0.01[c]
60 wk	1.82±0.31[e]	6.25±1.09[b]	0.78±0.13[b]	0.07±0.01[b]	0.02±0.00[b]

[a]Turkey rolls were from process A, formulated with 1.2% salt, stuffed into 101 mm diameter casings and processed using a short smokehouse schedule. Expressed as mean ± standard deviation of three replicates.
[b-e]Means in the same column followed by the same letter are not different (P>0.05).

D. Conclusion

A sandwich LDH ELISA could be used to determine if precooked turkey rolls were processed to the minimum USDA required internal temperature of 71.1 °C. Extractable protein and LDH activity were not suitable methods as these tests could not differentiate between products cooked to 70.0 °C and 71.1 °C. Results suggested that turkey rolls made from frozen and thawed turkey meat would not influence the effectiveness of the sandwich ELISA to detect compliance with the USDA minimum endpoint processing temperature of 71.1 °C.

CHAPTER SIX: ENZYME-LINKED IMMUNOSORBENT ASSAY FOR ENDPOINT COOKING TEMPERATURE OF UNCURED TURKEY ROLLS BY QUANTIFICATION OF LACTATE DEHYDROGENASE, SERUM PROTEINS AND IMMUNOGLOBULIN G

6.1 Abstract

The efficacy of using an LDH sandwich enzyme linked immunosorbent assay (ELISA), a turkey serum protein sandwich ELISA and an immunoglobulin G (IgG) indirect competitive (IC) ELISA for determining endpoint cooking temperature of uncured turkey thigh rolls were studied. Five LDH isozymes were found in raw turkey thigh rolls. The LDH ELISA could not differentiate endpoint temperature of rolls processed between 68.9 and 71.1 °C due to the presence of three heat stable isozymes. The intensity of a 66,000-Da band, identified as turkey serum albumin, decreased in extracts as processing temperature was increased. Serum protein content of extracts was assessed by IC-ELISA and were found to decrease as the cooking temperature increased. Serum protein ELISA differentiated between products cooked to the minimum required endpoint of 71.1 °C and those processed 2.2 °C below the minimum. The IgG content was low and did not differ in turkey thigh rolls processed between 68.9 and 72.2 °C.

6.2 Introduction

Title 9 of the Code of Federal Regulations requires thermal treatment of meat products to ensure the destruction of harmful microorganisms and viruses. The possibility of food borne disease outbreaks from precooked meats contaminated by pathogens such as *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* is a concern by meat processors, consumers and the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS). Rapid and accurate methods are needed to verify the adequacy of thermal treatment of meat products.

Presently, the USDA-FSIS uses a residual phosphatase assay (USDA-FSIS, 1986a), catalase test (USDA-FSIS, 1989), or a coagulation test (USDA-FSIS, 1986b) to verify endpoint cooking temperatures of beef and pork. These assays are time consuming, empirical and subjective (Townsend and Blankenship, 1989). When cooked under USDA-FSIS inspection, uncured poultry products are required to be cooked to an internal temperature of 71.1 °C (USDA-FSIS, 1992); however, the USDA does not have a standard assay for verifying processing temperatures in poultry.

The loss of solubility of water soluble or sarcoplasmic proteins (primarily glycolytic enzymes) is related to heating time, endpoint temperature and initial concentration of soluble proteins in muscle (Davis and Anderson, 1984). Residual enzyme activity has been

corelated to internal processing temperature of meat products in several studies (Davis et al., 1988; Townsend and Blankenship, 1989; Collins et al., 1991a; 1991b; Davis et al., 1991; Anonymous, 1992; Bogin et al., 1992; Townsend and Davis, 1992; Kormendy et al., 1992; Townsend et al., 1993; Ang et al., 1993; Hsu et al., 1993). It has been reported that lactate dehydrogenase (LDH) activity could be used as an endpoint processing indicator in beef, pork and turkey muscle (Collins et al., 1991a, 1991b, Stalder et al., 1991; Bogin et al., 1992; Townsend and Davis, 1991; Hsu et al., 1993). McCormick et al.(1987) also showed that LDH content in porcine muscle extracts decreased between 65 and 70 °C.

Our laboratory has previously shown that LDH concentration can be used as an endpoint cooking indicator in commercially prepared turkey breast rolls (Wang et al., 1992). A competitive indirect enzyme-linked immunosorbent assay (ELISA) (Wang et al., 1992) and sandwich ELISA (Abouzied et al., 1993) were developed to detect LDH in turkey breast rolls. Both ELISAs accurately differentiated the endpoint cooking temperature within ±1.1-1.2 °C between 68.3-72.1 °C. Salt concentration, cooking schedule and product casing diameter of turkey breast rolls did not have a marked influence on sandwich LDH ELISA in determining endpoint cooking temperature; a maximum concentration of 0.31 μg LDH/g meat was found to indicate proper processing of turkey breast rolls (Wang et

al., 1993).

Townsend and Davis (1991) and Townsend et al. (1993) reported that LDH from dark meat was more heat stable than that from light meat. Thus, we questioned whether the above described ELISA was applicable to dark meat. The purpose of this study was to investigate the feasibility of using ELISAs for LDH and various serum proteins to monitor endpoint cooking temperature of uncured turkey thigh rolls. As turkey thigh muscle contains LDH isozumes different from breast muscle, the ability of LDH antibodies to detect different LDH isozymes was also studied.

6.3 Materials & Methods

A. Materials

Polyoxyethylenesorbitan monolaurate (Tween 20), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), gelatin (type A, from porcine skin), turkey serum proteins, turkey serum albumin (TSA), chicken immunoglobulin G (IgG), polyclonal antibodies to turkey serum proteins (PAb-TS), polyclonal antibodies to turkey IgG whole molecule (PAb-IgG), biotinamido-caproate N-hydroxysuccinimide ester, dimethyl sulfoxide and avidin peroxidase conjugate were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) (Fraction V) was from Ameresco (Solon, OH). Goat anti-rabbit and

anti-mouse IgG conjugated to horseradish peroxidase were obtained from Cappel Laboratories (West Chester, PA). Microtiter plates (Immunolon-2 Removawells) were from Dynatech Laboratories (Alexandria, VA). Turkey muscle LDH was purified using ammonium sulfate and acetone precipitation (Wang et al., 1992). All other chemicals were of reagent grade or better.

B. Conjugation of Biotin to PAb-TS

PAb-TS was purified using a protein G column according to the manufacturer's instructions (Pierce, Rockford, IL) and conjugated to biotin (Harlow and Lane, 1988). Two milliliters antibody solution (3 mg/mL) was prepared in 0.1 M sodium borate buffer (pH 8.8). Biotinamidocaproate N-hydroxysuccinimide ester was dissolved in dimethyl sulfoxide to 5 mg/mL, then 0.12 mL biotin ester solution was added dropwise to the antibody solution (0.1 mg of biotin ester/mg of antibody) with vortexing. After incubation at 25 °C for 4 hr, 48 μL 1 M NH_4Cl (0.08 μL 1 M NH_4Cl/μg of biotin ester) was added and incubated at 25 °C for 10 min. The final antibody solution was dialyzed against PBS.

C. Processing of Turkey Thigh Rolls

Turkey rolls were formulated using 45.4 kg fresh skinless turkey thigh meat, 7.4 kg water, 1.23 kg NaCl, 0.73 kg sugar and 0.23 kg Na tripolyphosphate (Rhone-

Poulenc, Washington, PA). Turkey thigh rolls were processed according to Wang et al. (1993) except that ground meat was mixed with all the ingredients for 25 min after turkey thigh meat was ground though a 24x28 mm kidney plate (Model 4146, Hobart Mfg. Co., Troy, OH). Turkey thigh rolls were smokehouse processed using the schedule in Table 6.1. Each roll weighed about 3.63 kg and measured 10.16 cm in diameter by 35.56 cm in length. Calibration of recorders and probes were described previously (Wang et al., 1993). Smokehouse runs were performed in triplicate. The moisture, protein and fat content of turkey thigh rolls were 73.5±0.31, 16.2±0.11 and 3.9±0.12%, respectively (AOAC, 1990); the pH of turkey rolls was 6.3±0.02.

D. Extraction of Protein and LDH Activity

Meat (25 g) was excised from the geometric center of each turkey roll, adjacent to a thermocouple location, and homogenized and extracted in 0.15 M NaCl, 0.01 M Na phosphate buffer, pH 7.2 (PBS) (Wang et al., 1992). Protein concentration of extracts was determined by the biuret method with BSA as the standard (Gornall et al., 1949). Percentage extractable protein was determined by dividing the protein content of the extract by the protein content of the turkey rolls and multiplying by 100. The LDH activity in each extract was determined using an LDH diagnostic kit (DG 1340-K, Sigma) at 25 °C. One unit of

Table 6.1. Processing schedule for turkey thigh rolls[ab]

Stage	Cycle time (min)	Internal temp.(°C)	Dry bulb temp.(°C)	Wet bulb temp.(°C)
1	60	(30.8±2.0)	60.0	60.0
2	120	(56.3±0.3)	65.6	65.6
3	60	(61.6±0.6)	71.1	71.1
4	60	(67.0±0.5)	75.6	75.6
5	(73±7)	71.7	79.4	79.4

[a]Total cook time for turkey rolls processed to an internal temperature of 72.2 °C was about 6.5 hr.
[b]Values in parentheses indicate mean ± standard deviation of internal product temperature at the end of a cycle or cycle time to reach internal product temperature of 71.1 °C.

LDH activity was expressed as 1 μmol of NADH oxidized per minute.

E. Electrophoresis and Western Blot Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE was performed as described by Wang et al. (1992). SDS-PAGE gels were stained with Coomassie Brilliant Blue R 250. Native PAGE gels were stained for the presence of LDH using an LDH-specific staining solution (Wang et al., 1992).

To determine the binding ability of antibodies to LDH isozymes, turkey roll extract proteins were transferred electrophoretically (1 hr at 100 V) from the native gel to nitrocellulose membrane (0.45 μm, Schleicher & Schuell, Keene, NH) using 25 mM Tris, 192 mM glycine buffer (pH 8.3) in a Mini Trans-Blot unit (Bio-Rad, Laboratories, Richmond, CA). After transferring, the membrane was washed with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween), blocked with 10 mL 3% (w/v) BSA in PBS (BSA-PBS) for 30 min at 25 °C, and rinsed with PBS-Tween. Ten milliliters polyclonal LDH antibody F (1/2000) (Wang et al., 1992) or monoclonal LDH antibody D5E (1/400) (Abouzied et al., 1993) diluted in 3% BSA-PBS was added to the membrane and incubated at 25 °C for 30 min. Unbound antibody was removed by washing with PBS-Tween, and 10 mL goat anti-rabbit IgG peroxidase conjugate (for polyclonal antibody) or goat anti-mouse IgG peroxidase conjugate (for

monoclonal antibody) diluted (1/2000) in 3% BSA-PBS was added to the membrane and incubated at 25 °C for 10 min. The membrane was washed with PBS-Tween, and bound peroxidase was determined (Wang et al., 1992).

To detect turkey serum proteins or IgG in meat extracts, turkey roll extract proteins were transferred electrophoretically (1 hr at 100 V) from the SDS-PAGE gel to nitrocellulose membrane using 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol buffer (pH 8.3). After transferring, the membrane was washed with PBS-Tween, blocked with 10 mL 1% gelatin (w/v) in PBS (gelatin-PBS) for 30 min, and rinsed with PBS-Tween. Ten milliliters of PAb-TS or PAb-IgG diluted (1/1000) in gelatin-PBS was added to the membrane and incubated at for 30 min. Unbound antibody was removed by washing with PBS-Tween, and 10 mL goat anti-rabbit IgG peroxidase conjugate diluted (1/2000) in gelatin-PBS was added to the membrane and incubated for 10 min. The membrane was washed with PBS-Tween, and bound peroxidase was determined (Wang et al., 1992).

F. Sandwich ELISA for LDH and Serum Protein

LDH sandwich ELISA was performed according to Wang et al. (1993) using antibodies against turkey and chicken muscle LDH (Wang et al., 1992; Abouzied et al., 1993). Purified turkey LDH was used to prepare a standard curve in each plate, and results were expressed as nanograms LDH

per gram of turkey roll.

For serum protein ELISA, microtiter wells were coated with 100 μL PAb-TS diluted (1/3000) in 0.1 M carbonate buffer (pH 9.6) and dried overnight at 40 °C in a forced air oven. Wells were washed 4 times with PBS-Tween, and 300 μL 1% gelatin (w/v) in PBS-Tween (gelatin-PBS-Tween) was added to each well and incubated for 30 min at 37 °C to minimize nonspecific binding. After washing 3 times with PBS-Tween, muscle extracts or standard TSA diluted in gelatin-PBS-Tween (100 μL) was added and incubated at 37 °C for 1 hr. Plates were washed 4 more times with PBS-Tween, and 100 μL biotin labeled PAb-TS diluted (1/500) in gelatin-PBS-Tween was added. After incubation for 1 hr at 37 °C and washing 4 times with PBS-Tween, 100 μL avidin peroxidase conjugate diluted (1/1500) in gelatin-PBS-Tween was added to each well. Plates were incubate for 30 min at 37 °C and washed 8 times with PBS-Tween. Bound peroxidase activity was determined with ABTS substrate (Pestka et al., 1982) at 405 nm using a Minireader II (Dynatech, Alexandria, VA). Standard turkey serum proteins were used to prepare a standard curve in each plate, and results were expressed as milligrams turkey serum proteins per gram of turkey roll.

G. Competitive Indirect (CI) ELISA for IgG

Microtiter wells were coated overnight (4 °C) with 100 μl chicken IgG (5 μg/ml) in 0.1 M sodium borate buffer

(pH 9.6). Plates were washed 4 times with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween). Wells were incubated with 300 µl gelatin-PBS at 37 °C for 30 min. After washing 3 times with PBS-Tween, 50 µL meat extract diluted in PBS-gelatin was added to each well followed by 50 µL PAb-IgG (1/5,000) diluted in gelatin-PBS. After incubation for 1 hr at 37 °C and washing 4 times with PBS-Tween, 100 µL goat anti-rabbit IgG peroxidase conjugate (1:500 in gelatin-PBS) was added to each well and incubated for 30 min at 37 °C. Plates were then washed 8 times with PBS-Tween, and bound peroxidase activity was determined as described earlier. Chicken IgG was used to prepare a standard curve in each plate, and results were expressed as nanograms IgG per gram of turkey roll.

H. Statistics

Basic statistics and one-way analysis of variance were performed using MSTAT software (MSTAT, 1989). Mean separations were performed using Tukey's test with the mean square error term at the 5% level of probability.

6.4 Results & Discussion

A. Identification of Protein Indicators

Two protein bands (A and B) in meat extracts decreased in intensity when processing temperature of turkey thigh rolls was increased (Figure 6.1). LDH (band

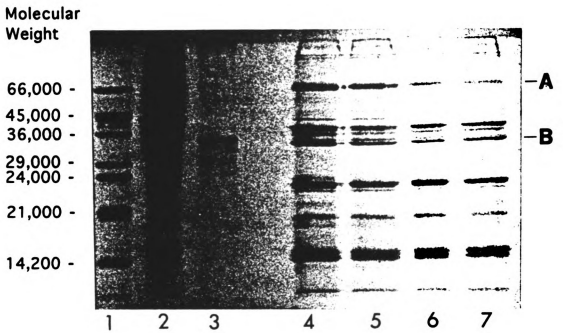


Figure 6.1. Representative sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of muscle extracts from turkey rolls heated to different endpoint temperatures. A is turkey serum albumin (TSA) and B is lactate dehydrogenase (LDH). Lane 1-molecular weight marker; lane 2-raw turkey roll; lane 3- chicken muscle LDH (from Sigma); lane 4-68.9 °C; lane 5-70.0 °C; lane 6-71.1 °C; lane 7-72.2 °C. Twenty micrograms of protein was loaded on each gel lane for turkey rolls.

B) was identified based on work of Wang et al. (1992). The molecular mass of LDH subunit is 35,000 (Holbrook et al., 1975). SDS-PAGE of turkey thigh rolls (Figure 6.1) showed that a single LDH band was observed in cooked turkey thigh rolls (lanes 4-7). Similar band patterns were observed in meat processed to 68.9 and 70.0 °C (Figure 6.1, lanes 4 and 5).

The intensity of a 66,000 Da band (band A) decreased in meat processed to 71.1 °C (lane 6). Changes in intensity of a 66,000-Da band was also found in turkey breast rolls processed from 68.3 to 72.1 °C (Wang et al., 1992). To identify the 66,000 Da protein, three commercially available purified proteins with similar molecular masses were run on SDS-PAGE (Figure 6.2). The relative mobilities of TSA (66,000 Da), chicken IgG heavy chain (66,000 Da) and phosphoglucomutase (PGM) (61,600 Da) (Ray et al., 1983) were compared to the unidentified 66,000 Da protein. TSA and IgG had relative mobilities similar to the unknown protein (Figure 6.2). There was one major band (55,000 Da) and 2 minor bands in chicken muscle PGM (Figure 6.2, lane 4). PGM is a monomeric enzyme, therefore, the two minor bands in chicken muscle PGM were probably impurities; regardless, results indicated the 66,000 Da band was not PGM.

SDS-PAGE of turkey serum proteins also showed that TSA was the predominant serum protein (Figure 6.2, lane 5) which agreed with the literature (Berne and Levy, 1988).

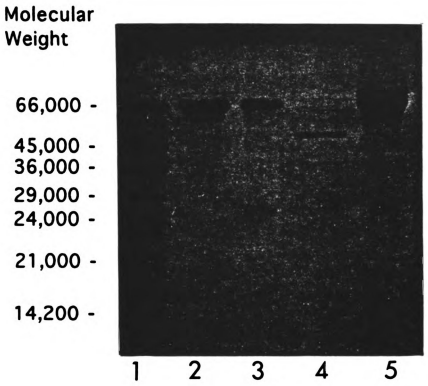


Figure 6.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of standard poultry muscle and serum proteins. Lane 1-molecular weight marker; lane 2-turkey serum albumin (TSA); lane 3-chicken immunoglobulin G (IgG); lane 4-chicken muscle phosphoglucomutase (PGM); lane 5-turkey serum proteins.

Western blot analysis of SDS gels showed that PAb-TS reacted with TSA and turkey thigh roll extracts (Figure 6.3). A major band with same mobility as standard TSA was observed in cooked turkey thigh rolls. These results indicated that the major band in cooked turkey thigh rolls detected by PAb-TS was TSA. Furthermore, the intensity of the TSA band identified by Western blotting decreased as processing temperature of the turkey thigh rolls increased.

IgG is the second most abundant serum protein (Berne and Levy, 1988). Western blot analysis of SDS gels revealed that PAb -IgG reacted with standard chicken IgG and raw turkey thigh roll extracts (Figure 6.4, lanes 1 and 2), but only faint bands were observed in cooked meat extracts (lanes 3-6). Thus, the 66,000 Da protein found in SDS-PAGE of cooked turkey thigh rolls was primarily TSA.

B. LDH as Endpoint Indicator

Extractable protein decreased from 18.3% in raw turkey rolls to 4.2% at an internal processing temperature of 68.9 °C (Table 6.2). A decrease ($P<0.05$) in extractable protein between 68.9 and 71.1 °C was observed. However, extractable protein content did not differ ($P>0.05$) in turkey thigh rolls processed to 70.0 °C and above. LDH activity differed ($P>0.05$) in rolls processed to 68.9 and 71.1 °C. LDH activity did not differ in

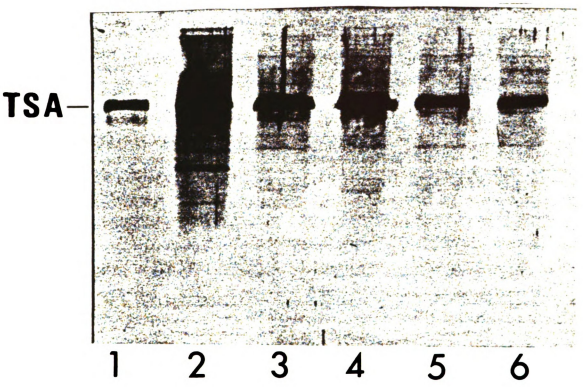


Figure 6.3. Western blot of sodium dodecyl sulfate polyacrylamide gel of turkey serum albumin (TSA) and muscle extracts from turkey thigh rolls heated to different endpoint temperatures. Lane 1-TSA; lane 2-raw turkey roll; lane 3-68.9 °C; lane 4-70.0 °C; lane 5-71.1 °C; lane 6-72.2 °C. The amounts of protein loaded on the gel lane were 0.1 and 10 μg for TSA and turkey rolls, respectively. Polyclonal antibodies to turkey serum proteins (PAb-TS) were used.

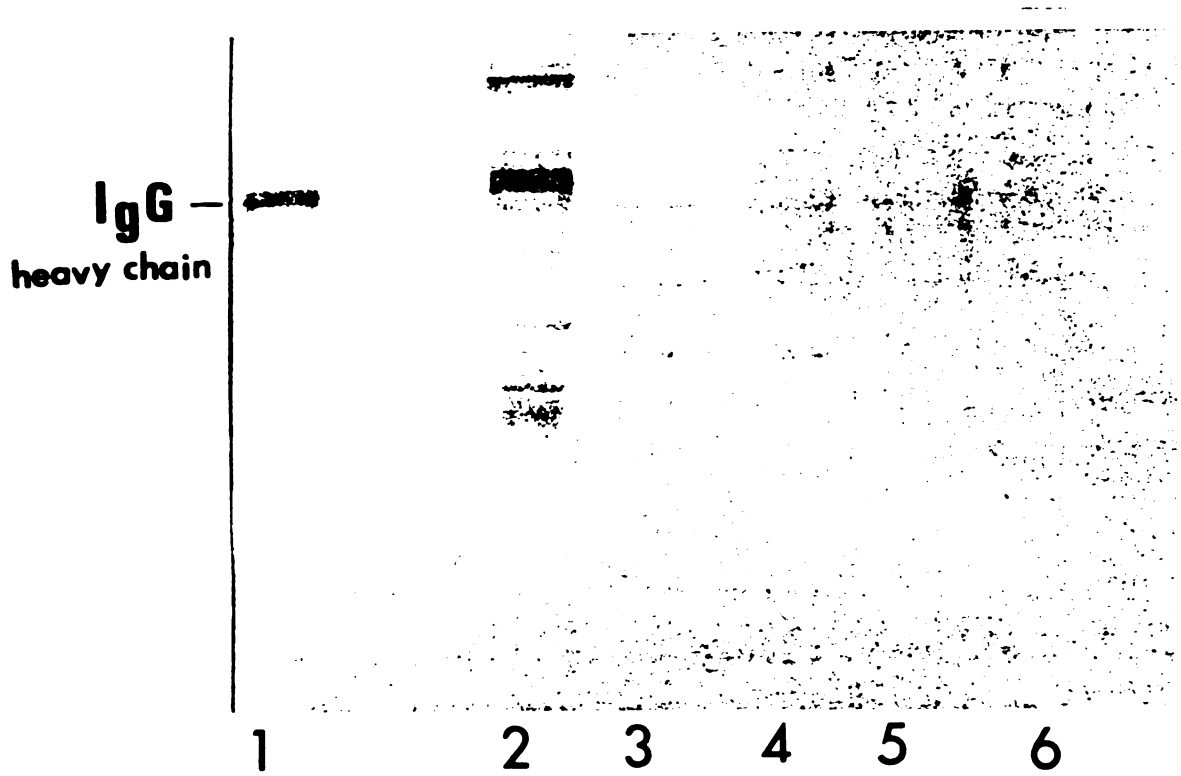


Figure 6.4. Western blot of sodium dodecyl sulfate polyacrylamide gel of chicken immunoglobulin G (IgG) and muscle extracts from turkey thigh rolls heated to different endpoint temperatures. Lane 1- chicken IgG; lane 2-raw turkey roll, lane 3-68.9 °C; lane 4-70.0 °C; lane 5-71.1 °C; lane 6-72.2 °C. Protein loaded on the gel lanes were 0.2, 10 and 20 μg for chicken IgG, raw turkey roll, and heated turkey rolls, respectively. Polyclonal antibodies to turkey IgG (PAB-IgG) were used.

Table 6.2. Influence of processing temperature on extractable protein content and lactate dehydrogenase (LDH) activity of turkey thigh rolls[a]

Internal temp. (°C)	Extractable protein (%)	LDH activity (U/g of sample)
Raw	18.3±1.0[b]	274.5±30.3[c]
68.9±0.3	4.2±0.4[c]	108.0±21.2[d]
70.0±0.3	3.8±0.3[cd]	81.6±7.2[de]
71.1±0.3	3.3±0.2[d]	70.2±2.7[e]
72.2±0.3	3.3±0.3[d]	43.7±7.8[f]

[a]Expressed as mean ± standard deviation of three replicate values.
[b-f]Means in the same column followed by same letter are not different (P>0.05).

turkey rolls processed to 70.0 and 71.1 °C; however, a decrease ($P<0.05$) in LDH activity was observed between 71.1 and 72.2 °C.

Five turkey thigh LDH isozymes were observed on polyacrylamide gels stained with LDH specific stain (Figure 6.5, lane 1). LDH is a tetrameric molecule. The five isozymes consist of five different combinations of two different polypeptide chains called H and M; the electrophoretic labels LDH-1, 2, 3, 4 and 5 refer to the H_4, H_3M, H_2M_2, HM_3 and M_4 tetramers, respectively (Holbrook et al., 1975). Only LDH-1, LDH-2 and LDH-3 were observed in turkey thigh rolls processed to 68.9, 70.0 and 71.1 °C (Figure 6.5, lanes 2, 3 and 4). These results indicated that LDH-1, LDH-2 and LDH-3 were more heat stable than LDH-4 and LDH-5. LDH-3 was not found in turkey thigh rolls processed to 72.2 °C (Figure 6.5, lane 5).

The LDH activity was 70.2 and 43.7 U/g in extracted turkey thigh rolls processed to 71.1 and 72.2 °C, respectively. However, less than 2 U/g of LDH activity remained in turkey breast rolls processed to the same temperatures (Wang et al., 1992, 1993). Wang et al. (1992) showed that LDH-5 was the predominant isozyme in turkey breast rolls. This isozyme was not detected when thigh rolls were heated to 68.9 °C and above. Thus, higher LDH activity in cooked turkey thigh rolls was attributed to the presence of more heat stable isozymes in thigh muscle.

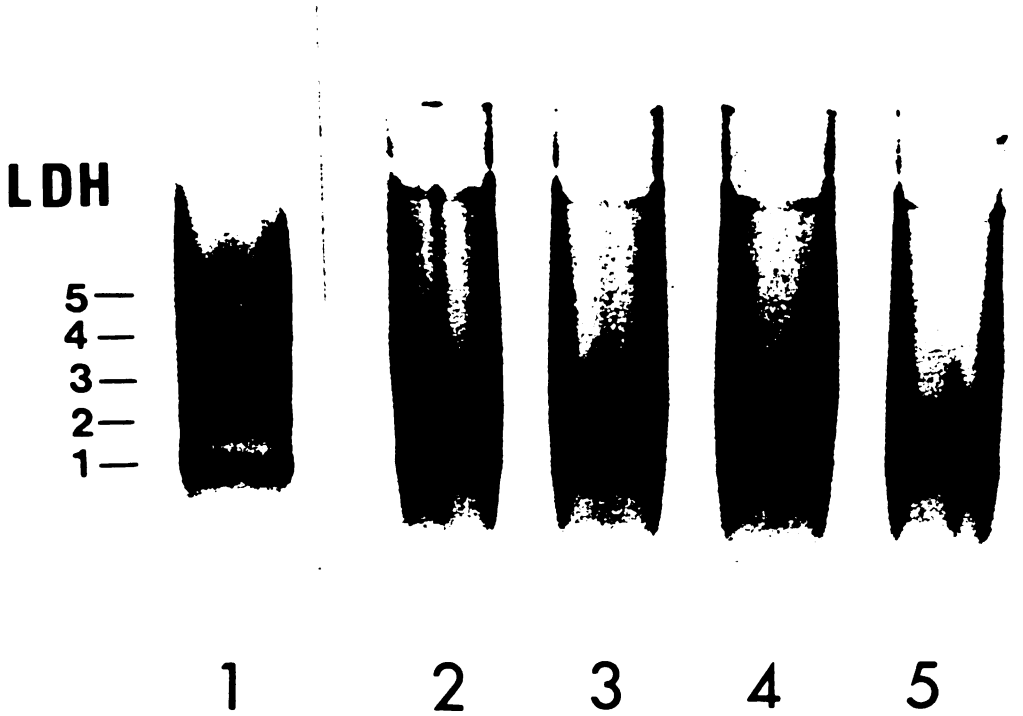


Figure 6.5. Representative native polyacrylamide gel electrophoretogram with lactate dehydrogenase (LDH)-specific stain of muscle extracts from turkey thigh rolls heated to different endpoint temperatures. Lane 1-raw turkey roll; lane 2- 68.9 °C; lane 3-70.0 °C; lane 4-71.1 °C; lane 5-72.2 °C. Ten and twenty micrograms of protein was loaded for raw and heated samples, respectively.

Western blot of native polyacrylamide gel of turkey thigh roll extracts with polyclonal antibodies (Figure 6.6) showed that there were three major bands (LDH-3, 4 and 5) and a faint band (LDH-2) in raw turkey thigh rolls (lane 2). These results indicated that polyclonal antibodies raised against turkey muscle LDH only recognized isozymes containing the M form. The intensity of LDH-3 and LDH-2 bands were similar at processing temperatures of 68.9, 70.0 and 71.1 °C (lanes 3, 4 and 5), whereas a less intense LDH-3 band was observed at 72.2 °C. LDH-3 band was detected at 72.2 °C in Western blot, but not observed on polyacrylamide gels stained to detect enzyme activity. Some LDH-3 molecules were probably partially denatured, which caused a conformational change in the region of active site and loss of enzyme activity; but such change did not influence the binding of antibodies to another portion of the LDH-3 molecule.

The LDH content measured by sandwich ELISA for raw turkey thigh rolls was 217,120 ng (Table 6.3), which was about 1/10 of that in turkey breast rolls (Wang et al., 1993). The LDH content did not differ ($P>0.05$) between thigh rolls processed to 70.0 and 71.1 °C. Two major bands (LDH-4 and LDH-5) and 1 faint band (LDH-3) were detected on Western blots (Figure 6.7, lane 2) of native polyacrylamide gel containing raw turkey thigh roll extracts with LDH MAb D5E. These results suggested that MAb D5E mainly bound LDH isozymes with at least 2 subunits

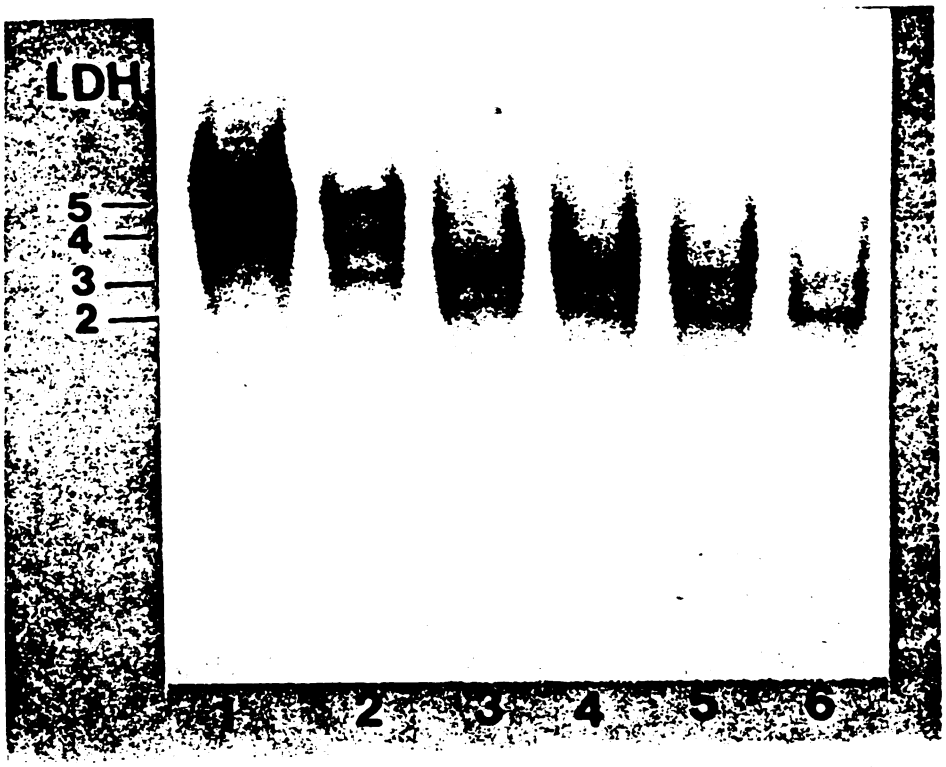


Figure 6.6. Western blot using polyclonal antibodies of native gel of turkey muscle lactate dehydrogenase (LDH) and muscle extracts form turkey thigh rolls heated to different endpoint temperatures. Lane 1-isolated turkey muscle LDH; lane 2-raw turkey roll, lane 3-68.9 °C; lane 4-70.0 °C; lane 5-71.1 °C; lane 6-72.2 °C. Protein loaded on the gel lanes were 0.2, 2.5 and 20 μg for isolated turkey muscle LDH, raw turkey roll, and heated turkey rolls, respectively.

Table 6.3. Influence of processing temperature on lactate dehydrogenase (LDH) content in turkey thigh rolls as measured by sandwich enzyme-linked immunosorbent assay (ELISA)[a]

Internal temp. (°C) of turkey rolls	LDH content (ng/g of sample)
Raw	217,124±46,778[b]
68.9±0.3	461±80[c]
70.0±0.3	346±38[cd]
71.1±0.3	305±51[d]
72.2±0.3	146±45[e]

[a]Expressed as mean ± standard deviation of three replicate values.
[b-e]Means in the same column followed by same letter are not different (P>0.05).

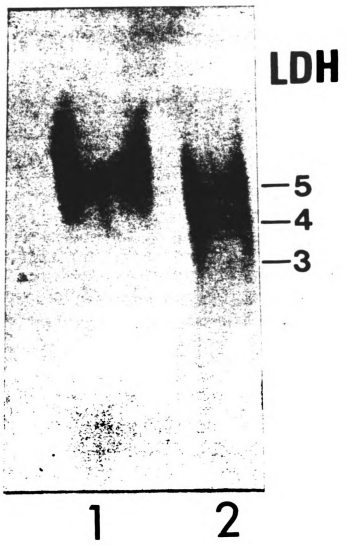


Figure 6.7. Western blot using monoclonal antibody of native gel of isolated turkey muscle lactate dehydrogenase (LDH) and muscle extract form raw turkey thigh rolls. Lane 1-isolated turkey muscle LDH; lane 2-raw turkey roll. Protein loaded on the gel lanes were 0.4 and 5 μg for isolated turkey muscle LDH and raw turkey roll, respectively.

of M form. Thus, the lower LDH content of raw turkey thigh rolls as compared to breast rolls was due to the differential binding ability of MAb D5E to LDH isozymes.

Changes in LDH activity, LDH content, and LDH-3 intensity were found between turkey thigh rolls processed to 71.1 and 72.2 °C. Results suggested that these assays could differentiate turkey thigh rolls processed to these temperatures. Moreover, turkey thigh rolls are usually cooked commercially to a minimum internal temperature of 73.9 °C (165 °F) to extend shelf life (Weiner, 1987; Weiner, 1992) and to insure safety in products cooked in food service establishments (FDA, 1976). Because of higher stability of thigh LDH isozymes, LDH activity and LDH ELISAs were better suited for detection of higher endpoint cooking temperatures in products containing thigh meat.

C. Serum Proteins as Endpoint Indicators

A sandwich ELISA with PAb-TS as capture antibodies and biotin labeled PAb-TS as detector antibodies was developed to detect turkey serum protein in meat products. Turkey serum protein content decreased as internal processing temperature of turkey thigh rolls was increased (Table 6.4). A decrease ($P>0.05$) in turkey serum proteins content was found between 68.9 and 71.1 °C, and 70.0 and 72.2 °C. There was no difference ($P>0.05$) in serum protein content between turkey rolls processed to 70.0 and

Table 6.4. Influence of processing temperature on the content of serum proteins and immunoglobulin G (IgG) in turkey thigh rolls measured by enzyme-linked immunosorbent assay (ELISA)[a]

Internal temp. (°C) of turkey rolls	Turkey serum protein content (mg/g sample)[b]	IgG content (ng/g sample)[c]
Raw	39.22±13.43[d]	658,243±289,102[d]
68.9±0.3	2.36±0.37[e]	1,258±389[e]
70.0±0.3	1.82±0.30[ef]	1,097±193[e]
71.1±0.3	1.23±0.27[fg]	771±287[e]
72.2±0.3	0.89±0.23[g]	605±254[e]

[a]Expressed as mean ± standard deviation of three replicate values.
[b]Measured by sandwich ELISA.
[c]Measured by competitive indirect ELISA.
[d-g]Means in the same column followed by same letter are not different (P>0.05).

71.1 °C. These results suggested that serum protein ELISA could differentiate turkey thigh rolls processed to 71.1 C and 2.2 °C below the temperature. The IgG content in cooked meat extracts determined by CI-ELISA was low and did not differ in rolls processed between 68.9 and 72.2 °C (Table 6.4). This was consistent with Western blot analysis described earlier.

D. Conclusion

High residual LDH activity in cooked turkey thigh rolls was due to heat stable isozymes. The LDH ELISA could not be used to determine endpoint temperature of turkey thigh products processed between 68.9 and 71.1 °C due to the presence of heat stable LDH isozymes in thigh muscle. LDH ELISA might be better suited to differentiate turkey thigh rolls processed to temperatures above 71.1 °C. The decrease in the intensity of a 66,000 Da protein was attributed to the loss of TSA during thermal processing of turkey thigh rolls. Serum protein ELISA could be used to differentiate turkey thigh rolls processed between 68.9 and 71.1 °C. TSA was the major serum protein remaining in cooked turkey thigh rolls. Only small quantities of IgG remained in turkey thigh rolls processed to 68.9 °C and above.

CHAPTER SEVEN : LACTATE DEHYDROGENASE MONOCLONAL ANTIBODY SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY FOR EPITOPE MAPPING AND DETECTION OF TURKEY MUSCLE IN RAW BEEF AND PORK

7.1 Abstract

A monoclonal-based lactate dehydrogenase (LDH) sandwich enzyme-linked immunosorbent assay (ELISA) was developed to detect turkey muscle in beef and pork. Relative epitope binding positions on turkey muscle LDH for four monoclonal antibodies (MAbs) was determined by comparing the absorbance of sandwich ELISA from different combinations of capture MAbs and biotin-labeled detector MAbs. Epitopes for MAbs B3C and G4D were separated from D5E by a considerable distance, and epitopes for MAbs E6B and B3C were close to each other. The LDH MAb sandwich ELISA detected 1% adulteration of turkey breast or thigh muscle in raw beef and pork using D5E as capture MAb and biotin-labeled B3C as detector MAb.

7.2 Introduction

Immunoassays are used to detect adulteration, additives, microorganisms, mycotoxins, pesticides, drugs, and other constituents in foods (Rittenburg, 1990;

Samarajeewa et al., 1991; Morgan et al., 1992). Moreover, enzyme-linked immunosorbent assays (ELISAs) with monoclonal antibodies (MAbs) have been developed to study changes in structure and epitope (antigenic determinant) regions of proteins, such as ovalbumin (Varshney et al., 1991; Ikura et al., 1992) and ß-lactoglobulin (Kaminogawa et al., 1989) during heat treatment.

Detecting adulteration of meat products with unlabeled meat species is important for economic, health and religious reasons (Andrews et al., 1992). Antibodies against whole serum, serum albumins, muscle proteins, and heat-resistant antigens have been used in ELISAs to identify adulteration of raw and cooked meat products (Whittaker et al., 1982, 1983; Dincer et al., 1987; Kang'ethe and Gathuma, 1987; Berger et al., 1988; Martin et al., 1988a,b; Martin et al., 1991; Andrews et al., 1992; Sherikar et al., 1993).

Polyclonal antibodies (PAbs) against chicken muscle lactate dehydrogenase (LDH) and turkey muscle LDH, and four MAbs against chicken muscle LDH have been produced in our laboratory (Wang et al.,1992; Abouzied et al., 1993). Turkey muscle LDH PAbs cross-reacted with chicken muscle LDH and vice versa, but not with LDH from bovine, porcine or rabbit muscle (Wang et al., 1992). Chicken muscle LDH MAbs cross-reacted with turkey muscle LDH, but not with LDH from other species (Abouzied et al., 1993).

Epitope mapping is used to study whether individual

MAbs raised against the same antigen bind to identical or overlapping epitopes (Harlow and Lane, 1988). If epitopes are located in relatively close proximity on the antigen's surface, mutually competitive binding for MAbs will occur (Tzartos et al., 1981; Kordossi and Tzartos, 1987). Competitive ELISAs have been used for epitope mapping to investigate the relative position among the epitopes recognized by different monoclonal antibodies (Smith-Gill et al., 1984; Pfeiffer et al., 1987; Smith and Wilson, 1992). For example, Smith and Wilson (1992) found that twenty-three MAbs against rat brain hexokinase were divided into nine groups on defined surface regions of the N- or C-terminal domains of the enzyme using competitive epitope mapping methods.

Sandwich ELISAs are reproducible and sensitive assays for detecting adulteration of meat products (Berger et al., 1988; Martin et al., 1988a,b; Martin et al., 1991; Andrews et al., 1992); however, these assays were based on PAbs or a combination of MAbs and PAbs. Skerritt and Hill (1990) have developed a reliable and quantitative sandwich ELISA based on MAbs to detect gluten in foods. Because of the species-specificity of LDH MAbs and unlimited supply of MAbs, it is highly desirable to develop a LDH MAb sandwich ELISA to detect meat adulteration. The purpose of this study was to determine the relative position of MAb epitopes on turkey muscle LDH to design an effective monoclonal-based sandwich ELISA,

and to investigate the feasibility of using this ELISA for detecting turkey muscle in beef and pork products.

7.3 Materials & Methods

A. Materials

Polyoxyethylenesorbitan monolaurate (Tween 20), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), biotinamido-caproate N-hydroxysuccinimide ester, dimethyl sulfoxide and avidin-horseradish peroxidase conjugate were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) (Fraction V) was from Ameresco (Solon, OH). Microtiter plates (Immunolon-2 Removawells) were from Dynatech Laboratories (Alexandria, VA). Turkey breast muscle LDH was purified using ammonium sulfate and acetone precipitation (Wang et al., 1992). All other chemicals were of reagent grade or better.

B. Conjugation of Biotin to LDH MAbs

Production of MAbs against chicken muscle LDH was described previously (Abouzied et al., 1993). Four MAbs (D5E, B3C, G4D and E6B) were purified with 50% ammonium sulfate precipitation (Abouzied et al., 1993) and a protein G affinity column (Pierce, Rockford, IL). Biotin was conjugated to purified MAbs according to Harlow and Lane (1988).

C. Titration for Biotin-Labeled MAbs

Biotin-labeled MAbs were titrated to determine the appropriate concentration to use in sandwich ELISA. Microtiter wells were coated overnight (4 °C) with 100 µl turkey muscle LDH (5 µg/ml) in 0.1 M sodium borate buffer (pH 9.6). Plates were washed four times with 0.15 M NaCl, 0.01 M Na phosphate buffer, pH 7.2, containing 0.05% (v/v) Tween 20 (PBS-Tween). Wells were incubated with 300 µL 1% BSA (w/v) in 0.15 M NaCl, 0.1 M Na phosphate buffer, pH 7.2, containing 0.2% (v/v) Tween 20 (BSA-PBST) at 37 °C for 30 min to minimize nonspecific binding. After washing 3 times with PBS-Tween, 50 µL biotin-labeled MAbs diluted (1:50-1:800) in BSA-PBST was added to each well. After incubation for 1 hr at 37 °C and washing five times with PBS-Tween, 100 µL avidin peroxidase conjugate (1/1500 in BSA-PBST) was added to each well and incubated for 30 min at 37 °C. Plates were then washed eight times with PBS-Tween. Bound peroxidase activity was determined by adding 100 µL substrate solution (0.1 M citrate buffer, pH 4.0, containing 0.025% (w/v) ABTS and 0.02% (v/v) H_2O_2) to each well. After 10 min at 25 °C, the reaction was stopped by adding 100 µL 0.3 M citric acid containing 0.1% (w/v) Na azide to each well. Absorbance was read at 405 nm using a Minireader II (Dynatech).

D. Sandwich ELISA for Epitope Mapping

Microtiter wells were coated with 100 µL MAbs (as

capture antibodies) diluted (1/400 for D5E, 1/100 for B3C and G4D, and 1/50 for E6B) in 0.1 M carbonate buffer (pH 9.6) and dried overnight at 40 °C in a forced air oven. Wells were washed 4 times with PBS-Tween, and 300 μL BSA-PBST was added to each well and incubated for 30 min at 37 °C. After washing 3 times with PBS-Tween, 100 μL turkey muscle LDH in BSA-PBST was added and incubated at 37 °C for 1 hr. Plates were washed 4 more times with PBS-Tween, and 100 μL biotin-labeled MAbs (as detector antibodies) diluted in BSA-PBST was added. After incubation for 1 hr at 37 °C and washing 4 times with PBS-Tween, 100 μL avidin peroxidase conjugate diluted (1:1500) in BSA-PBST was added to each well. Plates were incubated for 30 min at 37 °C and washed 8 times with PBS-Tween. Bound peroxidase activity and absorbance was determined as described earlier. Relative position of epitopes was determined by comparing the absorbance of sandwich ELISA from different combinations of capture antibodies and detector antibodies.

E. Detection of Turkey in Meat

Pork sirloin, lean ground beef, turkey thigh and skinless ground turkey breast were purchased from a local market. Pork or skinless turkey thigh was ground through the 4-mm plate of a KitchenAid grinder (Hobart Corp., Troy, OH). Fifty grams of meat containing 1, 3, 10 and 20% (w/w) ground turkey breast or thigh were homogenized

with 150 mL 0.15 M NaCl, 0.01 M Na phosphate buffer (pH 7.2) in a Waring blender for 90 sec. The homogenate was centrifuged at 16000 x g for 20 min at 4 °C, and the supernatant was filtered through Whatman No. 1 filter paper. Meat extracts were diluted (1:100-1:10000) in BSA-PBST and the amount of turkey muscle LDH was determined by sandwich ELISA. Purified turkey muscle LDH (0-100 ng/ml) was used to prepare a standard curve in each plate, and results were expressed as micrograms LDH per gram of meat. Experiments were performed in triplicate.

F. Statistics

Simple linear regression analysis was performed with Lotus 1-2-3 program (Lotus Development Corporation, Cambridge, MA). All experiments were performed in triplicate.

7.4 Results & Discussion

A. Epitope Mapping

For epitope mapping, each biotin-labeled detector MAb must be used at a dilution that produced equal absorbance in ELISA. The concentration of biotin-labeled MAbs used in our study was based on the titration curves shown in Figure 7.1. A similar ELISA absorbance (about 1.5) was observed for biotin-labeled D5E, B3C, G4D and E6B at 400, 200, 125 and 50-fold dilution, respectively. Thus, these

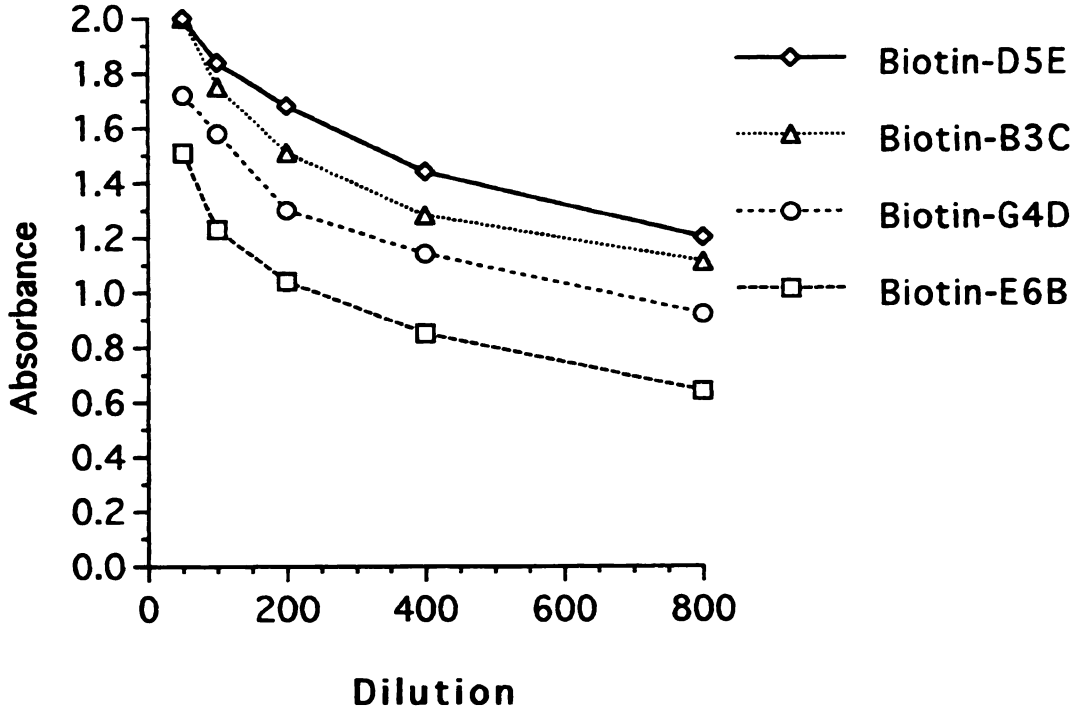


Figure 7.1. Titration curves of biotin-labeled lactate dehydrogenase (LDH) monoclonal antibodies (MAbs). Microtiter wells were coated with turkey muscle LDH. Serial dilutions of biotin-labeled LDH MAbs were used. Results are average of three replicate values.

dilutions of biotin-labeled MAbs were used in our study for epitope mapping.

When D5E was used as capture antibody, ELISA absorbance for biotin-labeled B3C and G4D increased from about 0.3 to 1.6-1.7 as LDH concentration was increased to 100 ng/ml (Figure 7.2). These results suggested that the epitopes for B3C and G4D were separated from D5E such that binding of LDH to capture MAb (D5E) did not inhibit the binding of detector MAbs (biotin-labeled B3C or G4D) to the same LDH molecule. Therefore, peroxidase-conjugated avidin could bind biotin-labeled MAb and color development was observed via the enzyme reaction with substrate.

ELISA absorbance for biotin-labeled E6B increased from about 0.2 to 0.55 as LDH concentration was increased to 100 ng/ml when D5E was used as capture antibody (Figure 7.2). This result suggested that epitopes for E6B and D5E were close to each other, and binding of D5E to LDH inhibited access for biotin-labeled E6B. Since both unlabeled and labeled D5E recognized the same epitope, ELISA absorbance for biotin-labeled D5E as detector antibody was very low (about 0.5 at 100 ng/ml of LDH) as expected (Figure 7.2). It has been reported that an average protein epitope covers an area of about 2-7 nm^2, and the antigen binding region of immunoglobulin G binds to a circular area of about 10 nm^2 with a diameter of about 3.5 nm on the surface of the antigen (Tzartos et al., 1981; Kordossi and Tzartos, 1987). Hence, the

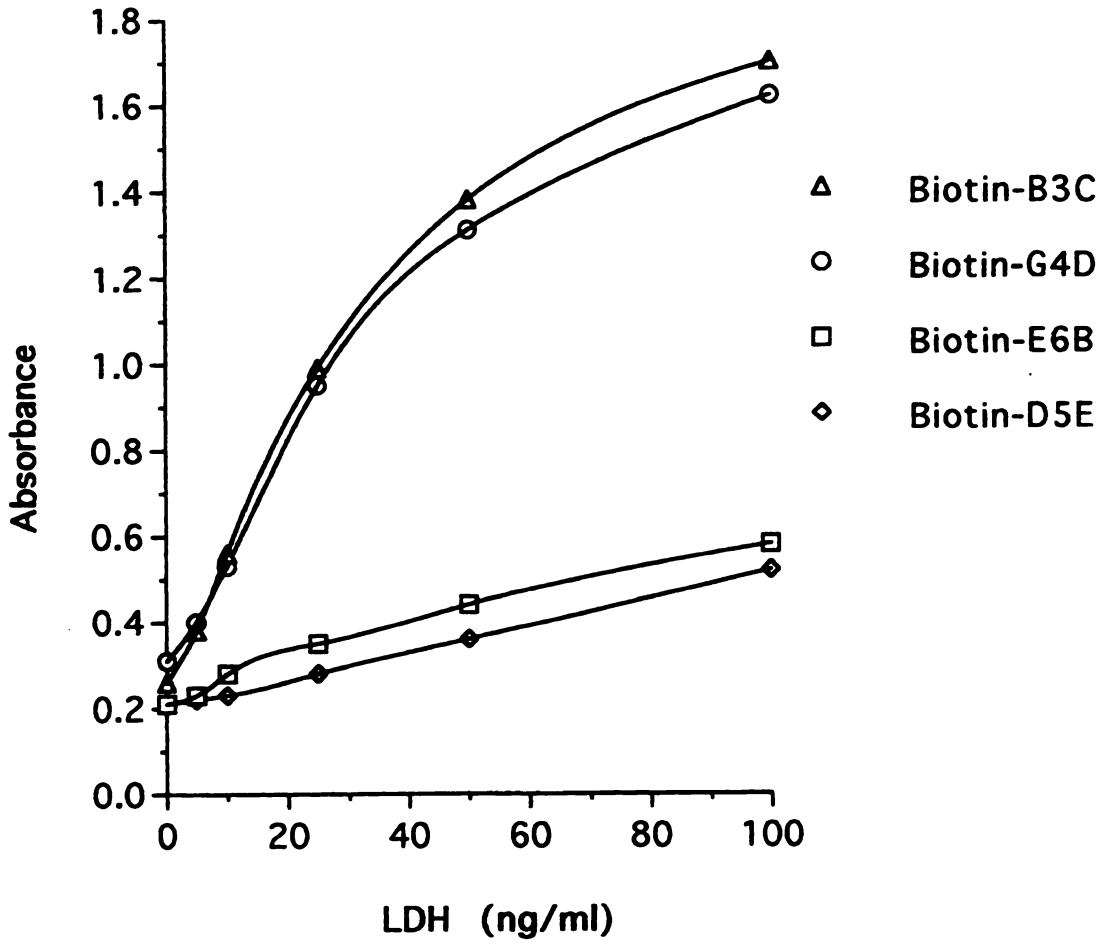


Figure 7.2. Sandwich enzyme-linked immunosorbent assay for epitope mapping of lactate dehydrogenase (LDH) monoclonal antibodies (MAbs). Microtiter wells were coated with MAb D5E as capture antibody. Biotin-labeled D5E, B3C, G4D and E6B at 400, 200, 125 and 50-fold dilution, respectively, were used as detector antibodies. Results are average of three replicate values.

distance between epitopes for E6B and D5E is probably less than 3.5 nm; the binding sites for B3C and G4D are probably separated from D5E by a distance greater than 3.5 nm.

The ELISA absorbance measured at 100 ng/ml of LDH was about 0.70 for the pairs B3C and labeled B3C, and G4D and labeled G4D (Figure 7.3); the absorbance was about 0.5-0.6 for the pairs D5E and labeled D5E, and E6B and labeled E6B at 100 ng/ml of LDH. The background absorbance (without LDH) was about 0.2-0.3. These results indicated that when the capture MAb was bound to turkey muscle LDH, there were available binding sites for the same biotin-labeled detector MAb. We previously demonstrated that turkey breast muscle LDH used in our study contained four M subunits (Wang et al., 1992, 199X). It is possible that the same epitopes were found on different M subunits of turkey muscle LDH, and capture antibodies did not bind to all the epitopes on the surface of LDH. Hence, some epitopes were still available for biotin-labeled MAbs after LDH molecules were bound by the same capture MAbs.

When B3C was used as capture MAb, the difference in ELISA absorbance between biotin-labeled G4D and biotin-labeled B3C was about 0.1-0.2 (Figure 7.3). This result suggested that the epitopes for B3C and G4D were close to each other and the distance between these two epitopes was less than 3.5 nm. A similar result was also obtained using G4D as capture MAb. High ELISA absorbance (>1.30)

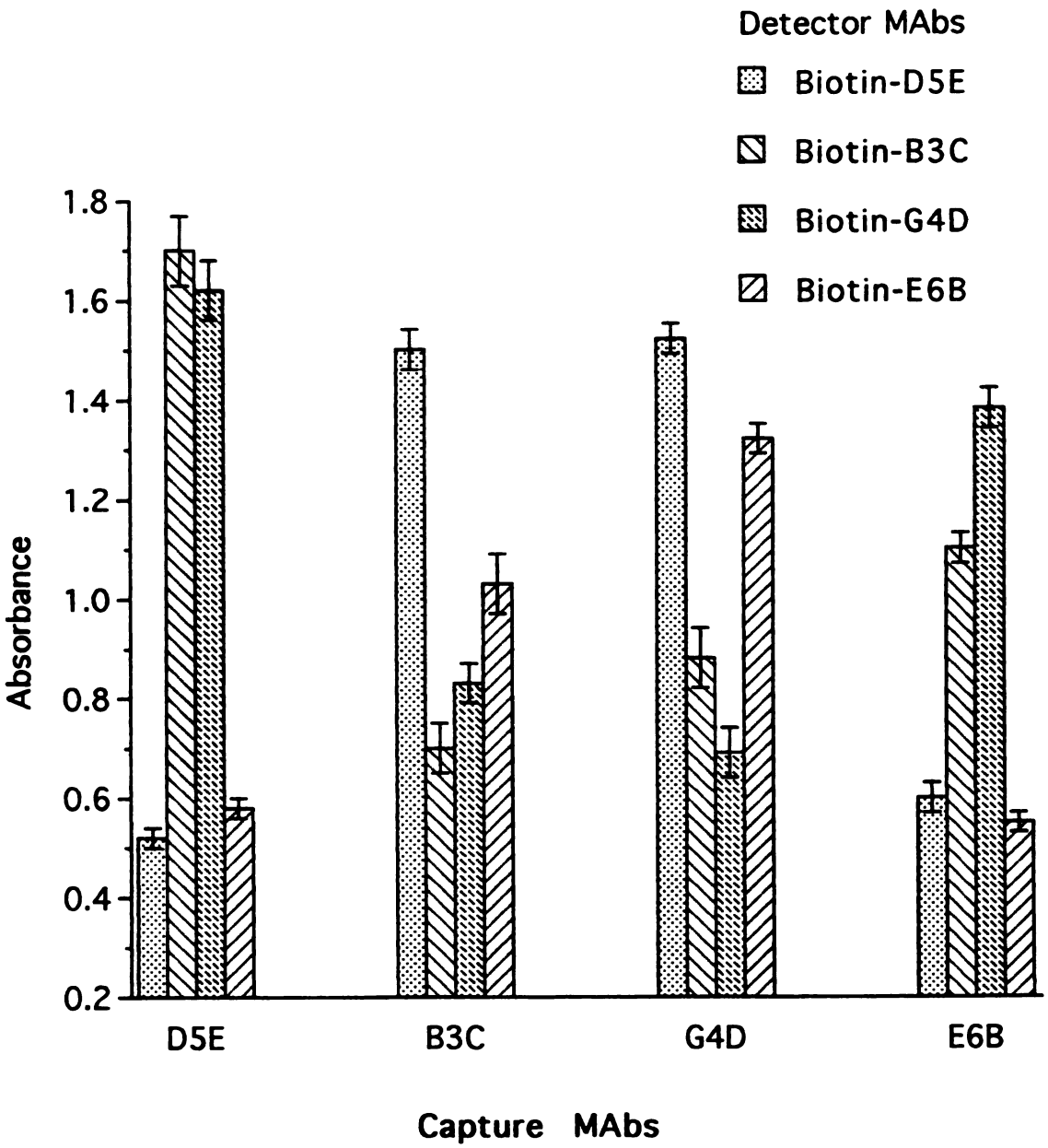


Figure 7.3. Sandwich enzyme-linked immunosorbent assay (ELISA) for epitope mapping of lactate dehydrogenase (LDH) monoclonal antibodies (MAbs). Microtiter wells were coated with different MAbs as capture antibodies. Biotin-labeled D5E, B3C, G4D and E6B at 400, 200, 125 and 50-fold dilution, respectively, were used as detection antibodies. ELISA absorbance was measured at 100 ng/ml of turkey muscle LDH. Means ± standard deviation of three replicates are shown.

was observed for the pairs G4D and biotin-labeled E6B, and E6B and biotin-labeled G4D. These results indicated that the epitopes for G4D and E6B were not close to each other.

The ELISA absorbance for biotin-labeled B3C was 1.1 when E6B was used as capture antibody. This value was between those observed for an epitope distance <3.5 nm (about 0.6) and those for an epitope distance >3.5 nm (about 1.5). These results suggested that steric hindrance occurred and binding of LDH to E6B partially inhibited the subsequent binding of biotin-labeled B3C. Therefore, an intermediate ELISA absorbance was observed. It has been reported that when epitopes are close enough (about 3.5 nm), steric hindrance will occur, and binding of one antibody partially prevents binding of a second antibody (Preiffer et al., 1987; Wilson, 1991). Thus, the distance between epitopes for B3C and E6B was probably about 3.5 nm. A similar result was also found using B3C as capture MAb and labeled E6B as detection MAb. From the above results, the relationship between epitopes for each LDH MAb could be determined, and a possible model depicting the relative position of each epitope was postulated in Figure 7.4.

B. Detection of turkey muscle in meat

Since epitopes for D5E and B3C were not close to each other, an LDH sandwich ELISA with D5E as capture antibody and biotin-labeled B3C as detector antibody was used to

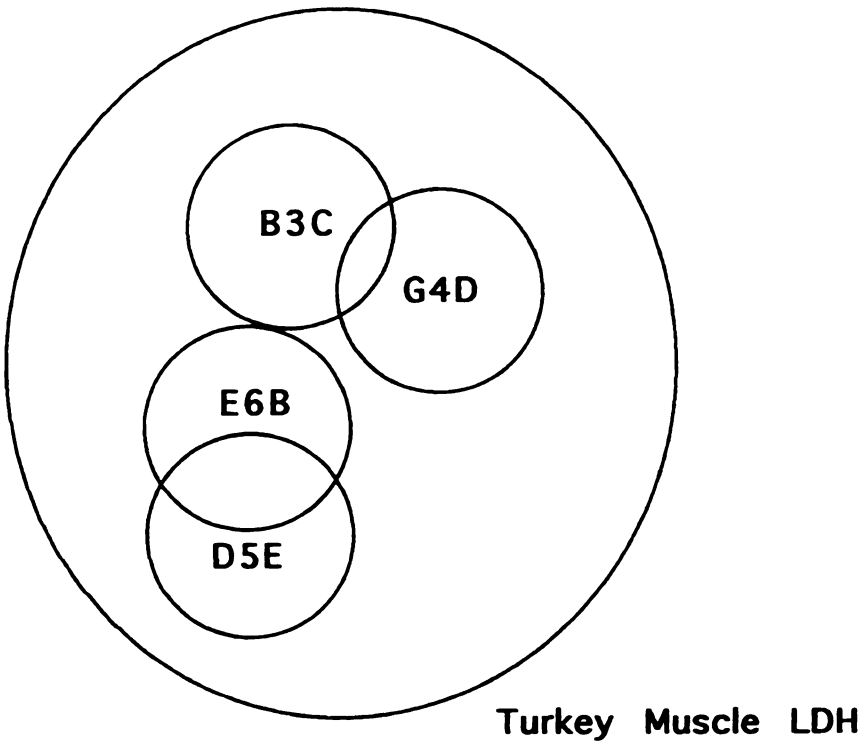


Figure 7.4. Schematic diagram indicating relative position of epitopes for lactate dehydrogenase (LDH) monoclonal antibodies (MAbs) deduced from sandwich enzyme-linked immunosorbent assay. Circles represent the MAbs epitopes on turkey muscle LDH. Overlapping circles suggest that binding of one MAb precludes binding of second MAb.

quantify turkey muscle in beef and pork. The amount of turkey muscle LDH in beef increased as the content of turkey breast or turkey thigh was increased (Figure 7.5). A linear relationship between the amount of turkey muscle LDH and turkey content in beef was observed with correlation coefficients for turkey breast and thigh of 0.992 and 0.975, respectively. A linear relationship between turkey muscle LDH and turkey breast content (r=0.992) and turkey thigh content (r=0.991) in pork was also observed (Figure 7.6). These results implied that the amount of turkey muscle LDH measured by sandwich ELISA might be used to estimate the amount of turkey muscle in pork and beef.

The LDH content of turkey thigh was lower than turkey breast due to the differential binding ability of MAb D5E to LDH isozymes (Wang et al., 199X). Since the amount of LDH in turkey breast and turkey thigh is different, the type of turkey muscle must be known for accurate quantification. Nevertheless, LDH MAbs sandwich ELISA can detect small concentrations of turkey muscle in beef and pork (Figure 7.7). ELISA absorbance for the extracts of beef and pork containing 1% turkey breast or thigh muscle was about 6 and 3 fold higher, respectively, than those containing no turkey. These results indicated LDH MAbs sandwich ELISA could be used to screen for adulteration of raw beef and pork by turkey.

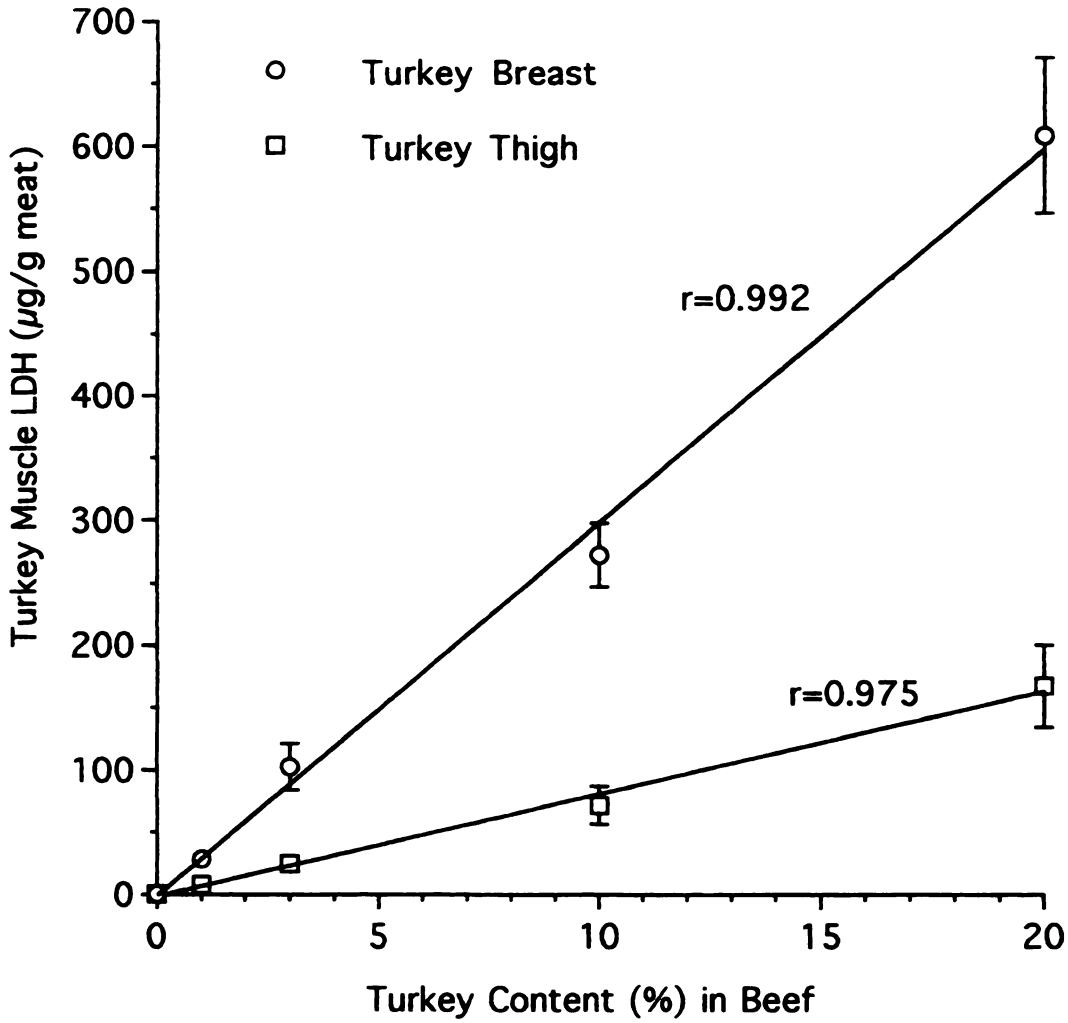


Figure 7.5. Detection of turkey muscle lactate dehydrogenase (LDH) in beef containing different concentrations of turkey breast or thigh muscle using sandwich enzyme-linked immunosorbent assay. Microtiter wells were coated with monoclonal antibody D5E (400-fold dilution) as capture antibody. Biotin-labeled B3C (200-fold dilution) was used as detector antibody. Bars indicate standard deviation of the mean for three replicate values. Correlation coefficient (r) was determined by linear regression.

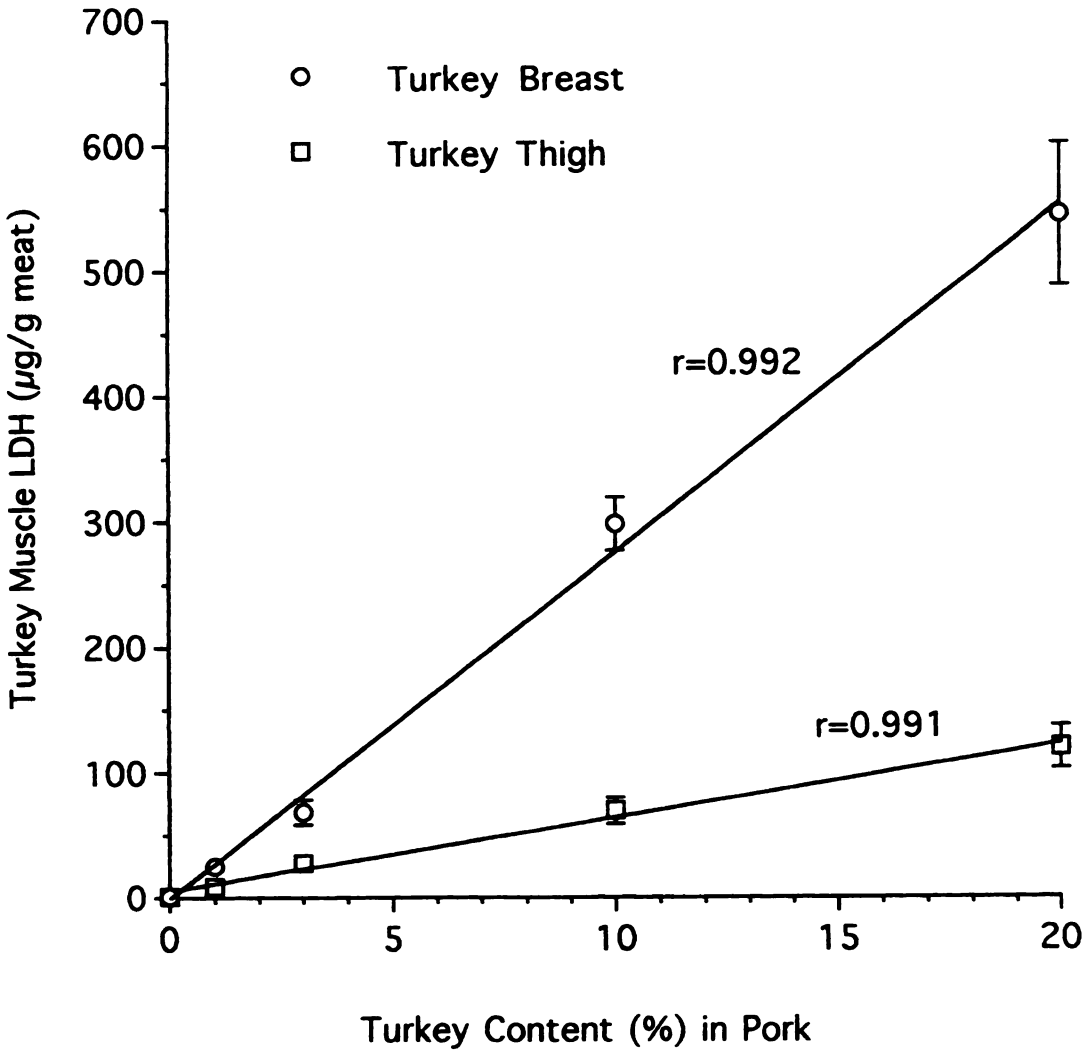


Figure 7.6. Detection of turkey muscle lactate dehydrogenase (LDH) in pork containing different concentrations of turkey breast or thigh muscle using sandwich enzyme-linked immunosorbent assay. Microtiter wells were coated with monoclonal antibody D5E (400-fold dilution) as capture antibody. Biotin-labeled B3C (200-fold dilution) was used as detector antibody. Bars indicate standard deviation of the mean for three replicate values. Correlation coefficient (r) was determined by linear regression.

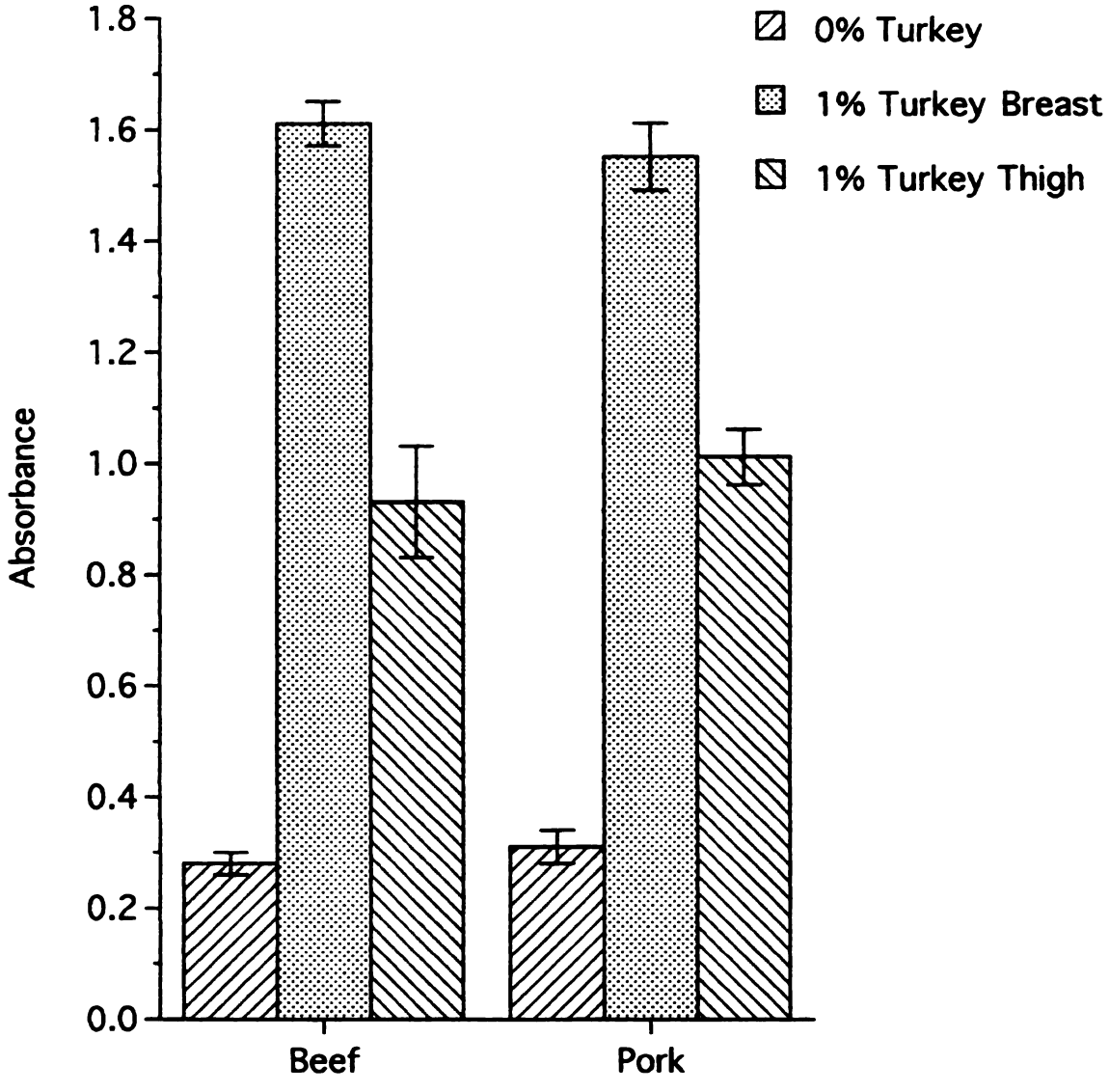


Figure 7.7. Lactate dehydrogenase monoclonal antibody sandwich enzyme-linked immunosorbent assay (ELISA) for detecting turkey muscles in beef and pork. Microtiter wells were coated with monoclonal antibody D5E (400-fold dilution) as capture antibody. Biotin-labeled B3C (200-fold dilution) was used as detector antibody. One hundred-fold dilution of meat extracts from beef and pork containing 0 and 1% turkey muscles were used for comparison of ELISA absorbance. Means ± standard deviation of three replicates are shown.

C. Conclusion

The relative epitope binding positions of four LDH MAbs was defined by sandwich ELISA. Epitopes for MAbs D5E and B3C were separated by a distance greater than 3.5 nm, therefore, D5E was used as capture MAb and biotin-labeled B3C as detector MAb in a sandwich ELISA for detection of turkey muscles in beef and pork. The LDH MAb sandwich ELISA detected 1% adulteration of turkey muscle in raw beef and pork.

CHAPTER EIGHT : CONCLUSION

Lactate dehydrogenase (LDH) was identified as an indicator protein using electrophoresis, enzyme assay, and LDH-specific stain on native polyacrylamide gels. The LDH content in turkey breast roll extracts as measured by indirect competitive enzyme-linked immunosorbent (ELISA) and sandwich ELISA decreased as the cooking temperature increased. This decrease resulted from heat denaturation and insolubilization of the enzyme. While both competitive and sandwich ELISA differentiated between products cooked to 70.0 and 71.1 °C, the sandwich ELISA showed higher sensitivity. Detection limits of sandwich ELISA for turkey and chicken muscle LDH were 1 ng/ml.

Formulation, process, and storage conditions of turkey breast rolls did not have a significant effect on the sandwich ELISA to detect compliance with the USDA minimum endpoint processing temperature of 71.1 °C. Results suggested that turkey rolls made from frozen and thawed turkey breast meat would not influence sandwich LDH ELISA in determining endpoint cooking temperature of turkey breast rolls.

Five LDH isozymes (LDH-1, 2, 3, 4 and 5) were found in raw turkey thigh rolls. However, LDH-5 was the major

isozyme in turkey breast rolls. LDH-1, 2 and 3 were more heat stable and caused high residual LDH activity in turkey thigh rolls cooked to 71.1 °C. Differential binding of polyclonal and monoclonal antibodies to LDH isozymes was observed using Western blot technique. Because of the presence of heat stable LDH isozymes in thigh muscle, the LDH ELISA might be better suited for detection of higher endpoint cooking temperatures (>71.1 °C) in products containing thigh meat.

The decrease in the intensity of a 66,000-Da protein was due to the loss of turkey serum albumin during thermal processing of turkey thigh rolls. Serum protein ELISA was used to differentiate turkey thigh rolls processed between 68.9 °C and 71.1 °C. The immunoglobulin G content in cooked thigh roll extracts measureed by ELISA was low and did not differ in rolls processed between 68.9 and 72.2 °C.

ELISA can screen a large number of samples at the same time by using 96-well microtiter plates. LDH ELISA and serum protein ELISA could serve as simple and rapid methods to determine adequacy of processing of turkey breast rolls and turkey thigh rolls, respectively.

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