DEVELOPMENT OF MONOCLONAL ANTIBODIES FOR A MULTIPLE ANTIGEN ELISA TO VERIFY SAFE COOKING END-POINT TEMPERATURES IN BEEF AND PORK

A Dissertation

by

BRIAN SCOTT HAFLEY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Food Science and Technology

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Approved by:

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ABSTRACT

Development of Monoclonal Antibodies for a Multiple Antigen

ELISA to Verify Safe Cooking End-point Temperatures

in Beef and Pork. (December 2005)

Brian Scott Hafley, B.S., Texas A&M University**;**

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Four proteins exhibiting different rates of denaturation or precipitation with increasing cooking temperature from 63 to 73 °C for beef and 67 to 79 °C for pork were selected for developing a ratio model and incorporating the results into a mathematical expression. Monoclonal antibodies (Mabs) against lactate dehydrogenase isozyme 5 (LDH-5), bovine serum albumin (BSA), porcine enolase, and bovine myoglobin were developed for use in a sandwich enzyme-linked immunosorbent assay (ELISA) to simultaneously investigate changes in protein concentration with incremental increases in temperature.

Four groups of mice were immunized separately with commercially available or purified protein (LDH-5, BSA, enolase, or myoglobin). After reporting ample blood serum titers, spleen cells were harvested and fused with SP2 myeloma tumor cells using an electro fusion cell manipulator. Hybridoma containing wells were screened against their respective protein to isolate hybridomas secreting protein specific Mabs.

Tissue culture flask produced Mabs were used initially in sandwich ELISA assay testing. Mabs were tested against ground beef and pork cooked to instantaneous end-point temperatures (EPTs). A 6 g section removed from the geometric center of each sample was homogenized in phosphate buffer, centrifuged, and a 1 ml aliquot collected for analysis.

Microtiter plates were coated with goat anti-mouse IgG antibody (2 μg/ml) to act as a capture antibody for the protein specific monoclonal antibody concentrated from cell culture supernatant. Serial diluted muscle (beef or pork) extract (10 μl) from each EPT was applied to a microtiter plate. A protein A/G purified polyclonal antibody (Pab) was applied, followed by a goat anti-rabbit IgG peroxidase conjugated antibody. Concentration was determined by comparison to a standard curve.

After multiple cell fusions, 24, 29, 66, and 12 cell lines secreting protein specific Mabs against LDH-5, BSA, enolase, and myoglobin, respectively, were produced. Six Mabs against LDH-5 reported R^2 values > 0.9 indicating high specificity and affinity for LDH-5. Sandwich ELISA assays development with Mabs against BSA, enolase, and myoglobin was not as successful. Mouse ascites produced Mabs against BSA, enolase, and myoglobin were also unsuccessful when used in a sandwich ELISA. However, preliminary data suggested a multiple antigen ratio model still remained a viable option.

DEDICATION

This manuscript is dedicated in honor of the author's parents, Mark and Carolyn Hafley, and his sister Malinda Hafley, who provided the inspiration for pursuit of a further degree, and whose love and encouragement have sustained the author throughout his college education.

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CHAPTER I

INTRODUCTION

An estimated 76 million cases of food-borne illness occur in the United States each year resulting in 325,000 hospitalizations and 5,000 deaths (Mead and others 1999). The Centers for Disease Control Emerging Infectious Disease Program reported that a sustained decrease in *Campylobacter* and *Listeria* had occurred from 1996-2002 (CDC 2002). This finding indicates that progress in reducing the incidence of food-borne infections is being made to achieve the 2010 goals set forth by the national health objectives (NCHS 2000). The report did note that *Escherichia coli* O157:H7 and *Salmonella* showed no sustainable decline from 1996-2002 indicating the need for additional efforts to reduce the occurrence of these organisms (CDC 2002). From 1996-2004, the incidence of *Escherichia coli* O157:H7 infections decreased 42 percent reaching the goal set forth by the Healthy People 2010 goal (USDA-FSIS 2005a). In 2004 *Salmonella* incidence was 14.7 per 100,000 people, exceeding the national objective of 6.8 cases per 100,000 (USDA-FSIS 2004). However, a decrease in incidence of 8 and 41% was reported for *Salmonella* and *Salmonella Typhimurium*, respectively (USDA-FSIS 2005b).

This dissertation follows the style of Journal of Food Science.

In 1996, the United States Department of Agriculture (USDA) issued a final regulation that created the Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) System (USDA-FSIS 1996). HACCP was implemented to reduce the occurrence and numbers of pathogenic microorganisms in meat and poultry products as evidenced by the previous declines in food-borne illness, thus reducing the risk of food-borne disease.

In addition to the Pathogen Reduction program, various categories of meat products must now meet minimal thermal processing (time/temperature) and chilling requirements to control *Salmonella* and *E. coli* O157:H7 (USDA-FSIS 1999a, 2005b), *C. perfringens* and *C. botulinum* (USDA-FSIS 1999b), and *Listeria monocytogenes* (USDA-FSIS 2004). Under Appendix A (USDA-FSIS 1999a) the Food Safety and Inspection Service requires a minimum internal temperature/time of 55 °C (131 °F) for 121 min or > 70 °C (158 °F) instantaneously for domestic cooked beef and roast beef products to meet a 7 $\log_{10}$ reduction of *Salmonella*. Current methods of monitoring end-point temperatures (EPTs) have been deemed inadequate (Townsend and Blankenship 1989; Stalder and others 1991, 1997;). For example, the acid phosphatase test (USDA-FSIS 1986a), used to determine the adequacy of heat-treatment for canned hams, canned picnics, and canned luncheon meat, requires 16 different reagents and considerable time to perform. For beef and pork products cooked below 65 °C, a protein "Coagulation Test" (USDA-FSIS 1986b) can be used but is considered empirical and subjective. Seven time/temperature processing schedules have been established by the USDA for fully cooked, uncured meat patties ranging from 66.1 °C for 41 s to 69.4 °C for 10 s,

however, no reliable testing methods are available to verify adequacy of thermal processing (USDA-FSIS 1999c). In addition, no assay exists for determining the end-point temperature (EPT) achieved in poultry products, but according to USDA-FSIS guidelines poultry products must reach an internal temperature of 71.1 °C for uncured and 68.3 °C for cured/smoked products (USDA-FSIS 1999a). Thus, meat products produced under HACCP require verification of the EPT achieved and the use of verifiable monitoring devices that ensure the safety of these products.

The objectives of this study were to:

1. Purify previously identified proteins (lactate dehydrogenase isozyme 5, serum albumin, myoglobin, and enolase) from beef and pork that could potentially serve as EPT indicators and to quantify their immunologic reactivity at various heating temperature end-points.

2. Produce or acquire a polyclonal antibody (Pab) against each purified protein for initial studies over the heating range of interest.

3. Produce and characterize three to five monoclonal antibody (Mab) sets to the protein antigens that satisfy the appropriate temperature requirements and that are suitable for use in a sandwich enzyme-linked immunosorbent assay (ELISA).

4. Test the ELISA assays developed on extracts from ground beef and pork and develop a predictive ratio model (calibration curve) with an accuracy of ± 0.5 °C of a given EPT.

5. Compare the multiple ELISA assay's accuracy with the EPTs achieved (as monitored by calibrated thermocouples inserted into the geometric center of a product) by testing the expressed juice of serially cooked ground beef and pork for residual immunologic activity.

CHAPTER II

LITERATURE REVIEW

Current testing methods for predicting the EPT achieved in cooked meats rely upon a loss of protein solubility resulting from protein denaturation or coagulation during heating. Loss of protein solubility as a result of denaturation or coagulation has been thoroughly investigated (Hamm and Deatherage 1960; Bendall and Wismer-Pedersen 1962; Paul and others 1966; Trautman 1966; Lee and others 1974; Crespo and Ockerman 1977a, 1977b; Cheng and Parrish 1979). Current methods of monitoring EPTs currently in use have been deemed inadequate (Townsend and Blankenship 1989; Stalder and others 1991, 1997;), therefore, other alternatives to the "Coagulation Test" and residual acid phosphatase activity have been explored in an effort to provide greater accuracy and precision for predicting actual heating end-points achieved.

The "Coagulation Test" currently used by FSIS is based on a decline in or loss of protein solubility. It was initially developed for monitoring the destruction of *Trichinella spiralis* in smoked pork products. The internal EPT achieved in the product is determined by the first appearance of cloudiness in a heated, filtered meat extract. Variation between the actual product temperature and reported test results is less than 2.0 to 3 °C for products heated to an internal temperature of 54 to 57 °C. However, with products heated to higher degrees of doneness (63 to 71 °C), test results can vary as much as 8 to 10 °C from the true product internal temperature. Standardized procedures to determine the first appearance of turbidity (cloudiness) have been tested since 1957

(Coretti 1957; Visacki and others 1966; Olsman 1968; Lyon 1972; Popescu and Din 1982). The lack of consistent correlations with the temperature of coagulation and the actual product temperature were due to variations in sample preparation or filtration treatment. This indicates the "Coagulation Test" to be empirical and subjective (Townsend and others 1984, 1985).

Loss of protein solubility, as measured by the biuret assay, was investigated to estimate the EPT of heat processed pork (Davis and Anderson 1983). It was noted that muscle quality, as defined by raw muscle pH, had considerable impact on protein solubility during cooking and that time/temperature relationships might also affect overall protein solubility at a given temperature. Use of the biuret method to quantify water soluble protein extracts from porcine and bovine muscles has shown protein solubility generally to be a time/temperature dependent process through 70 °C (Davis and others 1985). Difficulties in establishing a specific EPT were confounded with the length of time a muscle had been heated. The biuret-positive water-extract method illustrated the difficulties for determining specific EPTs without establishing mathematical models for each species at specific critical temperature values. Additional investigations determined that water soluble biuret-positive values were lower in canned cured pork picnic shoulders as compared to uncured pork (Davis and others 1987). The study confirmed the difficulties of establishing a method for determining EPTs based on declining protein solubility. It was concluded that extractable biuret positive compounds could establish a yes/no test, but separation of undercooked products would require more accurate and precise measurement technique.

Currently, the residual acid phosphatase activity method is approved for estimating the internal temperature of cooked canned hams, canned picnics, and canned luncheon meat. In an evaluation of the residual acid phosphatase testing method it was revealed that an increase in salt content reduced the heat of inactivation thus causing an increase in residual activity and an erroneous prediction of EPT (Kormendy and Gantner 1960). Based on low correlation values (Visacki and others 1966) and results from tests conducted by Lind (1965), Suvakov and others (1967) recommended that the acid phosphatase test not be adopted for monitoring the maximum internal temperature of canned hams. Olsman (1968) determined that temperature, muscle type, and addition of polyphosphates affect acid phosphatase activity. Their studies showed differences in regression slopes between hams and pork shoulders for denaturation of acid phosphatase. This indicated the necessity for different equations to calculate the EPT from residual acid phosphatase activity. Kormendy and others (1986) determined that acid phosphatase activity was not only dependent on temperature but on time/temperature combinations. They found that container size also affected the heat transfer rate and that the USDA guide book for determining phosphatase activity of cooked hams was only applicable for a single container size.

A variety of other techniques to determine the EPT achieved in cooked meat products have been explored. Loss of protein solubility in bovine muscle as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was evaluated by Lee and others (1974). Water extracts of muscle samples that displayed the presence of four distinct electrophoretic protein bands were determined to be cooked to a

temperature of 70 °C or greater, but only with an accuracy of ± 5 °C. Additional work has shown that low-salt soluble proteins from bovine muscle cooked between 60 to 80 °C gradually disappear and become undetectable above 80 °C (Caldironi and Bazan 1980). Using gradient SDS gel electrophoretic techniques, Steele and Lambe (1982) concluded that protein band intensity decreased with increasing temperature. It was also noted that similar protein conformation states are required when using gel electrophoresis techniques to characterize reference and extracted proteins. Differentiation of deamidated actin components from myofibrillar proteins using isoelectric focusing also has been considered as a potential technique for verification of thermal processing in cooked meat (King 1978).

Dominant spectral wavelengths of beef juices (Nusimovich and others 1979); color of cooked tissue (Marksberry and others 1993); protein conformational changes denoted by fluorescence (Oreshkin and others 1986); near infrared spectroscopy (Isaksson and others 1989; Ellekjaer and Isaksson 1992; Marks and Chen 1996); and differential scanning calorimetry (Wright and Wilding 1984; Ellekjaer 1992) also have been evaluated as potential techniques for verifying the EPT achieved in cooked meat products.

Using reverse phase high performance liquid chromatography, McCormick and others (1987) determined that lactate dehydrogenase, pyruvate kinase, and myoglobin comprised the bulk of the soluble protein fraction from porcine muscle extracts heated to 60 °C for 0 min or to 55 °C for 30 min. Investigating the potential for using proteins as EPT indicators, Wang and others (1996) determined that acid phosphatase, bovine serum

albumin (BSA), glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, phosphoglycerate mutase, and triose phosphate isomerase might be useful in assays to verify thermal processing. These proteins or enzymes were selected based on their high concentrations in raw ground beef and subsequent decline in SDS-PAGE gel intensity with incremental heating.

Selection of proteins or enzymes as EPT markers should be based on several characteristics: (a) their sensitivity to heating and ability to meet the time/temperature criteria; (b) their abundance and specificity in the specific tissues evaluated; (c) their resistance to change or modification by ingredients, cold storage, processing, etc.; and (d) their response in a verification test should be rapid, accurate, and easy to perform (Bogin and others 1992). Bogin and others (1992) evaluated 12 enzymes from turkey breast meat, and determined aspartate aminotransferase, creatine kinase, malic dehydrogenase, lactic dehydrogenase, isocitric dehydrogenase, and aldolase to have high initial specific activities and potential for use as EPT indicators.

Evaluation of enzymes as potential EPT indicators has resulted in numerous investigations. Acid phosphatase (Cohen 1969; Kormendy and others 1987, 1992) and pyruvate kinase (Davis and others 1988) were evaluated as potential indicators in canned hams and pork products, respectively. Spanier and others (1990) reported that catalase from beef homogenates heated up to 74 °C retained more than 50% of its activity. Further investigations for improvement of the "bovine catalase test" resulted in an improved assay procedure for monitoring catalase activity in chicken meat heated between 69 and 71 °C with an accuracy of ± 0.5 °C (Ang and others 1994). However,

the presence of catalase activity could possibly be the result of bacterial catalase not tissue catalase as noted from preliminary data collected at Kansas State University. Their work indicated that catalase activity was lost in ground beef heated to 65 °C for 2 min (Ang and others 1994).

Triose phosphate isomerase was evaluated as an EPT indicator in ground beef (Sair and others 1999) and roast beef cuts (Hsu and others 1999). Orta-Ramirez and others (1997) reported that *Salmonella* lethality curves were similar to thermal inactivation curves for triose phosphate isomerase and that the enzyme had potential as an EPT indicator for roast beef (Hsu and others 1999). Decreases in triose phosphate isomerase activity and concentration were reported for ground beef *semimembranosus* muscle extracts heated from 48.9 to 76.7 °C at approximately 5.5 °C intervals. It was noted that an ELISA was less sensitive to changes in triose phosphate isomerase as compared to enzyme activity. Hsu and others (2000) determined that a maximum residual triose phosphate isomerase activity of about 2 units per gram of meat could potentially be used to differentiate adequately processed roast beef from under-processed roast beef. However, the study also determined that muscle type and sex both influenced triose phosphate isomerase activity in beef roasts.

N-acetyl-β-D-glucosaminidase activity has been identified as a potential EPT indicator for beef, pork, and turkey (Townsend and others 1993). However, the authors concluded that the use of an N-acetyl-β-D-glucosaminidase activity assay to monitor EPTs in beef, pork, and turkey may be limited to samples heated to < 71.1 °C due to residual enzyme activity. Townsend and others (1994) studied the presence of creatine

phosphokinase activity and found it to be product dependent. For commercially prepared chicken and turkey products, creatine phosphokinase levels ranged from 0 to 10.6 Sigma units/ml. However, creatine phosphokinase levels in commercially prepared meat products were more variable with values ranging from 0 Sigma units/ml for beef frankfurters to 258 Sigma units/ml for hard salami. Based on these observations, creatine phosphokinase activity may be useful for quality control, but not as regulatory verification test.

Further studies using enzyme activity to monitor EPT have evaluated glutamic oxalacetic transaminase (GOT). Due to its abundance in muscle tissue (Cornelius and others 1959; Hamm and others 1969; Bogin and Sommer 1976), its stability during several weeks of storage at 0 to 4 °C (Hamm and others 1969), and heat stability during cooking (Hamm 1977; Klinger and others 1982), GOT was considered as a potential EPT indicator in meat. Townsend and Davis (1992) determined that the currently available test kit for determining GOT activity was not adequate for evaluating the EPTs achieved in domestic-type meat products (patties, nuggets, etc.). However, it was suggested that GOT activity might serve as an indicator for imported cooked beef heat processed to 79.4 °C. Searcy and others (1995) confirmed the findings of Townsend and Davis (1992) and reported that GOT activity could be used to confirm heat processing of beef cooked to 79.4 °C. However, the study did question the reliability of the assay in its ability to distinguish cooked beef samples heated to 79.4 and 80 °C. Further research revealed differences in GOT activity between white and dark chicken meat processed to

EPTs of 70 to 75 °C, thus suggesting different activity ranges based on muscle type (Senter and others 1996).

Many studies have explored the potential of lactate dehydrogenase activity as an end-point processing indicator in beef, pork, and turkey (Collins and others 1991a, 1991b; Stalder and others 1991; Townsend and Davis 1991; Bogin and others 1992; Hsu and others 1993). Collins and others (1991a) first noted potential for an enzymatic detection assay based on lactate dehydrogenase (LDH) activity due to observed loss of activity that paralleled the EPT requirements for roast beef products. Upon heating from 55 to 66 °C, Collins and others (1991a) discovered that bovine lactate dehydrogenase enzyme activity declined substantially in cooked bovine muscle tissue. Further investigations of ham muscles revealed that LDH activity was variable based on muscle, age, and storage conditions (fresh vs. frozen). Aging increased LDH activity in *biceps femoris* and *rectus femoris* ham muscles while freezing dramatically decreased activity. In addition, Collins and others (1991b) demonstrated that curing of ham muscles significantly decreased LDH activity when heated to 63.8 and 68.8°C compared to uncured and cured raw ham muscles suggesting that LDH activity would not be a potential indicator of EPT in canned, cured hams. Bovine tissue slurries heated to 63 °C declined in LDH activity at pH 5.6 and 6.4 with no activity reported at pH 4.8 (Stalder and others 1991). The study also concluded that increased salt and phosphate concentrations decreased LDH activity. Similar to the findings of Collins and others (1991b), LDH activity was noted to vary greatly from muscle to muscle, but it was

recommended as a potential indicator for monitoring minimum heating end-points in precooked beef.

An alternative technique to enzyme activity for monitoring proteins is ELISA. Species adulteration, non-meat proteins, microorganisms, bacterial toxins, drugs, hormones, pesticides, and mycotoxins have successfully been detected and quantified through the use of immunological test kits (Krysinski and Heimsch 1977; Griffiths and others 1981, 1984; Hitchcock and others 1981; Skerrit and Smith 1985; Breton and others 1988; Fukal 1991; Samarajeewa and others 1991). Use of immunoassay methods to detect adulteration from different species was first demonstrated by Uhlenhuth in 1901. The method was further utilized in the detection of adulterated ground beef and beef products by species specific serum albumin and myoglobin (Hayden 1978, 1979). Immunoassays are a continually emerging technology replacing traditional chemical methods, and they offer high specificity, sensitivity, ease of use, speed, and adaptability to automation. Due to the inadequacy of current testing methods and the need for a rapid, sensitive, and accurate assay, studies have focused on the use of ELISA techniques for the development of a temperature end-point monitoring device for meat products. Antibodies can be prepared against indicator proteins (antigens) and can recognize either native or denatured epitopes, and amino acid sequences that appear in conformational or linear arrangement. An ELISA could potentially incorporate protein specific antibodies to monitor end-point cooking temperatures by quantitatively determining the appearance or disappearance of targeted protein epitopes.

Early investigations have revealed a partial or complete loss of antigenicity for globular proteins that have been denatured (Crumpton 1974; Reichlin 1975). Ovalbumin heated at 100 °C for 20 to 30 min displays a complete loss of antigenicity by immunoprecipitation (Grabar and Kaminski 1950). However, mild denaturation results in an increase in antigenicity between anti-BSA antibodies and BSA heated to 70 °C for 10 min (Wolberg and others 1970). Early immunological assays were restricted to qualitative (Oudin 1952; Grabar and Williams 1953; Ouchterlony 1961) or quantitative (Boyden 1951; Stavitsky 1954; Kabat 1961) immunoprecipitation. Initial immunological assays lacked sensitivity allowing detection of antigens in the order of 1 mg/ml. Solid phase immunoassay techniques on the other hand have increased the sensitivity of antigen detection to 1 to 10 nanograms of protein (Engvall and Perlmann 1972).

Several immunoassay techniques are currently approved for detecting the presence of adulterated meat products. The Overnight Rapid Bovine Identification Test and Poultry Rapid Overnight Field Identification Test are agar-gel immunodiffusion techniques that are approved by the Association of Official Analytical Chemists International for the detection of raw beef and poultry as adulterants constituting 10% or more of the meat product. Additional immunodiffusion identification tests have been developed for the detection of pork, sheep, horse, and deer meat (Cutrufelli and others 1988, 1989, 1991, 1992). Detection of species adulteration using Pabs against serum albumin or sarcoplasmic proteins required immunosorbent affinity chromatography to reduce species cross-reactivity of the Pabs (Jones and Patterson 1986; Martin and others 1988; Ayob and others 1989). Using Mabs in a sandwich ELISA assay has enabled

detection of turkey and chicken in beef and pork products at a level of 1% (Martin and others 1991). With detection limits of 1 to 2% of a specific contaminant, commercially available test kits configured onto a microplate or immunostick assay are available for 11 animal species (Ritter 1993). In addition to raw meat adulteration, assays for the detection of species contaminates in cooked meat products have been developed based on heat-resistant antigens (Kang'ethe and Gathuma 1987; Berger and others 1988; Andrews and others 1992). Although detection limits were determined at levels as low as 1 to 2%, Pabs used in the assay did display cross-reactivity between species.

ELISA to measure lactate dehydrogenase concentration as a potential EPT indicator in turkey products (Wang and others 1992, 1993; Abouzied and others 1993), roast beef (Morris 1995) and beef patties (Wang and others 1996) have been investigated. Lactate dehydrogenase concentration decreased to less than 4 µg/g of meat at the USDA recommended minimum temperature of 69 °C in ground beef heated at 2 °C intervals between 62 and 74 °C and arrayed in a Pab sandwich ELISA (Wang and others 1995). Further testing of ground beef patties indicated that a critical concentration of 5.0 µg of LDH per gram of patty could be detected in patties heated to at least 68.3 °C. Miller and others (2003a) demonstrated that declining log curves of lactate dehydrogenase isozyme 5 (LDH-5) immunogenicity at different time/temperature combinations paralleled lethality curves for *E. coli* in samples of ground beef. Additional studies revealed significant, but parallel declines in LDH-5 in incrementally heated beef extracts with 0 to 3% salt (Miller and others 2003b).

Pabs are heterogeneous population of antibodies that can require extensive purification to eliminate cross-reactivity. Unlimited production, increased sensitivity, and unique specificity are a few of the advantages of using a homogeneous population of Mabs as compared to Pabs. To measure LDH-5 concentration more accurately monoclonal antibodies have been used in sandwich ELISA assays. Using a Mab developed against chicken lactate dehydrogenase as a capture antibody and a polyclonal anti-LDH chicken or turkey antibody for detection, LDH was detectable at concentrations of 1 ng/ml of chicken or turkey extract (Wang and others 1992; Abouzied and others 1993). Antigen capture with a Mab increased sensitivity 100 times as compared to an indirect competitive ELISA using Pabs for determining LDH concentration in turkey rolls processed to 70 and 71 °C (Wang and others 1993). Turkey rolls processed to 70 and 71 °C were quantifiably differentiated by sandwich ELISA whereas measurements of extractable protein or LDH activity were not significantly different at these temperatures. In addition, salt concentration, cooking schedule or product casing diameter did not influence the sandwich ELISA used to determine LDH concentration. This study did show that LDH concentration, as measured by sandwich ELISA, declined in frozen uncooked turkey rolls indicating the possible need for different testing parameters to distinguish EPTs in fresh vs. frozen products.

Multiple marker proteins are required for different species due to different minimum processing temperature requirements by USDA and the heat stability of proteins that may vary between muscles, and more likely between species (Smith 1995). Miller and others (2003b) suggested that the use of two or more proteins expressed as a

ratio (which tends to reduce the inherent error in absolute measurements more than either component alone) may be more productive as an "end-point temperature assay." Hsieh and others (2002) used a Mab developed for species adulteration to measure the concentration of a thermally stable muscle protein in beef and pork extracts that increased when heated to 100 °C. The presence of a heat-stable protein could serve as a constant in a multiple antigen ELISA assay.

Many of the immunoassay studies that examined a single protein revealed that the accuracy of the assay was limited and indicated that only a maximum EPT temperature achieved in a product could be measured meaningfully. At 69 °C, LDH-5 may become undetectable; therefore, beef cooked to an instantaneous EPT of 71.1 °C could not be distinguished from beef cooked to 69 °C. Therefore, this study proposes a new approach to EPT testing by evaluating the reactivity of three (or more) proteins and expressing them as a ratio to accurately assess (± 1 °C) the EPT achieved in cooked beef and pork extracts.

Three to four proteins with molecular weights ranging from approximately 16 to 64 kilo-Daltons and exhibiting different rates of denaturation or precipitation with increasing cooking temperature (63 to 73 °C for beef and 67 to 79 °C for pork) were selected for developing a ratio model and incorporating the results into a mathematical expression (calibration curve) to achieve a target accuracy of ± 0.5 °C for a given EPT. Previous research focused on a single protein for determining EPT in cooked meat products with varied results. Therefore, the goal of this study was to investigate changes in simultaneous multiple protein concentrations, with incremental increases in

temperature, to determine if a consistent relationship existed among them. Previous studies of protein concentration or enzyme activity have shown potential based on the assay methodology for determining EPT, however, current results have indicated that only a final EPT could be determined due to a lack of precision and accuracy. To increase precision and accuracy, this study will develop Mabs to selected proteins for use in the multiple antigen ratio model. As stated above, ELISA assays that incorporated Mabs showed a significant increase in sensitivity and precision. In addition, the literature has shown that protein concentration can vary between muscles, animals, and species. Therefore, the selected proteins will be evaluated as a ratio and compared to absolute amounts to account for the potential variability.

Compared to Pabs, Mabs offer three distinct characteristics: specificity of binding, homogeneity, and production in unlimited quantities. Köhler and Milstein (1975) developed a technique to grow clonal populations of cells secreting antibodies with a defined specificity. Their work introduced hybridoma technology, fusion of an antibody secreting cell, isolated from an immunized animal, with a myeloma cell, a type of B-cell tumor.

To develop Mabs, animals (mice) are injected with an antigen (protein) followed by routine booster injections (Harlow and Lane 1988). These injections usually incorporate an adjuvant to increase phagocytosis that keep the antigen trapped in a local deposit. After 2 to 3 injections a small sample of blood is collected from the immunized animal to determine blood titers. Blood titers determine the relative level of antibodies present in the blood sera, or indicate the dilution level of sera against a given amount of

antigen. Blood titers provide an indication level of the animal's immune response to the injected antigen and the need for continued immunization boosters to potentially raise blood serum level antibodies against the injected antigen. Upon reaching a useable titer of antibodies in the serum, a final injection series is administered without adjuvant prior to spleen removal and harvesting of the spleen cells.

After removal of the spleen, splenocyte cells are combined in a 1 to 1 ratio with SP2 myeloma mice cancer cells (Harlow and Lane 1988). Cell fusion can be accomplished using electro-fusion as compared to chemical fusion with polyethylene glycol. Following cell fusion, cells are diluted and plated in multiwell tissue culture plates. Screening of hybridoma containing wells commences 1 to 2 weeks later, or after sufficient growth. Positive wells are expanded, frozen, then undergo clonal dilution. Cells from the original fusion plates are transferred to a second multiwell plate, serially diluted, and allowed to grow. Wells with single clonal colonies are re-screened, and positive clones are expanded and frozen. Individual clones are used for Mab production using *in vitro*, static cell culture flasks, or *in vivo*, mouse ascites fluid techniques.

CHAPTER III

MATERIALS AND METHODS

Isolation, purification and testing of proteins and Pabs

Protein isolation and purification. Bovine LDH-5 (L 0508), porcine LDH-5 (L 5762), and BSA (A 3059) were acquired from Sigma-Aldrich Inc. (St. Louis, MO). These proteins, without further purification, were used for mouse inoculation, Mab production, and ELISA sandwich assays. Porcine serum albumin (PSA), enolase, and myoglobin were not available commercially and had to be purified from fresh raw muscle extracts of beef or pork. Fresh beef *semimembranosus* (IMPS #169A) and pork *longissimus dorsi* (IMPS #412E) cuts were acquired from the Rosenthal Meat Science Center at Texas A&M University.

Enolase was purified as described by Merkulova and others (1997). A 95g sample from fresh beef top round and pork loin was homogenized (30 sec on, 10 sec off, 3 times) in 475 ml of cold (4 °C) extraction buffer (15 mM sodium phosphate pH 7.2 containing 4 mM magnesium acetate and a proteinase inhibitor, aprotinin 1 mg/100 ml), in a Waring blender (model 31BL92, Dynamics Corporation of America, New Hartford, CT). Homogenates were centrifuged at 1,500 x g for 15 min at 2 °C (Avanti J-25 using a JA-25.50 rotor, Beckman Instruments Inc., Fullerton, CA). The supernatant and protein precipitates were separated and the supernatant centrifuged for a second time at 75,000 x g for 90 min at 2 °C. Following the second centrifugation step, saturated ammonium

sulfate was added to the supernatants to achieve a concentration of 50, 60, and 80%. Centrifugation was performed between each saturation level at 20,000 x g for 15 min at 2 °C. Based on molecular weight, muscle-specific enolase was recovered in the 60 and 80% fractions of saturated ammonium sulfate. Pellets from the 80% saturation were resuspended in extraction buffer and dialyzed (Spectra/Por® Membrane MWCO: 3500, Spectrum Laboratories Inc., Rancho Dominguez, CA) against large volumes (2 X 4000 ml for 24 h) of 15 mM Tris/HCl buffer, pH 9.0, containing 4 mM magnesium acetate (4 °C).

Dialyzed beef and pork extracts were chromatographed on a DEAE-Sepharose (DFF-100, Sigma) column (2.5 cm x 35 cm) and bound proteins eluted (5 ml fractions) stepwise (pH 9.0 down to 7.0, 0.5 pH units per step, with 15 mM Tris/HCl at 4 °C). Fractions were measured with a spectrophotometer (Cary 300 Bio, Varian Instruments, Sugar Land, TX) at 280 nm. Concentrated pooled fractions were evaluated by SDS-PAGE and Western blotting as described below. The stained SDS gel and Western blot were evaluated to select fractions for further purification with phenyl-sepharose (P-2459, Sigma) chromatography.

Selected fractions were pooled, concentrated, and brought to a 30% ammonium sulfate saturation then chromatographed on a phenyl-sepharose column (2.5 cm x 6.5 cm) at 4 °C. A stepwise elution from 30 to 0% ammonium sulfate was conducted at 10% intervals. Fractions were analyzed at 280 nm on a spectrophotometer (Cary 300 Bio, Varian Instruments, Sugar Land, TX), pooled and then evaluated by SDS-PAGE and Western blotting as described below. The stained gel and Western blot confirmed

muscle specific enolase fractions that were aliquoted and frozen (-20 °C) for later use in the study.

Myoglobin was purified as described by Quinn and others (1964). The 80% saturated ammonium sulfate supernatants from beef and pork extracts that yielded enolase were further saturated to 100% using ammonium sulfate crystals. The 100% saturated beef and pork extracts were centrifuged at 20,000 x g for 15 min (2 °C), the pellets (containing beef or pork myoglobin) resuspended in distilled deionized water (pH 7.0), and dialyzed (Spectra/Por® Membrane MWCO: 3500) in a 75% solution of ammonium sulfate overnight at 4 °C. After 24 h, the precipitate was discarded and the remaining supernatant was dialyzed (Spectra/Por® Membrane MWCO: 3500) against large volumes of 0.1 M triethanolamine (TEA) buffer at pH 7.0 containing 0.2 M sodium chloride (4 °C) until free of ammonium sulfate. The myoglobin extract was transferred to dialysis tubing (Spectra/Por® Membrane MWCO: 3500) and concentrated to approximately 50 ml using Aquacide III (17852, Calbiochem, San Diego, CA) to remove excess media and components less than 3,500 molecular weight. Five ml aliquots were then applied to a polyacrylamide (Bio-Gel P-150, 1502240, Bio-Rad, Hercules, CA) molecular weight chromatography column (2.5 cm x 35 cm) and myoglobin eluted with TEA-NaCl buffer at 4 °C. Sequential fractions (5 ml) were read spectrophotometric-ally at 280 and 525 nm. Ratio values of absorbance at 280 divided by absorbance at 525 were calculated. A ratio index of 4.5 or less was considered purified myoglobin. Purified fractions from beef and pork muscle extracts were identified and confirmed by SDS-PAGE gel electrophoresis and Western blots as described below.

Porcine serum albumin was isolated and purified by fractionation using saturated ammonium sulfate to create a salt gradient for separation of proteins based on molecular weight. The primary fractionation was used for isolation of PSA, while a secondary fractionation gradient was used for purification. For the primary fractionation gradient saturated ammonium sulfated was added to porcine serum (5 ml) at 20% intervals until a final saturation of 80% was achieved (Figure 1A). After each 20% increase with saturated ammonium sulfate, the sample was centrifuged at 25,000 x g for 20 min at 2 °C. The precipitate from the 60 and 80% ammonium sulfate fractionation steps were resuspended in 5 ml of phosphate buffered saline (PBS) for SDS-PAGE evaluation. The precipitates from the 60 and 80% ammonium sulfate primary fractionation steps were subjected to a second ammonium sulfate salt gradient for further purification. The precipitate from the 60% primary fractionation step was taken to 40, 50, 60, and 70% saturation using saturated ammonium sulfate (Figures 1B). Likewise, the precipitate from the 80% primary fractionation step was taken to 60 and 70% saturation using saturated ammonium sulfate (Figures 1C). At each ammonium salt gradient the sample was centrifuged at 25,000 x g for 20 min at 2 °C. The precipitate from each secondary ammonium sulfate fractionation step was resuspended in 5 ml of phosphate buffered saline (PBS) for SDS-PAGE evaluation. Precipitates from the secondary ammonium sulfate fractionation of the 60% precipitate from the primary fractionation step were pooled, dialyzed (Spectra/Por® Membrane MWCO: 3500) at 4 °C in 40% ammonium sulfate and further purified on a phenyl-sepharose column (2.5 cm x 6.5 cm) at 4 °C. A stepwise elution from 40 to 10% ammonium sulfate was conducted at 10% intervals.

Fractions (5 ml) were analyzed spectrophotometrically at 280 nm to identify tubes containing eluted protein (high absorbance > 0.5) and grouped according to elution order. These pooled protein fractions based on elution order were then evaluated by SDS-PAGE.

SDS-PAGE. Gel electrophoresis was performed using 4 to 15% linear gradient Ready Gels from Bio-Rad (161-1104) and run in Tris/Glycine/SDS buffer (161-0732, Bio-Rad) at room temperature (25.5 °C) on a Mini-PROTEAN II electrophoresis unit (Bio-Rad) set at 105 volts. A sample aliquot of the indicator protein was combined with equal volumes (1:1) of Laemmli sample buffer, 62.5 mM Tris-HCl, pH 6.8; 2.0% SDS (w/v); 25% glycerol; 2.5% β-mercaptoethanol (2-mercaptoethanol); and 0.01% (w/v) Bromphenol Blue then heated (90 to 100 °C for 5 to 10 min) to denature and reduce the proteins in the presence of SDS. Gel lanes were loaded based on equal amount of total protein or equal volume. After completing the separation, the gel was placed in a plastic container, rinsed with distilled deionized water, stained using GelCode staining reagent (24590, Pierce, Rockford, IL) for 1 h, then destained using distilled deionized water. The gels were then dried for 10 to 20 min using 50 ml of Gel-Dry solution (LC4025-4, Invitrogen, Carlsbad, CA) and mounted between two DryEase cellophane sheets (NC2380, Invitrogen).

Western blotting. After SDS-PAGE protein separation, the gel was placed in a dish and rinsed with distilled deionized water. The gel was then loaded into a Mini Transblot Cell (Bio-Rad) and the protein bands transferred overnight (4 °C) in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.05% (w/v) SDS) to a

polyvinylidene difluoride membrane (PVDF) (Trans-Blot PVDF 162-0180, Bio-Rad) using a setting of 30V. To prevent non-specific binding, the membrane was placed in a plastic container with 50 ml of Tris-Buffered Saline (TBS) containing 5.0% (w/v) non-fat dry milk (NFDM) and incubated for 2 h at room temperature (25.5 °C) on an orbital shaker. After blocking, the membrane was rinsed three times with 50 ml of TBS plus 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) (TTBS) and incubated for 2 h with rabbit anti-porcine enolase diluted 1:2000 or rabbit anti-human myoglobin (M 8648, Sigma) diluted 1:1000 in 50 ml of TTBS plus 0.5% (w/v) NFDM. The membrane was rinsed a second time as described above to remove any unbound antibody then incubated for 1 h with a peroxidase conjugated goat anti-rabbit IgG antibody (170-5046, Bio-Rad) diluted 1:3000 in 50 ml of TTBS plus 0.5% (w/v) NFDM. The blot was rinsed two times with 50 ml aliquots of TTBS and one final rinse in 50 ml of TBS prior to color development. Development of the membrane was done colorimetricaly using an Opti 4CN substrate detection kit from Bio-Rad (170-8235). The membrane was incubated in 50 ml of color development solution until the desired color was reached. Variation in color development intensity was accounted for by the presence of a protein standard. The intensity of the standard would be proportional to the intensity of the purified proteins.

Production of Pabs. In addition to the purified proteins described, cross-reactive (beef and pork) Pabs against LDH-5, serum album, enolase, and myoglobin were required. Anti-bovine serum albumin (B 1520) and anti-human myoglobin (M 8648) serum antibodies produced in rabbits were purchased from Sigma, while M.D. Anderson

Veterinary Center, Bastrop, TX, produced Pabs against LDH-5 in rabbits (as described below). Anti-enolase Pabs were produced in rabbits at the Laboratory Animal Research Resource, Texas A&M University, College Station, TX. Production of Pabs was conducted as described by Harlow and Lane (1988).

At least two rabbits (New Zealand White) were injected subcutaneously with 100 to 500 µg of protein in neutral saline mixed 1:1 with RIBI adjuvant (M 6661, Sigma) in a volume of 1.0 ml. Booster injections were administered 5 to 10 weeks later and at 15 weeks a final boost of 100 µg of protein minus adjuvant was given prior to sacrifice (16 weeks). Blood titers, using blood harvested from the marginal ear vein, were performed by indirect ELISA, as described below, at four weeks after initial injection and every two weeks thereafter. Approximately 500 µl of blood was collected in a microcentrifuge tube (1.7 ml, 20170-610, VWR International Inc., Sugarland, TX) and centrifuged (model 5410, Eppendorf, Hamburg, Germany) at 12,800 x g for 5 min at room temperature (25.5 °C) to separate sera from whole blood cells. Tubes of serially diluted sera were tested against 2 ng of each protein.

Production and characterization of Mabs

Immunization. Mabs were produced as described by Harlow and Lane (1988). Four groups (n=4) of 6 to 8 week old female BALB\c mice (Harlan Teklad, Madison, WI) were immunized separately with commercially available or purified protein (e.g., bovine LDH-5, BSA, porcine enolase or bovine myoglobin) dissolved in 0.01 M, pH 7.2, phosphate buffered saline (PBS) at a concentration of 0.5 mg per ml and added to RIBI

adjuvant (R-700, Corixa Corporation, Hamilton, MT). Injections were administered intraperitoneally at a rate of 100 µg/mouse initially followed by a 50 µg/mouse booster at two week intervals. After the third injection, approximately 100 µl of blood was collected from the tail vein in a microcentrifuge tube (1.7 ml, 20170-610, VWR) to determine antibody titers by indirect ELISA. Blood samples were centrifuged (model 5410, Eppendorf, Hamburg, Germany) at 12,800 x g for 5 min at room temperature (25.5 °C) to separate sera from whole blood cells. Serially diluted serum was tested against 2 ng of each protein blotted onto a nitrocellulose membrane. Booster injections continued at two-week intervals as needed until titers of 1:2000 or greater were reached. After reporting ample blood serum titers a final set of booster injections (50 µg/mouse, minus adjuvant, 5 and 4 days prior to spleen removal) was administered in preparation for harvesting of spleen cells and cell fusion.

Production of Mabs. Mice were sacrificed by cervical dislocation and spleens removed using aseptic technique. A spleen cell suspension was formed using a scalpel to gently free the cells from the capsule into a petri dish containing 10 ml of serum-free Dulbecco's Modified Eagles Media (DMEM) (D 5796, Sigma). The spleen cell suspension, free from large particulates, was transferred to a 15 ml centrifuge tube and centrifuged at 500 RPM for 5 min. The cells were resuspended in 10 ml of red blood cell lysing solution (R 7757, Sigma) and placed in ice for 5 min. Three rinses in 10 ml of serum-free DMEM were performed to remove red blood cell lysing buffer. The final step was to suspend the spleen cells in 4 ml of fetal bovine serum (FBS) (F 2442, Sigma) containing 10% dimethyl sulphoxide (DMSO) (D 2650, Sigma), then 1 ml aliquots were

transferred to cryogenic freezer vials (66008-284, VWR). Cell vials were placed in a freezing container (55710, VWR) and slowly frozen to -80 °C over 24 h and then transferred to a liquid nitrogen cell storage unit until needed for hybridoma production.

Mouse spleen cells ($1x10^8$ to $1x10^{15}$) were fused with an equal number of SP2 myeloma tumor cells using an electro fusion cell manipulator (ECM 2001, BTX Instrument Division, Harvard Apparatus, Inc. Holliston, MA). Combined spleen cell and myeloma cells were rinsed three times in 5 mls of electrofusion media (0.255 M sucrose, 0.2 mM $CaCl_2$, and 0.2 mM $MgCl_2$). After the final rinse, the cells were resuspended in 1.0 ml of electrofusion media. A 0.5 ml aliquot of the cell suspension was transferred to a meander fusion chamber (model 454, BTX Instrument Division, Harvard Apparatus, Inc. Holliston, MA), connected to the ECM 2001, and fused using an alignment setting of 9V for 15 seconds and a fusion setting of 100V at a pulse length of 40µs for one pulse followed by a post fusion of 8 sec. The fused cell suspension was diluted in 20 ml of complete DMEM media (20% FBS, 1% L-glutamine G 7513, 1% Penicillin-Streptomycin P 4333, 1% Sodium Pyruvate S 8636, Sigma) plus 5ng/ml Interleukin-6 (IL-6) (I 1395, Sigma) and seeded (100 µl/well) onto two, 96-well tissue culture plates (167008, Nalge Nunc International). A second fusion was conducted on the remaining 0.5 ml cell suspension for a total of four, 96-well plates per fusion. Cell cultures were incubated at 37 °C with 5.2% CO_2 (model 3326, Forma Scientific Inc., Marietta, OH).

After 24 h, hypoxanthine, aminopterin, and thymidine (HAT) (H 0262, Sigma) selection media (4 ml/100 ml complete DMEM plus IL-6) was added (100 µl/well) to

fusion plates to terminate any remaining myeloma cells. After 7 d, 50 to 100 µl of media per well was replaced with hypoxanthine and thymidine (HT) (H 0137, Sigma) selection media (4 ml/100 ml complete DMEM plus IL-6) and cells were allowed to grow for another 7 d. Two weeks post fusion, HT cell culture media was replaced (100µl/well) with complete DMEM plus IL-6. Hybridoma containing wells were screened after sufficient growth. Indirect ELISA was used to determine antibody secreting hybridomas to the selected protein. Cell culture supernatant (100 µl) from each hybridoma containing well was transferred to a separate well on a microtiter plate that had been coated with antigen (2µg/ml). Following incubation of cell culture supernatant and coated antigen, a goat anti-mouse IgG labeled antibody was used for detection of monoclonal antibodies. Wells containing monoclonal antibodies to the antigens would bind the labeled detection antibody. Positive wells on the screening plate indicated antibody secreting hybridoma wells on the cell fusion culture plate. Prior to freezing and limiting dilution, antibody secreting hybridoma cells (100µl cell suspension) were transferred to 24-well tissue culture plates (143982-Nalge Nunc) for expansion in complete DMEM plus IL-6 (2 ml/well).

After sufficient growth, cells were screened against their respective antigen for a second time to confirm antibody secreting hybridomas. Positive hybridomas were cloned by limiting dilution. Hybridomas (100 µl cell suspension) were seeded onto a 96-well tissue culture plate in complete DMEM plus IL-6 media and serially (1:2) diluted across the plate to isolate a single hybridoma and establish true individual clone. During

the fusion process, multiple hybridomas could grow within the same well, so limiting dilution was necessary to establish an individual hybridoma and not a collection of hybridomas. In addition to clonal limiting dilution, a 1 ml cell suspension from the 24-well plate was transferred to a tissue culture flask (163371, Nalge Nunc International) in complete DMEM plus IL-6 media for further expansion and freezing to ensure hybridoma preservation. Single colonies on the dilution plate were identified and screened by indirect ELISA to confirm antigen specific antibody secreting hybridomas and the best three colonies per plate were selected for expansion, freezing, antibody production, and subsequent use in a sandwich ELISA.

Selected hybridomas were then grown in tissue culture flasks in complete DMEM plus IL-6 media to collect sufficient quantities (200 to 300 ml) of supernatant for use in sandwich ELISA assays. After complete cell coverage of the flask, 50 ml of media was added to the flask, the flask positioned vertically in the incubator and 3 to 5 d later, the media was collected. The process was repeated until sufficient (200 to 300 ml of cell culture supernatant) quantities of Mabs were harvested. Antibody-containing media was concentrated by ammonium sulfate precipitation.

Due to the incorporation of FBS in the cell culture media, anti-albumin hybridomas had to be grown in serum and protein-free medium (S 2897, Sigma). Following cell fusion, hybridomas from albumin immunized mice were grown in DMEM plus IL-6 culture media as stated above. Hybridoma containing wells were initially screened for IgG production by indirect ELISA versus testing against BSA. Expansion and freezing of antigen specific antibody secreting hybridomas (IgG) was

continued in DMEM plus IL-6 media on 24-well culture plates. At this stage, an identical 24-well plate was seeded with a 1 ml cell suspension from the 24-well expansion plate. This plate was used to slowly substitute serum containing media with serum and protein-free medium (1% L-glutamine G 7513, 1% Penicillin-Streptomycin P 4333, 1% Sodium Pyruvate S 8636, 1% ITS liquid media supplement I 3146, Sigma). Every 3 to 4 d, half the culture media was replaced with serum protein-free medium until minimal quantities of FBS remained. After removal of FBS from the cell culture media, clones could be tested against BSA by indirect ELISA. Cells from each confirmed well were transferred to a separate 96-well plate for clonal dilution in DMEM plus IL-6 media due to its higher growth and production capacity. Single colonies were transferred to a 24-well plate for expansion in DMEM plus IL-6 media. Complete cell culture media in the 24-well plate was slowly substituted with serum protein-free medium for final screening by indirect ELISA to identify a true clone. A 1 ml cell suspension was transferred to a cell culture flask for further expansion in complete cell culture media prior freezing. Selected clones from the 24 well plates, growing in serum protein-free medium, were expanded in tissue culture flasks in serum and protein-free medium for production of sufficient (200 to 300 ml) working quantities of Mabs. Due to the low concentration of protein in serum and protein-free medium, cell culture supernatatn from antibody secreting hybridomas were concentrated in lieu of purification by ammonium sulfate precipitation.

Purification and concentration of Mabs. Ammonium sulfate precipitation was the primary concentration method for separating Mabs from the cell culture supernatant

(200 to 300 ml) used for hybridoma growth. Precipitation of cell growth media was carried out according to Harlow and Lane (1988) by bringing the media to 50% saturation using saturated ammonium sulfate and then centrifuging at 3,000 x g for 30 min at 2 °C. The pellet was resuspended in PBS at 0.1 (40 to 60 ml) times the starting volume (400 to 600 ml). This suspension was dialyzed overnight (Spectra/Por® Membrane MWCO: 3500) against three changes (4 L) of PBS at 4 °C. Sodium azide (0.02% w/v) was added to the concentrated antibody sample and the sample stored at 4 °C until needed. Alternatively, antibody-containing cell culture supernatant was transferred to dialysis tubing (Spectra/Por® Membrane MWCO: 3500) and concentrated up to 75% using Aquacide III (17852, Calbiochem) to remove excess media and components less than 3,500 molecular weight. The remaining concentrates were stored at 4 °C with 0.02% (w/v) sodium azide until needed. A third method used to concentrate Mabs, was molecular weight separation under centrifugation. Using Macrosep centrifugation tubes containing a 100,000 molecular weight membrane filter, 15 ml aliquots of cell culture supernatant were centrifuged at 30,000 x g for 30 min at 2 °C. The process was repeated until the cell culture supernatant was concentrated to 75 to 80% of starting volume. Antibody concentrates were stored as above.

Mouse ascites fluid production. Mouse ascites fluid was produced as described by Harlow and Lane (1988). Four groups (n=4) of 6 to 8 week old female BALB\c mice (Harlan Teklad, Madison, WI) were primed by injecting 0.2 ml of pristine (2,6,10,14-tetramethyldecanoic acid) into the peritoneum. Pristane is an irritant that promotes nutrient secretion and recruitment of monocyte and lymphoid cells. These factors

contribute to a good environment for hybridoma growth. Selected hybridomas (n=4 from each protein) were removed from cryogenic storage, thawed, and transferred to a cell culture flask (163371, Nalge Nunc International) for growth and expansion in complete DMEM cell culture media. Approximately 7-14 days later and after hybridomas are in a rapid growth phase, cells were removed from the flask and centrifuged to separate cell culture media from hybridomas. Hybridomas cells were suspended in 0.5 ml of DMEM without fetal calf serum for injection. Cells (5 x 105 to 5 x 106) were injected into the peritoneum in the general area of the pristine injection site. Two to four weeks later ascites fluid was removed when the mouse peritoneum became noticeably large, but before the mouse had difficulty moving. Ascites fluid was harvested one time only; the animal was euthanized following extraction of the fluid. Using an 18-gauge needle, the peritoneum was punctured allowing the fluid to freely flow into a 15 ml centrifuge tube. Remaining fluid was removed by gently massaging the peritoneum. Mice were sacrificed by cervical dislocation. Ascites fluid was held overnight at 4 °C then centrifuged at 3000 x g for 10 minutes at room temperature (25.5 °C) to separate ascites fluid from the cell pellet. The supernatant (ascites fluid) was carefully removed and aliquoted to smaller microcentrifuge tubes and stored at -20 °C.

Indirect ELISA for blood titers and hybridoma screening

Blood titer determination. The following procedure was used to determine blood titers in immunized mice and rabbits. The procedure was identical for both assays

with the exception of the species specific detection antibody, anti-rabbit or anti-mouse IgG. Using a Bio-Dot (170-3938, Bio-Rad) blotting apparatus, 2 ng of respective protein was blotted onto a nitrocellulose membrane (162-0115 Bio-Rad). The membrane was soaked in TBS buffer prior to loading the Bio-Dot apparatus. After inserting the membrane, 100 µl of TBS buffer was vacuum filtered through each well to thoroughly wet the membrane. The respective protein, 2 ng/100 µl, was added to each well and the apparatus transferred to a shaker to aid gravity filtration. Following filtration of the protein through the membrane, 100 µl of TTBS was aspirated through the membrane. To prevent non-specific binding, the membrane was removed from the Bio-Dot apparatus, placed in a plastic container with 50 ml of TBS buffer containing 5.0% (w/v) NFDM and incubated for 2 h at room temperature (25.5 °C) on an orbital shaker. After blocking, the membrane was rinsed three times with 50 ml of TTBS. The membrane was returned to the Bio-Dot apparatus and collected sera (as described above) were then applied (100 µl/well) in serial dilutions (1:100, 250, 500, 1000, 2000, and 4000) in TTBS. After filtration by gravity, the membrane was removed from the apparatus placed in a plastic container and rinsed a second time to remove any unbound antibody. After rinsing, the blot was incubated for 1 h at room temperature (25.5 °C) on an orbital shaker in 50 ml of TTBS buffer with goat anti-rabbit or anti-mouse IgG peroxidase conjugated antibody diluted 1:3000 (170-6515 GaR, 170-6516 GaM, Bio-Rad). The membrane was then rinsed twice with 50 ml of TTBS and finally with 50 ml of TBS. Antibody detection was determined colorimetrically using an Opti 4CN substrate detection kit

from Bio-Rad (170-8236 GaR, 170-8237 GaM). Immunization boosts of mice or rabbits continued until blood titers of 1:2000 or greater allowed for the detection of 2 ng of protein.

Hybridoma screening. Microtiter plates (3591, Costar, Corning Inc., Corning, NY) were coated overnight (4 °C) with 100 µl/well of respective antigen (2 µg/ml) in coating buffer (0.15 M sodium carbonate, 0.35 M sodium bicarbonate buffer, pH 9.6). After rinsing four times with phosphate buffered saline (PBST) (13.7 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.8 mM Na_2HPO_4, 0.05% Tween 20), supernatant (100 µl) from an identified hybridoma containing well was transferred to one of the wells on the screening plate and incubated overnight at 4 °C. The following day the plate was rinsed four times with PBST, and then 100 µl of a 1:3000 dilution of goat anti-mouse IgG peroxidase conjugated antibody (170-6516, Bio-Rad) in PBST was added to each well. After incubating for 30 min at room temperature (25.5 °C), the plate was rinsed four times with PBST. Bound peroxidase was determined using a tetramethylbenzidine (TMB) substrate kit from Pierce (34021). Mab secreting hybridomas to the selected proteins were indicated by a dark blue color reaction. Positive hybridomas were then transferred to a 24-well plate for further expansion in preparation for freezing and clonal dilution.

Preparation of ground meat samples

To test the antibodies immunologic activity, ground beef and ground pork were cooked to instantaneous EPTs. Use of the ground meat samples reduced sample

variability for the initial ELISA experiments. Fresh beef (*semimembranosus*, top round) and pork (*semimembranosus*, inside ham muscle) stored at 4 °C were trimmed free of fat and connective tissue then finely chopped in a food processor (Cuisinart Custom 11 Pro, Cuisinart Inc., Norwich, CT). Approximately 40 g of finely chopped muscle tissue was placed in sterile, 50 ml polypropylene, disposable, capped centrifuge tubes and centrifuged at 600 x g to remove air pockets. Samples were refrigerated (4 °C) prior to heating to minimize cooking temperature variations.

Samples previously equilibrated to a common chilled temperature (~ 4 °C) were heated in a water bath (Neslab EX-211, Neslab Instruments Inc., Newington, NH) maintained at 55 °C to reduce the effects of rapid heating. Internal core temperatures of the samples were monitored using an NIST-calibrated digital thermometer and type K thermocouple, (model HH82, Omega Engineering, Inc., Stamford, CT) that was placed in the geometric center of the samples. Upon reaching 55 °C, tubes were transferred to a water filled heating block (13259-032, VWR) for additional heating to the desired EPT (63, 65, 67, 69, 71, or 73 °C for beef; 67, 69, 71, 73, 75, 77, or 79 °C for pork). The heating block was maintained at 1 °C above the desired EPT and monitored by an NIST certified mercury thermometer (accurate to ± 0.1 °C). Sample internal temperatures were verified by probing the geometric center of the sample with a thermocouple, and tubes were transferred to an ice bath to cool when the EPT was achieved. The samples were then refrigerated (4 °C) prior to protein extraction.

The instantaneous regulatory mandated safe heating EPT for ground beef is 71.1 °C (160 °F), therefore, ground beef samples were evaluated for immunologic activity after cooking to EPTs of 63, 65, 67, 69, 71, and 73 °C. Instantaneous EPTs for cured and uncured pork ranged from 68.9 °C (156 °F) to 77.3 °C (171 °F), respectively. For this study, pork samples were evaluated after reaching EPTs of: 65, 67, 69, 71, 73, 75, 77, and 79 °C. All EPT sample sets included an uncooked (raw) control held under refrigeration (4 °C) prior to extraction. Upon completion of cooking, all chilled samples in an experiment were homogenized in ice cold sodium phosphate buffer (0.01 M, pH 7.0). A 6 g section was removed from the geometric center of each sample and homogenized (30 sec on, 10 sec off, 3 times) in 30 ml of cold (4 °C) buffer with a Waring blender (model 31BL92, Dynamics Corporation of America, New Hartford, CT). The meat slurry was transferred to a 50 ml polypropylene tube, centrifuged at 16,000 x g for 20 min at 2 °C (Beckman) and a 1 ml aliquot was collected in micro-centrifuge tubes containing 0.05% thimerosal as a preservative. Extracts were refrigerated (4 °C) until further analysis.

Development of ratio model for predicting EPT

Due to the limited quantity of Mabs produced in cell culture supernatant, an affinity purified goat anti-mouse IgG unconjugated antibody (610-1102, Rockland, Gilbertsville, PA) was used to coat the microtiter plates to maximize the availability of Mab in the sandwich ELISA. Microtiter plates were coated with 200 µl/well of goat anti-mouse IgG antibody diluted in coating buffer at a concentration of 2 µg/ml and

incubated overnight at 4 °C. After rinsing four times with PBST, concentrated cell culture supernatant was applied 200 µl/well at 1:50 to 1:1000 dilutions in PBST and incubated at room temperature (25.5 °C) for 60 min. Following four rinses with PBST, 190 µl of PBST was added to each well, then 10 µl of serial diluted (1:2, 4, 8, 16, 32, 64, 128, and 256 in PBST) muscle extract (beef or pork) from each EPT was applied to the microtiter plate and incubated for 60 min at room temperature (25.5 °C). In addition, a standard curve was run on each plate with the respective antigen. A duplicate curve was generated on each plate by applying 10 µl of serial diluted antigen (1:2), starting with 10 ng to 0.15625 ng, in 190 µl of PBST. The plate was rinsed four times with PBST. A protein A/G purified Pab was applied (200 µl/well) at 1:10,000 to 1:50,000 dilution in PBST and incubated for 45 min at room temperature (25.5 °C). After rinsing the plate four times with PBST, a goat anti-rabbit IgG peroxidase conjugated antibody (611-103-122, Rockland) was applied at 200 µl/well diluted 1:10,000 to 1:50,000 in PBST and incubated for 30 min at room temperature (25.5 °C). Four final rinses with PBST were performed to adequately remove any unbound label antibody prior to color development. Bound peroxidase was determined with a TMB substrate kit (Pierce). Absorbance was read at 450 nm using a Wallac 1420 Victor multilabel counter (Wallac Oy, Turku, Finland).

CHAPTER IV

RESULTS AND DISCUSSION

The goal of this study was to investigate the relative decline in concentration of selected bovine and porcine proteins with increasing temperature and to determine the simultaneous relationships among the proteins with time. Four sarcoplasmic proteins (LDH-5, BSA, enolase, and myoglobin) were selected based on their sequential disappearance with heating for development of a mathematical ratio model to predict the EPT achieved within ± 0.5 °C of a given EPT. Previous research has attempted to utilize a single protein in an effort to determine the EPT achieved in cooked meat products. Results from these studies were variable and lacking in accuracy and precision. In an effort to increase the precision and accuracy of an EPT assay, this study produced Mabs to the selected proteins for use in developing a predictive multiple antigen ratio model. ELISA assays that incorporate specific Mabs as the capture species show a significant increase in sensitivity and precision over other assays. Because the concentrations of the selected proteins vary between muscles, animals, and species, temperature endpoint predictors will be attempted using a response ratio comparing this to corresponding EPTs.

Isolation, purification and testing of proteins and Pabs

Protein isolation and purification. Porcine serum albumin was isolated and purified by fractionation using saturated ammonium sulfate to create a salt gradient for separation of proteins based on molecular weight. The primary fractionation procedure

(Figure 1A) was used to isolate PSA, while the secondary fractionation salt gradient was used for purification of the protein (Figure 1B and 1C). Primary fractionation of porcine serum albumin with saturated ammonium sulfate yielded two relatively pure precipitates from the 60 and 80% saturated fractions. Additional secondary fractionation of precipitates from the 60 and 80% ammonium sulfate primary fractionation steps yielded slightly purer fractions of PSA (Figure 2). The original 80% precipitate from the primary fractionation step taken to a secondary 60% saturation was dialyzed (Spectra/Por® Membrane MWCO: 3500) in distilled deionized water, lyophilized, and stored at 4 °C for later use in the study. The original 60% precipitate from the primary fractionation step was saturated further to 40, 50, 60, and 70% yielded similar protein separation bands on the gel (Figure 2). Therefore, these four fractions were pooled to increase the total amount of available protein and subjected to further purification by liquid chromatography. Secondary fractionation of the original 60% precipitate, yielded no visible benefit on SDS-PAGE (PSA present in all four fractions), therefore, the four pooled secondary precipitates from the secondary fractionation step were further separated on a phenyl-sepharose column starting with a 40% ammonium sulfate gradient. Phenyl-sepharose liquid chromatography separation using a step-wise gradient of ammonium sulfate from 40 to 10% at 10% intervals yielded little to no remaining PSA from the original 60% precipitate as determined by SDS-PAGE (Figure 3). Therefore, primary fractionation at a 60% saturated ammonium sulfate level was sufficient to isolate and purify PSA.

Purification of enolase from beef and pork extracts on a DEAE-Sepharose column yielded four distinct fractions. These fractions were identified and verified by SDS-PAGE and Western blotting (Figures 4 and 5). Fractions 1, 2, and 3 from the beef extracts and fractions 1 and 2 from the pork extracts were pooled, respective to species, and purified further on a phenyl-sepharose column. Three fractions were identified from each of the beef and pork extracts. These fractions were concentrated and again subjected to SDS-PAGE and Western blotting (Figures 6 and 7). All three fractions from both beef and pork yielded muscle specific enolase. Bradford soluble protein assay indicated that fraction 2 from the beef extracts had a higher protein concentration than fractions 1 or 3. Fractions 1 and 2 from pork extracts yielded similar protein concentrations. All fractions were aliquoted and frozen (-20 °C) for further testing.

Molecular weight chromatography of beef and pork extracts yielded distinct myoglobin fractions. These fractions were analyzed for myoglobin by SDS-PAGE and Western Blotting (Figures 8 and 9). Myoglobin was confirmed in fractions 1 and 2 and fraction 1 from beef and pork muscle extracts, respectively. Stained SDS-PAGE gels indicated a relatively pure extract of muscle specific myoglobin. Soluble protein concentration as determined by Bradford protein assays were 4.94 and 4.8 mg/ml in the first and second beef extract factions, respectively. However, a lower soluble protein concentration (1.8 mg/ml) was found for the first pork extract (myoglobin containing) fraction as compared to the second fraction (7.04 mg/ml other soluble proteins). This would be expected since the myoglobin content on a weight/weight basis of porcine

tissue is typically lower than that of bovine tissue. All fractions were aliquoted and frozen (-20 °C) for further testing.

Cross-reactivity of Pabs. The rabbit protein specific Pabs that were purchased or developed for use in this study demonstrated significant inter-species cross-reactivity for each respective protein from bovine or porcine muscle. Rabbit anti-LDH-5 and anti-enolase Pabs were produced against bovine or porcine purified proteins, respectively. Rabbit anti-BSA antibodies were available commercially. Previous research from this laboratory established the cross-reactivity of anti-human myoglobin, also available commercially, against bovine and porcine myoglobin. Figure 10 shows the cross-reactivity of the commercially available or laboratory produced antibodies across bovine and porcine proteins. The dot-blots were done using the blood titer protocol for rabbit serum. All four Pabs displayed exceptional species cross-reactivity reacting with both bovine and porcine proteins. All Pabs displayed a 1:2000 dilution rate against their respective protein (2 ng), both bovine and porcine, with the exception of anti-enolase Pabs which displayed a 1:1000 dilution rate. A blot appearing at the higher dilution rates indicated an increased affinity of the antibodies for their antigen and demonstrates increased detection sensitivity.

Production and characterization of Mabs

Mab production in mice (n=4 per protein) was initiated by immunization with bovine LDH-5 and BSA (100 µg/mouse initially followed by a 50 µg/mouse booster at two week intervals) due to their availability and purity. Immunization required a

minimum of five injections to obtain a blood serum titer of 1:2000 against 2 ng of protein. Blood titers indicated the level of Pabs, higher levels denote an ample population of B-cells required for a successful fusion. The blot from the blood titer (Figure 11) indicated that two of the four immunized mice for each protein were ready for harvest as indicated by a minimum blood serum titer of 1:1000. However, continued injections were needed for the remaining mice. Variable immunological responses to each antigen after immunization were to be expected since individual animals respond differently to the injected antigens. Additional booster injections of the antigen often increase blood titers of the respective proteins, but some mice simply fail to respond to increased injections. Low blood titers may indicate a decreased number of B-cells from the spleen resulting in a decreased number of potential hybridomas. The goal of immunization is to maximize B-cell production to increase the chance of forming successful antibody secreting hybridomas. Responses to immunization with porcine enolase were similar to LDH-5 and BSA in that a minimum of five injections were required to obtain blood titers of 1:2000 for 2 ng of protein (Figure 12). Three of the four mice immunized with porcine enolase reached a titer of 1:2000 after 5 injections; however, one mouse needed additional booster injections to reach a similar blood serum titer.

Mouse immunization with myoglobin was the last performed due to difficulties with protein isolation and purification. Myoglobin presented the greatest challenge due to the lack of available methods for the isolation and purification of the protein. Many isolation methods were attempted including ammonium sulfate precipitation at various

concentrations, extraction at low pH, extraction in various buffers, phenyl-sepharose liquid chromatography, and finally 100% ammonium sulfate precipitation followed by molecular weight liquid chromatography. Due to the unexpected delay in the purification of the protein, mice acquired for myoglobin immunization had reached six months of age (old). Therefore, a second group (young) of mice (6-8 wk of age) was acquired and both sets immunized with purified bovine myoglobin. Unlike the other three proteins, myoglobin required a minimum of six injections to obtain blood titers of 1:2000 for 5 ng of protein (Figure 13). As expected, the older mice (6 mo) lacked sufficient immune response to achieve the required blood titer; therefore, boosters were continued in an attempt to reach the desired blood titer level.

The second stage of Mab production for configuring a sandwich ELISA EPT assay was the generation of hybridomas from immunized mouse spleen cells. One of the original and traditional methods of hybridoma production for mammalian cells involves the use of polyethylene glycol (PEG) based on techniques of Galfre and others (1977), Gefter and others (1977) and Kennett (1978). PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei. However, electrofusion, a newer more efficient method of hybridoma production was chosen for this study. The electrofusion method has been shown to increase the number of hybridomas in comparison to one viable hybridoma per 10^5 starting cells with PEG (Harlow and Lane 1988). PEG is also detrimental to cell viability. Karsten and others (1988) determined that electrofusion resulted in a 3.8 to 33.0 times higher yield of hybridomas per unit number of spleen cells as compared to PEG-mediated cell fusion. A

hybridoma, as defined by Harlow and Lane (1988), is a cell that combines an antibody secreting cell with a myeloma cell, a type of B-cell tumor. The reason for producing a hybridoma is to produce one specific antibody, thus the term monoclonal antibody. An antibody secreting cell fused with a cancerous tumor cell theoretically provides an infinite supply of a specific antibody. However, a viable hybridoma does not necessarily produce a successful Mab secreting cell. Harvested cells from the spleen secrete a variety of antibodies against multiple antigens that have initiated an immune response in the animal. The motive behind multiple injections of a selected antigen is to increase the population of B-cells that have undergone expansion, thus increasing the chance of successfully generating a desired hybridoma. Many factors affect the fusion process often prolonging the period of time needed to successfully generate a Mab secreting clone.

A successful hybridoma must have the ability to replicate, or clone itself from the myeloma cell, thus making it immortal in addition to the ability to secrete an antibody. Although the process is theoretically simple the procedure is quite random, generating many combinations of hybridomas. During the fusion process, antibody secreting cells are randomly aligned with SP2 myeloma tumor cells in an array of combinations using an electrical current (9V for 15 s); however, the degree of a fusion association cannot be controlled. Therefore, some hybridomas are rapid growers, with a high degree of cellular division, but lack the ability to secrete antibodies. On the other hand, some hybridomas maintain the ability to secrete desired antibodies, but lack viable cell growth characteristics, thus leading to an ever decreasing population of the clone and eventually

death. Some of the hybridomas created will have the desired cellular characteristics; however, these cells can secrete undesired antibodies against other antigens. After a fusion many hybridomas are generated, thus a screening protocol is needed to select the desired cells against the respective antigen for further growth and storage. During this study, multiple fusions for each protein antigen were required to obtain a sufficient number of monoclonal secreting hybridomas.

After reporting ample blood serum titers ($\geq$ 1:2000) against bovine LDH-5 and BSA, a final set of booster injections (50 µg/mouse, minus adjuvant, 5 and 4 days prior to spleen removal) was administered in preparation for cell electrofusion. The first cell fusion was attempted with spleen cells harvested from bovine LDH-5 immunized mice. As expected many hybridomas were generated, but after an initial screening only a few cells were selected for Mab production. The first fusion of LDH-5 immunized mouse spleen cells generated 24 potential monoclonal secreting hybridomas. Upon completion of the first fusion for LDH-5, BSA immunized mice were given their final booster injections in preparation for production of viable hybridomas and anti-BSA Mabs.

Bovine serum albumin presented a unique challenge due to the cell culture media used for hybridoma production. The key ingredient in most cell culture media is some form of serum, usually from bovine fetal calves. Cell fusion, growth, and storage was carried out using media containing fetal calf serum; however, cell screening of hybridomas secreting antibodies against BSA could not be accomplished with such media. Media containing BSA could rapidly form an antigen-antibody complex thus inhibiting the antibody from binding with the BSA attached to the screening plate,

resulting in a negative signal or strong blue color reaction. Therefore, to obtain an accurate measurement of anti-BSA antibodies being produced by hybridomas, serum protein-free medium was used as an alternative to the complete serum cell culture media. Cell fusions using BSA immunized mouse spleen cells generated an unexpected number of viable hybridoma cells. Several cell fusions produced more than 200 hybridomas, however, very few secreted antibodies against BSA. Initial screenings for the presence of mouse IgG was notably indiscriminate for eliminating very few hybridomas. Only after serum protein-free cell culture media was substituted for media containing fetal calf serum could true anti-BSA antibody secreting cells be identified. Eliminating serum from the cell culture media to produce anti-BSA secreting hybridomas introduced physiological barriers for cell growth. Many potential cells could not adapt to the serum protein-free medium; therefore, a number of viable hybridomas that potentially secreted Mabs against BSA may have been lost resulting in a reduced number of clones. After multiple fusions and numerous months of testing of non-specific clones, 29 hybridomas were grown to produce Mabs specific to the original antigen.

Cell fusion and hybridoma production for enolase and myoglobin proceeded without the difficulties experienced with the BSA Mabs. Both enolase and myoglobin fusions were carried out in complete DMEM media without causing complications during screening. Like LDH-5, enolase, and myoglobin proteins resulted in numerous potential Mab secreting hybridomas. Fusions for enolase and myoglobin generated 66 and 12 hybridomas, respectively. With a sufficient number of clones to the various EPT

proteins, the production of antibody-containing cell culture supernatant by static culture flask could be initiated.

Due to the increased concerns of pain, discomfort, and distress of animals involved in the production of Mabs by *in vivo* techniques such as mouse ascites tumors, alternative *in vitro* methods have been developed for small scale production of Mabs without the use of animals. Some of the alternative methods that have been established include static cultures, agitated cell suspensions (Reuveny and others 1986; Tarleton and Beyer 1991) and hollow fiber cartridges (Evans and Miller 1988; Falkenberg and others 1995; Jackson and others 1996). Although alternative methods are available, some hybridoma cell lines (clones) do not adapt readily to *in vitro* conditions resulting in very low antibody concentrations (Hendriksen and others 1996; Varmus, 1997). In about 4% of the cases using *in vivo* methods, mouse ascites production is required to yield large concentrations of highly specific Mabs. Conversely, the alternatives identified above have been shown to reduce the yield of Mabs to only a few micrograms per milliliter (Vachula and others 1995; Heidel 1997; Peterson and Peavey 1998). Some researchers have noted however that if care is taken to maintain the integrity of the Mabs during concentration and purification, usable yields can be acquired.

Antibody production *in vitro* was not difficult with the exception of antibodies against BSA that were produced in serum protein-free medium. It has been shown that serum-free, cell culture methods currently do not produce sufficient amounts of Mabs for research applications. Aside from the *in vivo* conditions needed for serum-free hybridoma production, *in vitro* methods sometimes yield populations of Mabs that are

different from those harvested from mouse ascites fluids (Leibiger and others 1995). Recent workshops, forums, and publications have concluded that *in vitro* alternative methods can often provide an adequate means of generating most of the monoclonal antibodies needed by the research community (Center for Alternatives to Animal Testing and OPRR/NIH 1997; Marx and others 1997; de Geus and Hendriksen 1998). However, many of the *in vitro* produced antibodies cannot be readily concentrated from a cell culture supernatant using standard procedures, thus resulting in losses of antigen binding activity or other antigen-antibody characteristics (Underwood and Bean 1985; Lullau and others 1996). Provided desired concentrations and specificity of Mabs can be maintained, research has shown that alternative methods (*in vitro*) of producing Mabs are available (Knazek and others 1972; Boraston and others 1984; Reuveny and others 1986; Miller and others 1987; Evans and Miller 1988; Tarleton and Beyer 1991; Falkenberg and others 1995; Jackson and others 1996; Peterson and Peavey 1998). During this study, concerns were raised regarding the use of mice for ascites production; therefore *in vitro* production by static culture flask was the only alternative. Cell culture supernatant was deemed adequate pending further testing of potential Mab secreting hybridomas against their respective protein in beef and pork extracts.

Prior to ELISA testing, antibody-containing cell culture supernatant had to be concentrated to obtain sufficient concentrations of Mabs for use in an assay. Typically, ammonium sulfate precipitation of antibodies from serum or cell culture supernatant was acceptable; however Mabs can denature under such conditions or loose their ability to bind to their respective antigen. Concentration of anti-LDH-5 antibodies yielded useable

concentrations of an antibody that maintained affinity for LDH-5 as confirmed by indirect ELISA. Ammonium sulfate precipitation of anti-enolase and anti-myoglobin antibodies however was not adequate. After ammonium sulfate precipitation, these antibodies lost their ability to form an antigen-antibody complex as confirmed by indirect ELISA. A sufficient concentration of Mabs required for an ELISA plate coating was not achievable by this method. Other alternative concentration methods were attempted including protein A/G purification and molecular weight separation by centrifugation using a 100K membrane. Purification by these two methods was unsuccessful either due to very low concentration of Mabs present in the cell culture supernatant or destructive steps that denatured the antibody binding site. By avoiding the use of mouse ascites fluid, Mabs would have to be captured directly from cell culture supernatant by coating ELISA plates with an anti-mouse IgG specific antibody.

Variations in protein solubility of beef and pork extracts

Development of a multiple antigen assay into a ratio model required proteins possessing different solubility or antigenicity characteristics when heated to instantaneous EPTs. Preliminary studies by this laboratory identified the four proteins selected for this study. Ground *semimembranosus* beef extracts were heated to instantaneous EPTs from 25 to 73 °C at 2 °C intervals, then separated by SDS-PAGE, and characterized by densitometric methods (Figure 14). Previous research by this laboratory identified LDH-5, BSA, enolase, and myoglobin by comparison to standards or by immunological means and determined that they displayed unique protein solubility

curves (bands 1, 4, 6, and 8) over selected EPTs (Figure 15). In addition, PSA, enolase, and myoglobin was also identified by this laboratory as potential EPTs indicators in pork *semimembranosus* muscle extracts (Figure 16). Changes in protein solubility or specifically antigenicity profiles are the key factor in development of a multiple antigen ratio model. Declines in protein concentration were determined by loss of antigenicity and concentration between the antibody and its respective antigen (protein) or by denaturation of the protein epitope that is rearranged or destroyed with incremental heating. The Mab ratio is derived by dividing protein concentration values by one another. The concentration of myoglobin (M) divided by the concentration of BSA (B) divided by the concentration of LDH-5 (L) is denoted as the multiple protein ratio (M/B/L). Concentrations of individual proteins may vary between two separate beef samples at a given temperature, however, the relative concentrations between proteins at a given temperature offers a more accurate and precise measurement of EPT.

As stated above, factors such as muscle type, age, sex, species, storage conditions, and ingredients, can influence the concentration of protein within the muscle tissue. Miller and others (2003b) demonstrated that the addition of 0.5% sodium tripolyphosphate to a salt extraction buffer increased protein extraction approximately two fold compared to salt extraction alone (Figure 17). Their study also determined that increasing salt concentration from 0 to 3% decreased the amount of soluble LDH-5 (Figure 18).

Previous investigations describing the potential of proteins as EPT indicators were based solely on the concentration of a single protein at a given temperature.

However, this research has also indicated that quantification of a single protein at a given temperature to establish an EPT threshold is inadequate to achieve the sensitivity and precision required by the USDA-FSIS. Concentrations of soluble proteins as determined by the Bradford assay, from beef and pork extracts, were variable. The variation between samples from Tables 1 and 2 within an EPT are shown in Figures 19 and 20. Because of the inherent soluble protein variability, use of a single protein as an EPT indicator based on concentration at a given temperature would not be acceptable.

Development of ratio model for predicting EPT

Previous research using selected proteins as EPT indicators typically used an indirect ELISA assay with rabbit serum Pabs (Wang and others 1992, 1994 Morris 1995; Hsu and others 1999). Pabs are the total immunoglobin fraction of blood serum containing a wide array of antibodies against many different antigens or multiple epitopes. Although Pabs from immunized animals may detect a given antigen, the cross-reactivity of Pabs is significantly greater compared to Mabs. Multiple antibody secreting B-cells against a specific antigen are likely present in immunized animal serum. These multiple cells secrete antibodies against the antigen using random epitopes or recognition sites; however, some epitopes may share similarities with other proteins causing cross-reactivity and non-specific binding. Therefore, Mabs are used to ensure specific binding of respective antigens to generate accurate test results. Using a Mab as a capture antibody in a sandwich ELISA followed by a polyclonal serum detection antibody

improves assay accuracy and precision. A typical sandwich ELISA assay is displayed in Figure 21.

The assay requires a monoclonal primary antibody (usually attached to a fixed matrix) to capture a specific antigen, then a polyclonal primary serum antibody for detection of the antigen. A secondary antibody or species specific antibody against the polyclonal detection antibody that has some form of label (colorimetric, fluorimetric, etc.) is applied to determine the concentration of the specific antigen. A standard curve developed for incremental concentrations of the antigen enables quantification of the assay (the more antigen detected the lower the EPT of the cooked product). This model was the basis for a multiple antigen EPT assay against beef and pork extracts cooked to various degrees of doneness. Due to the relatively low concentration of Mabs, a mouse IgG specific secondary antibody was used to coat ELISA plates (Figure 22). Binding of mouse Mab was optimized with the additional capture antibody. Use of this model was thoroughly explored to avoid unnecessary production of mouse ascites fluid. In addition, incorporation of a mouse IgG specific secondary antibody would potentially capture anti-enolase and myoglobin Mabs directly from collected cell culture supernatant.

Initial testing of the model was implemented using LDH-5 in view of the fact that these monoclonals were the first available in the study. Preliminary sandwich ELISA results were inconclusive due to high background signal. High background refers to non-specific binding of the secondary species specific conjugated antibody (Goat anti-rabbit peroxidase labeled) resulting in a constant signal (absorbance) when microtiter plates are read. Immunological assays can be very complex and difficulties may arise

due to non-specific binding of antibodies within the assay. The detection antibody was tested against each individual antibody of the sandwich structure to determine if non-specific binding occurred. After further investigation, it was discovered that the detection Pab was binding even without the presence of bovine LDH-5 (Figure 23). Cross-reactivity of the rabbit polyclonal with the goat anti-mouse and the goat anti-rabbit with the goat anti-mouse (Figure 22) resulted in a constant high background signal. These findings suggested the presence of LDH-5 protein in one of the antibody components. It was concluded that the rabbit serum Pabs used as the primary detection antibody contained LDH-5 or might be reacting with the antigen specific mouse capture Mab. Lactate dehydrogenase has five isozymes, one of which is LDH-3 which is present in serum; therefore, isolation of anti-LDH-5 IgG from the rabbit serum was necessary. This problem was corrected by purification of the rabbit Pab using protein A/G chromatography. Purified rabbit Pabs specific to LDH-5 were isolated from serum resulting in a functional assay with minimum background noise (Figure 24). Standard curves generated from six of the 24 clones against LDH-5 are illustrated in Figure 25.

All six Mabs against LDH-5 reported a R^2 values greater than 0.9 indicating high specificity and affinity for LDH-5. In addition, these curves were generated using 0 to 10 nanograms of antigen demonstrating the sensitivity of a sandwich ELISA incorporating Mabs as the capture antibody. As stated previously, some studies were only able to establish a broad threshold value to distinguish adequately processed meat products from ones that were under cooked due to a lack of sensitivity and/or precision in indirect polyclonal or monoclonal sandwich assays. The standard curves shown in

Figure 25 would provide predicted concentration values of LDH-5 present in beef or pork extracts with a relatively high level of confidence (R^2 values greater than 0.9).

Sandwich ELISA assay development with Mabs against BSA was not as successful as LDH-5. Mabs against BSA were raised in serum protein-free medium; therefore, BSA contamination in cell culture media was not an issue. However, rabbit serum Pabs would have a substantial concentration of albumin. Although these antibodies were purified by protein A/G chromatography, antigen-antibody complexes could exist, resulting in a positive signal (Figure 26). If an antigen-antibody complex was responsible, the antigen would need to be removed freeing the antibody to function normally in the sandwich ELISA. Protein A/G purified rabbit serum was incubated in gentle antibody elution buffer (21027, Pierce) then subjected to molecular weight filtration by centrifugation. Testing continued with filtered purified polyclonal rabbit antibodies, but results were inconclusive. Thus, segregated testing was initiated similar to the LDH-5 sandwich ELISA development. Goat anti-rabbit peroxidase antibodies showed no cross-reactivity with anti-mouse or BSA Mabs. Cross reactivity existed between protein A/G purified rabbit antibodies and goat anti-mouse an antibody, thus a constant background signal was generated (Figure 27).

Initial trials assessed the blocking of excess binding sites on the goat anti-mouse IgG antibodies after application of the captive Mabs by incubation with mouse IgG (Figure 28). Trials using this technique to reduce background signal were successful using a commercially available anti-human myoglobin Mab, however, trials using Mabs for BSA, enolase, or myoglobin from cell culture supernatant were unsuccessful.

Continued assessment of the sandwich ELISA assay for BSA, enolase, and myoglobin considered the use of mouse, goat, and donkey IgG as a blocking component for goat anti-mouse and rabbit Pabs. These trials showed little to no improvement for reducing the background signal. Immunological assays can become very complex based on the number of antibodies and their interactions with one another. The use of a capture antibody, goat anti-mouse IgG, to isolate Mabs from cell culture supernatant for BSA, enolase, or myoglobin through a sandwich ELISA assays proved ineffective. Attempts at using *in vitro* produced Mabs introduced unforeseen immunological cross-reactivity between components; therefore, to produce truly antigen specific Mabs, the production of mouse ascites fluid *in vivo* was the only remaining option.

Mouse ascites fluid offers the advantage of significantly higher concentrations of Mabs compared to static cell culture supernatant but at the expense of sacrificing mice that produce the Mabs. Antibodies that are raised *in vivo* reduce stress on the hybridomas and receive vital growth factors from the host. Use of animals for production of Mabs has become unacceptable to some animal welfare advocate groups. In this case, alternative methods to produce Mabs were exhausted and no other means without the use of mice for ascites fluid production were available. Mabs from select hybridomas show high affinity for their respective antigen leaving little doubt that production of mouse ascites fluid and their resulting hybridomas would yield high concentrations of antigen specific Mabs against LDH-5, BSA, enolase, and myoglobin. These could then be used in a sandwich ELISA assay for determining EPT achieved in cooked meat products. Use of ascites produced Mabs would allow direct coating of the

ELISA plate (Figure 21) eliminating the need for a cell culture supernatant capture antibody and goat anti-mouse IgG. Because this work has thus far demonstrated that a multiple antigen EPT model by sandwich ELISA has great potential, ascites generated Mabs would likely give the best reactivity in a competitive immunoassay. Configuring the ELISA on a solid phase test strip without the additional antibodies would also be better suited for commercialization of a testing device.

Although a sandwich ELISA for each EPT protein was not necessary to construct the multiple antigen ratio model, the concept of such an assay still remains a viable option as previous research has shown (Table 3). Three proteins (BSA, LDH-5, and myoglobin) exhibited different rates of denaturation or precipitation with increased cooking temperature from 63 to 73 °C. Declines in protein concentration were determined by loss of antigenicity and concentration between the antibody and its respective antigen (protein) or by denaturation of the protein epitope. The Mab ratio is derived by the dividing protein concentration values by one another. The concentration of myoglobin (M) divided by the concentration of BSA (B) divided by the concentration of LDH-5 (L) is denoted as the multiple protein ratio (M/B/L). The ratio model demonstrated a significant increase in the M/B/L value with increasing temperature (Table 3). Concentrations of individual proteins may vary between two separate beef samples at a given temperature, however, the relative concentrations between proteins at a given temperature offers a more accurate and precise measurement of EPT.

CHAPTER V

CONCLUSIONS

Three to four proteins exhibiting different rates of denaturation or precipitation with increasing cooking temperature were selected for the development of a ratio model to determine the EPT achieved in cooked beef and pork extracts. Previous research has focused on single proteins for predicting EPT achieved in cooked meat products but with insufficient results. Therefore, the goal of this study was to investigate declines in protein concentration with increasing temperature by monitoring multiple proteins simultaneously and determining the ratio relationship among them. To increase the precision and accuracy of an EPT assay, this study produced Mabs to four proteins (BSA, LDH-5, enolase, and myoglobin) for use in a multiple antigen ELISA. It is known these protein concentrations can vary between muscles, animals, species, and processing ingredients. Therefore, the basis for an EPT assay was to evaluate the ratio of the selected proteins with incremental heating to account for the potential variability. Antigens (proteins) porcine serum albumin, bovine enolase, porcine enolase, bovine myoglobin, and porcine myoglobin that were not available commercially had to be isolated and purified prior to Mab production. In addition, Pabs against LDH-5 and enolase had to be produced, and further purification of all Pabs was required to reduce or eliminate cross-reactivity. Although configuration of a multiple antigen ELISA assay was initially unsuccessful, multiple clones were generated that produced antigen specific Mabs against bovine LDH-5, BSA, bovine myoglobin, and porcine enolase. Standard

curves with bovine LDH-5 did demonstrate the sensitivity and affinity of Mabs for specific antigens. Further research using Mabs from mouse produced ascites fluid and configuration into a competitive solid phase test strip without multiple antibodies could potentially prove successful.

Current USDA-FSIS methods of monitoring EPTs have been deemed inadequate. Meat products produced under HACCP require verification of EPTs using accurate and precise monitoring devices that ensure the safety of the products. Although not complete, the efforts made in this study indicate the potential for a multiple Mab ELISA as a rapid and potentially precise measurement tool for verification of thermal processing. This research demonstrates the proof of concept for a multiple Mab ELISA and warrants further investigation. An array of test kits that employ rapid and precise immunoassay technology are readily available and with continued research into construction of an immunosorbent assay for monitoring EPTs, a rapid and precise testing method could be developed and incorporated into food safety monitoring and verification protocols. Previous immunoassay EPT tests have established potential for the technology; but, the concept of establishing a concentration threshold at a given temperature using one protein (that may vary with sample, muscle, and species) is not sufficient to predict EPTs. On the other hand, a multiple antigen immunoassay has the potential to deliver a rapid, precise and accurate testing device to insure that meat products reach a safe thermal processing end-point.

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APPENDIX A

FIGURES

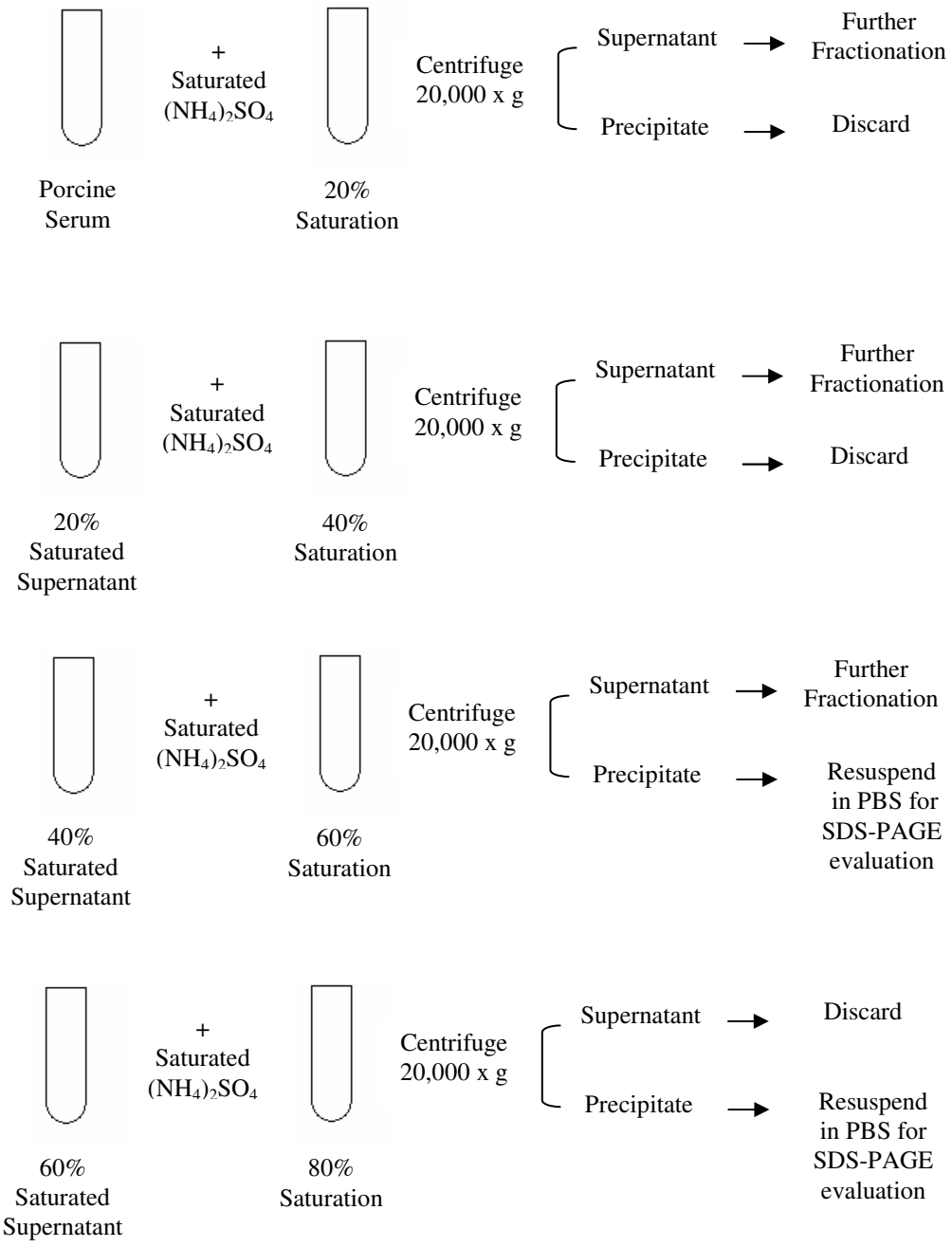


Figure 1A-Primary saturated ammonium sulfate precipitation salt gradient for isolation and purification of porcine serum albumin.

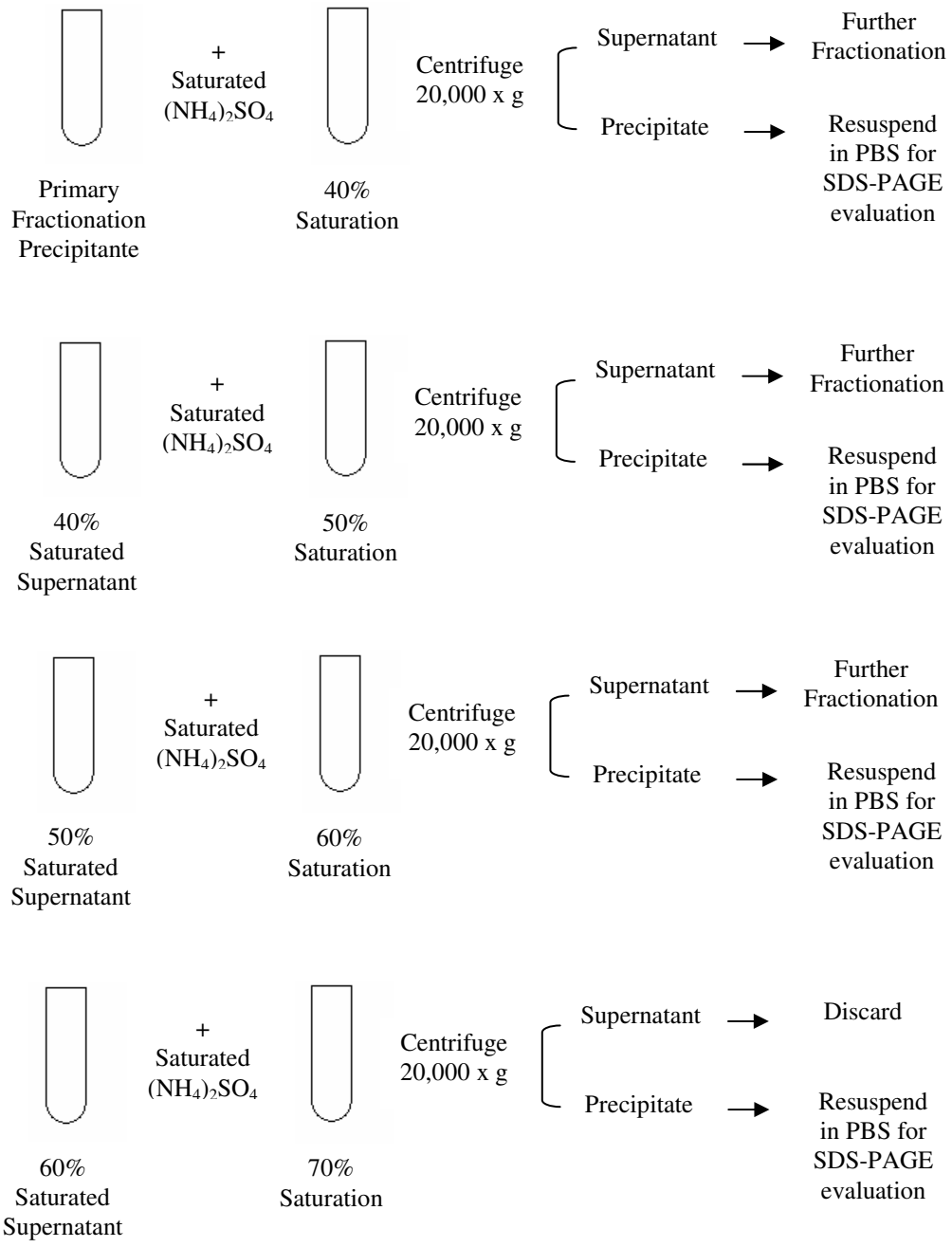


Figure 1B-Secondary fractionation of porcine serum albumin by a saturated ammonium sulfate salt gradient to further purifiy the primary 60% ammonium sulfate precipitate.

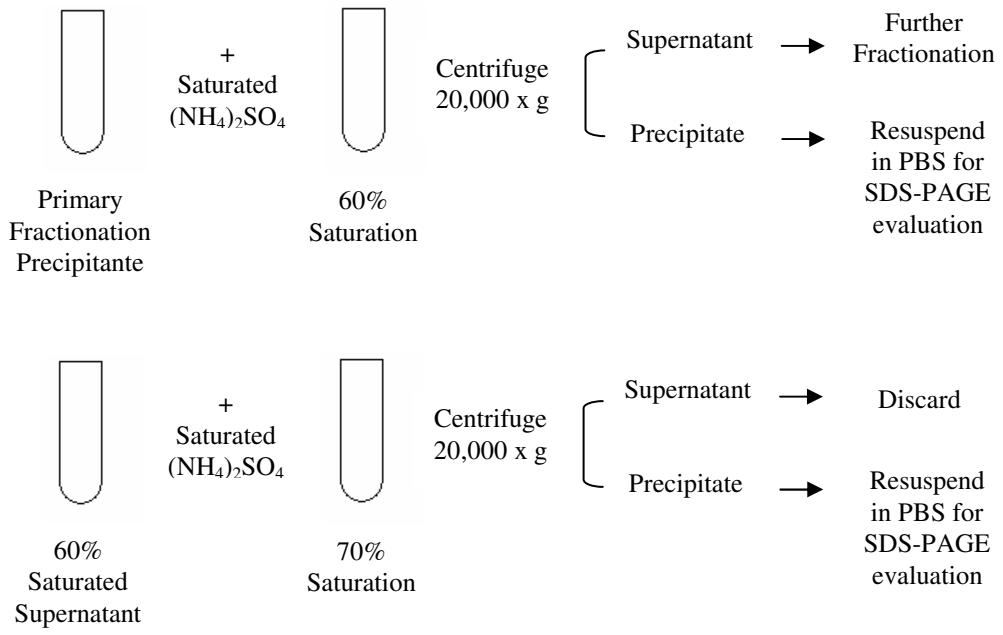


Figure 1C-Secondary fractionation of porcine serum albumin by a saturated ammonium sulfate salt gradient to further purifiy the primary 80% ammonium sulfate precipitate.

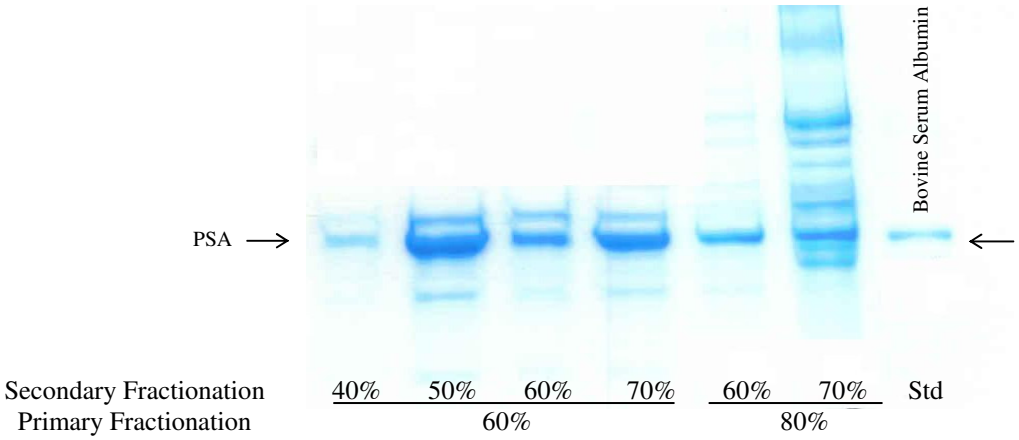


Figure 2-SDS-PAGE of secondary porcine serum albumin (PSA) fractions contained in the primary 60 and 80% ammonium sulfate precipitates.

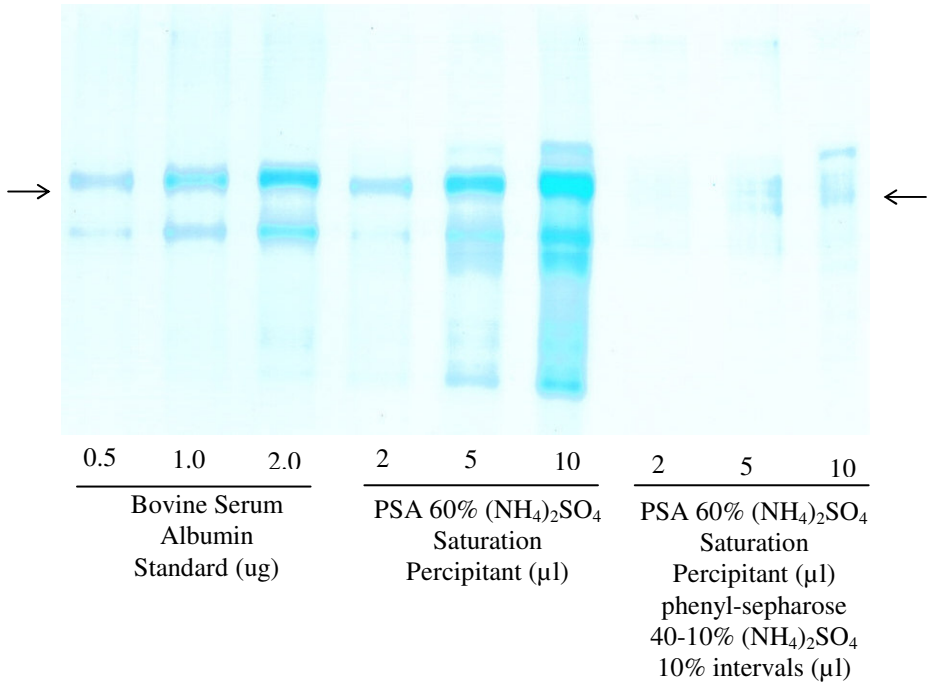


Figure 3-SDS-PAGE of porcine serum albumin (PSA) in a 60% ammonium sulfate precipitate before and after phenyl-sepharose liquid chromatography of a 40 to 10% ammonium sulfate fractionation separated at 10% intervals.

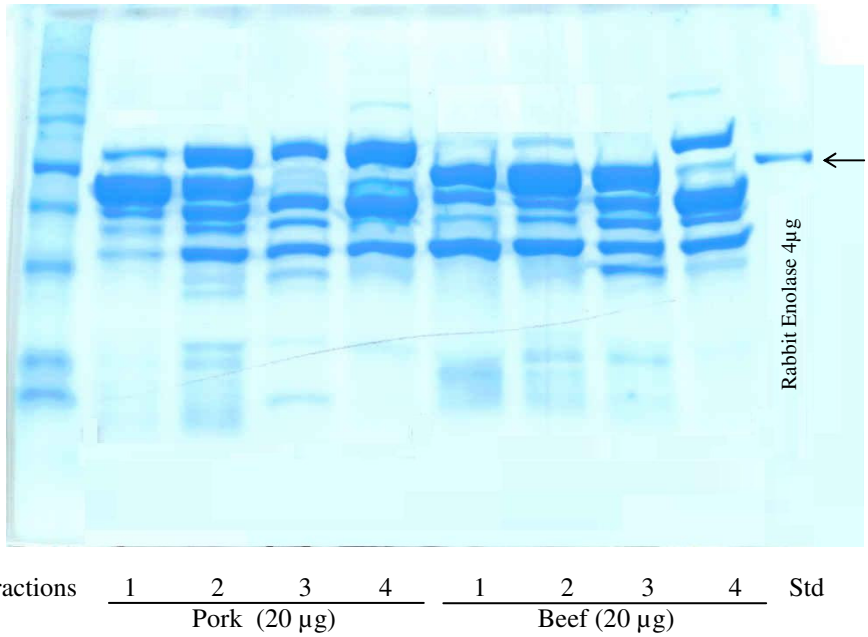


Figure 4-SDS-PAGE of muscle-specific enolase fractions after DEAE-sepharose chromatography (pH 9.0 to 7.0, in 0.5 pH increments).

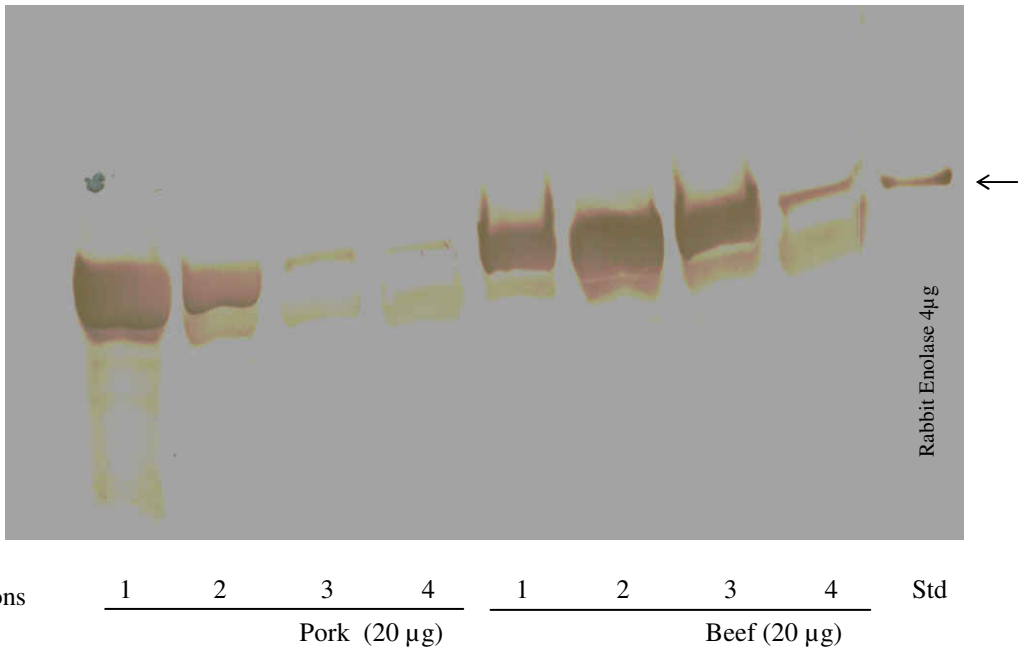


Figure 5-Western blot of muscle-specific enolase fractions after DEAE-sepharose chromatography (pH 9.0 to 7.0, in 0.5 pH increments).

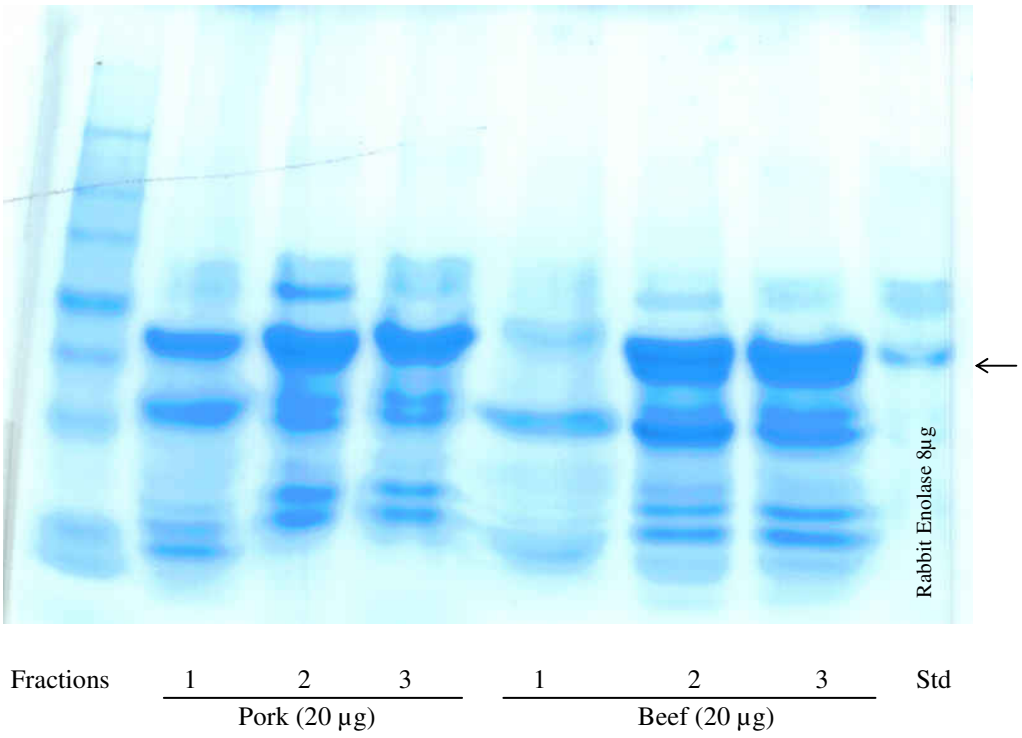


Figure 6-SDS-PAGE of muscle-specific enolase fractions after phenyl-sepharose chromatography (30 to 0% ammonium sulfate, 10% increments).

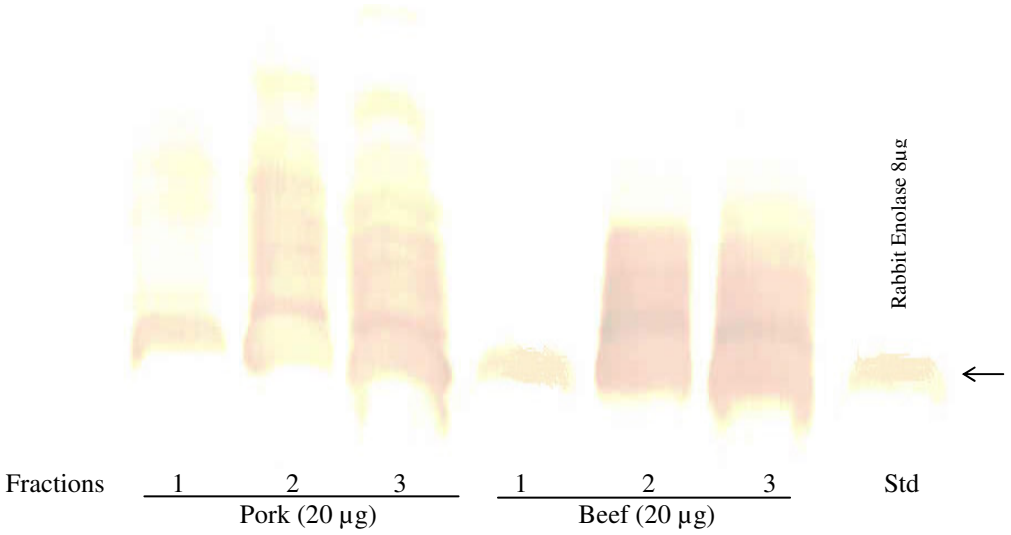


Figure 7-Western blot of muscle-specific enolase fractions after phenyl-sepharose chromatography (30 to 0% ammonium sulfate, 10% increments).

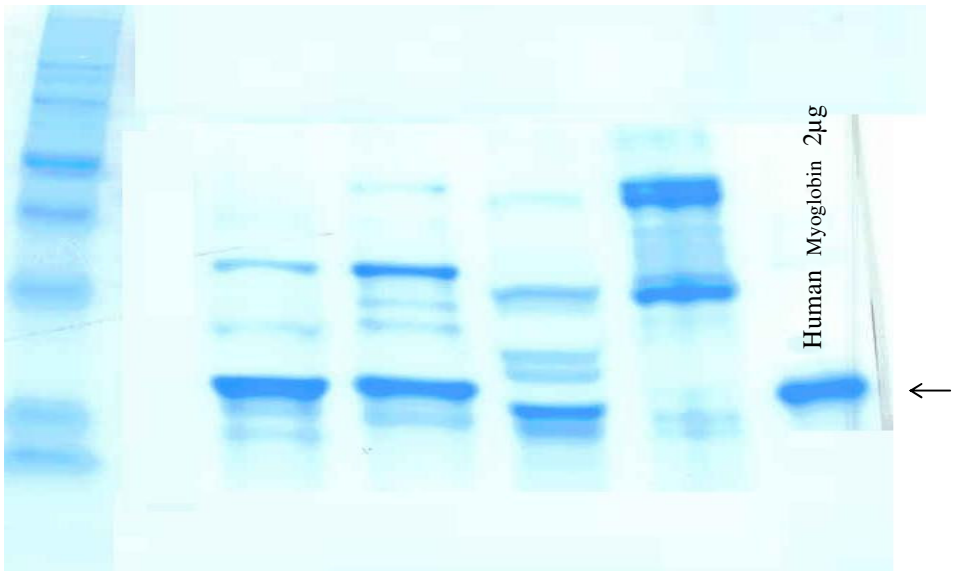


Figure 8-SDS-PAGE identification of muscle-specific myoglobin fractions after molecular weight chromatography.

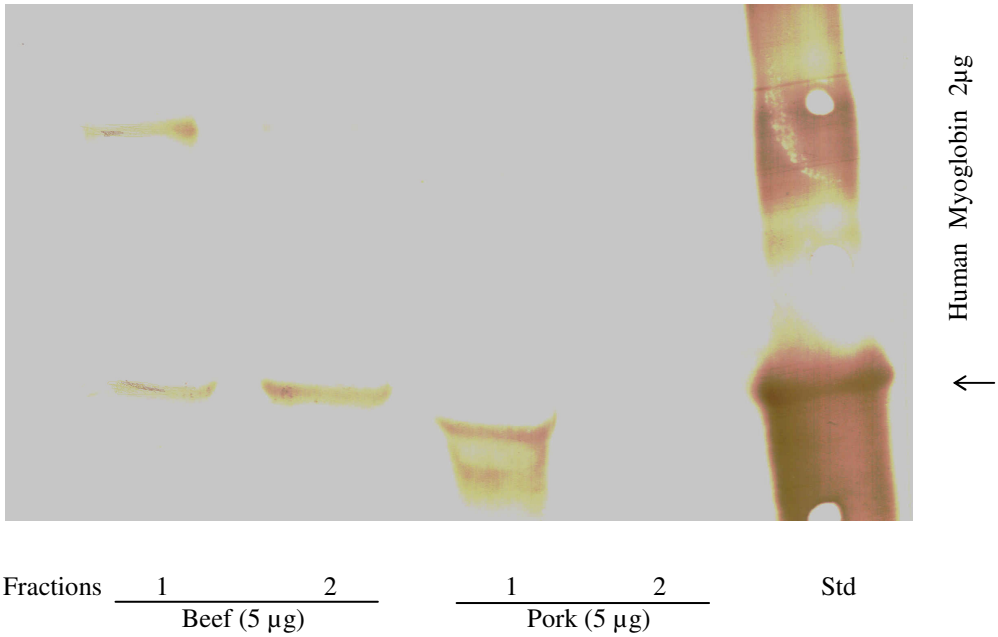


Figure 9-Western blot of muscle-specific myoglobin fractions after molecular weight chromatography.

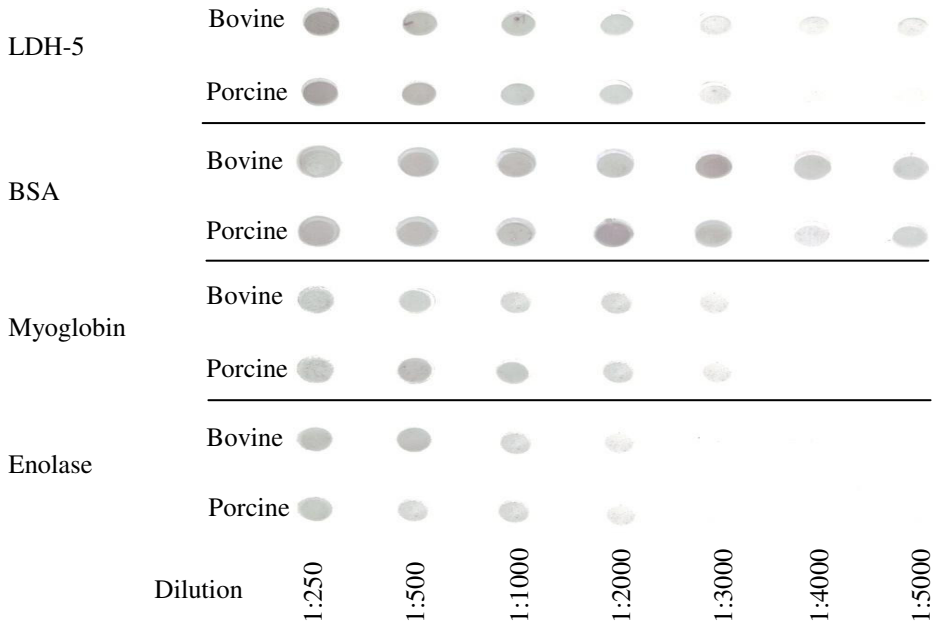


Figure 10-Dot-blots of diluted polyclonal rabbit antibodies (Pabs) demonstrating the degree of cross-reactivity against 2 ng of each respective protein from bovine or porcine muscle tissue proteins.

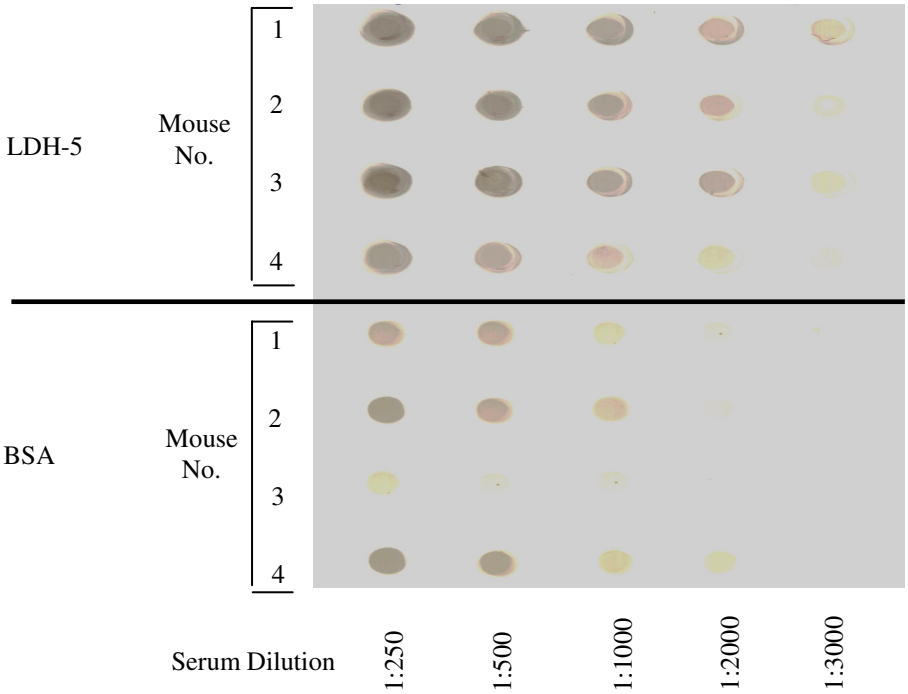


Figure 11-Indirect ELISA of blood serum titers against 2 ng of bovine LDH-5 and BSA - from immunized mice after the 5th injection.

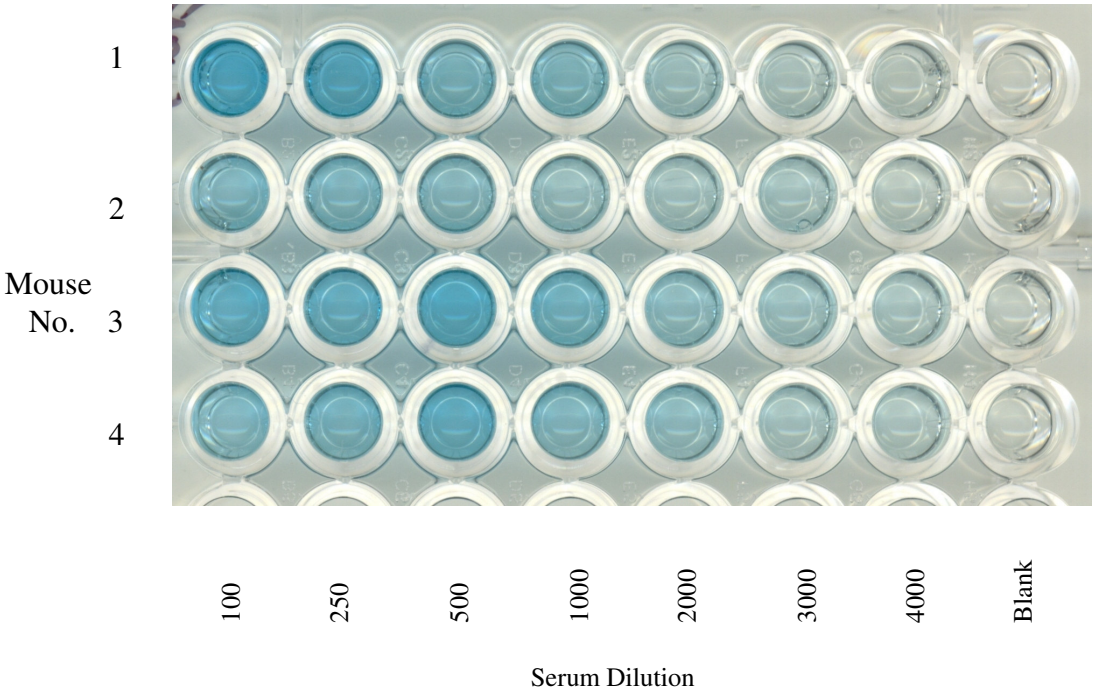


Figure 12-Indirect ELISA of blood serum titers against 2 ng of porcine enolase – from immunized mice after the 5th injection.

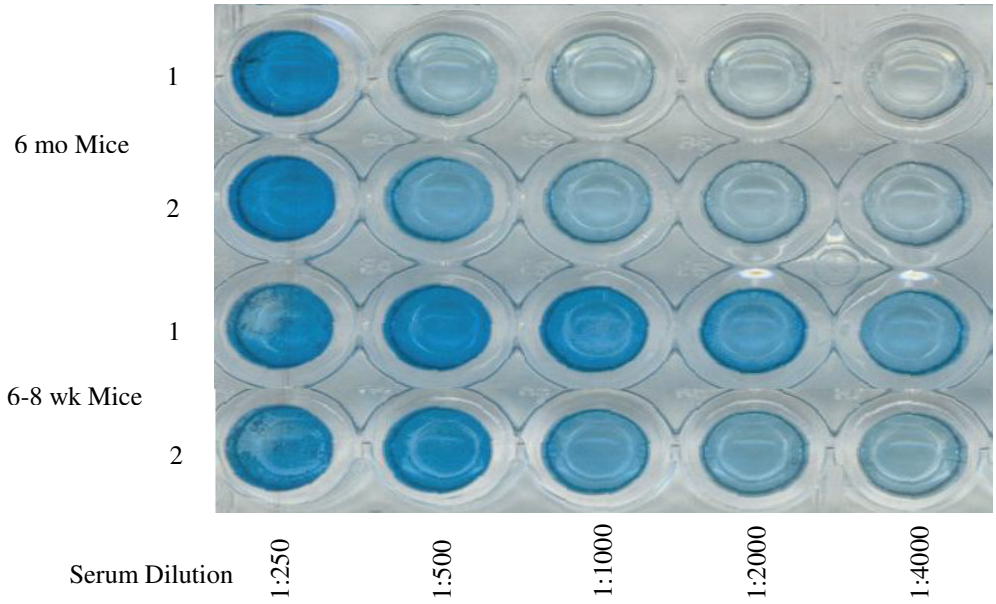
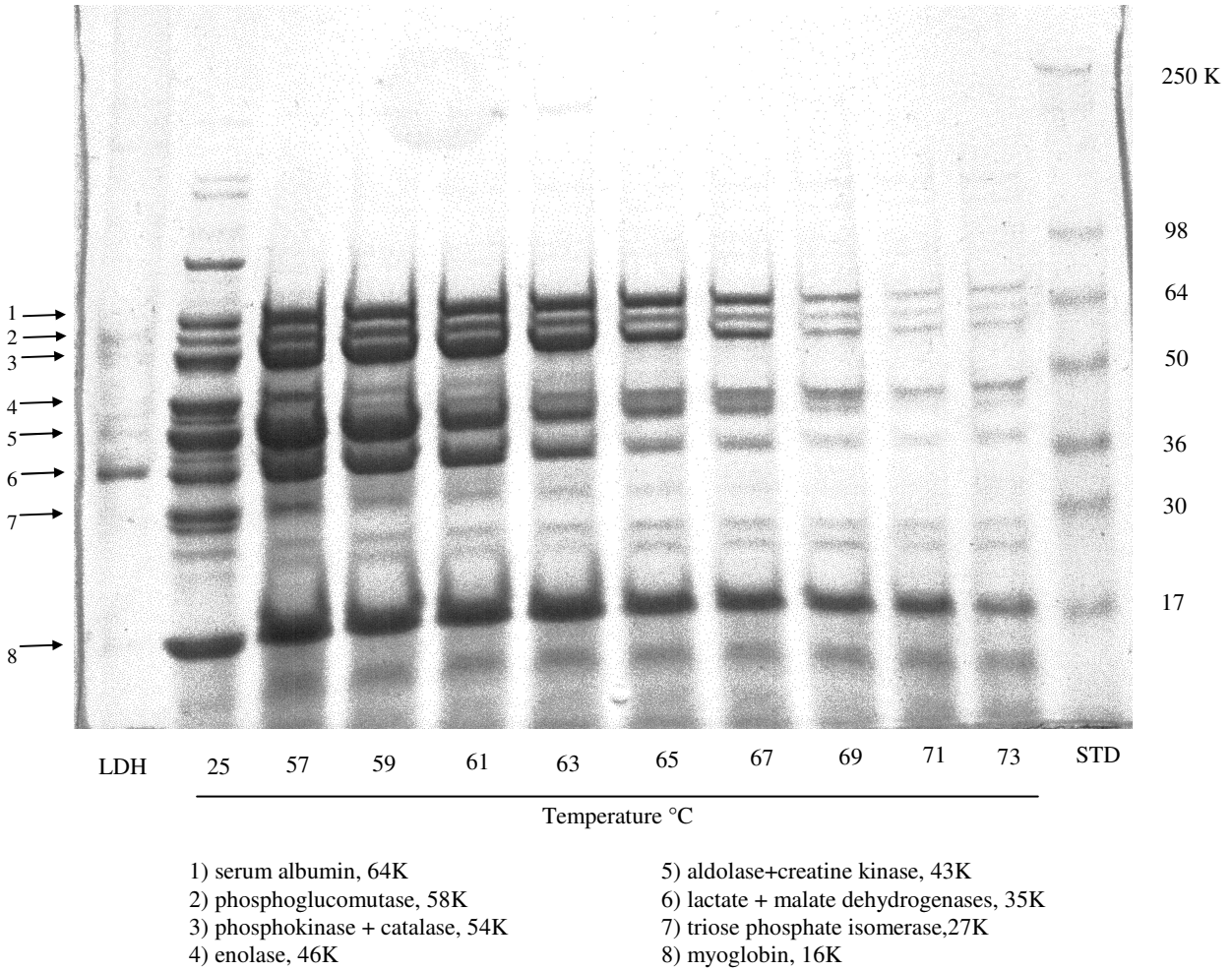


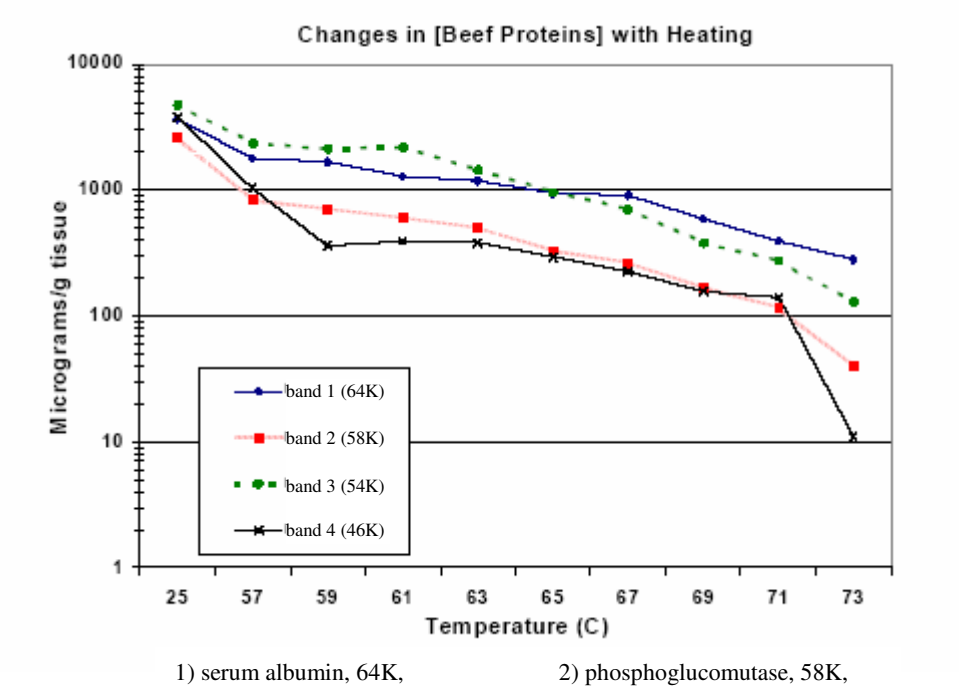
Figure 13-Indirect ELISA of blood serum titers against 5 ng of bovine myoglobin – from old (6 mo) and young (6 to 8 wk) immunized mice after the 6th injection.



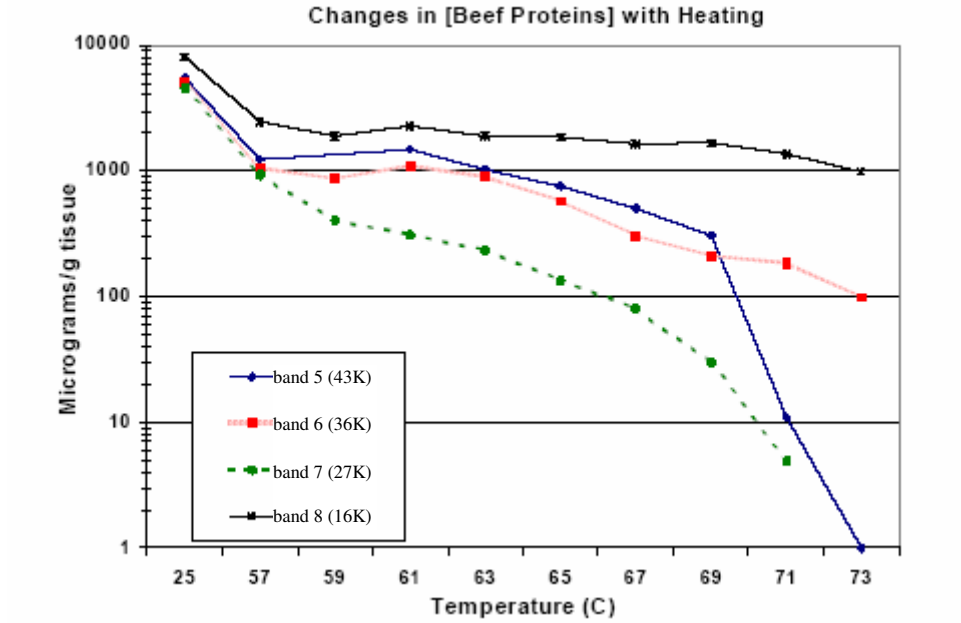
1) serum albumin, 64K
2) phosphoglucomutase, 58K
3) phosphokinase + catalase, 54K
4) enolase, 46K
5) aldolase+creatine kinase, 43K
6) lactate + malate dehydrogenases, 35K
7) triose phosphate isomerase,27K
8) myoglobin, 16K

[a] Preliminary data Texas A&M University, Department Animal Science, Dr. J.T. Keeton

Figure 14-SDS-PAGE profile of soluble, low ionic strength proteins from a beef *semimembranosus* muscle extract cooked to instantaneous EPTs of 25 (Raw) to 73 °C[a].



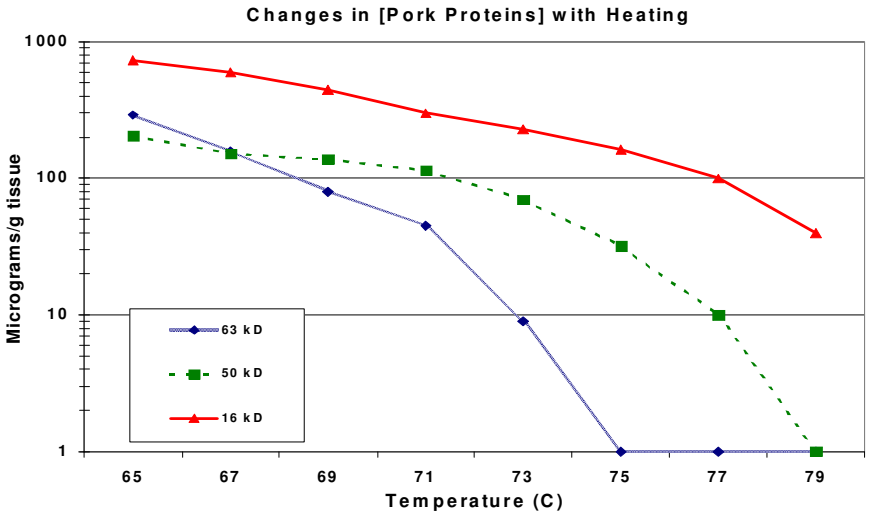
1) serum albumin, 64K, 2) phosphoglucomutase, 58K,
3) phosphokinase + catalase, 54K, 4) enolase, 46K



5) aldolase+creatine kinase, 43K, 6) lactate + malate dehydrogenases, 35K,
7) triose phosphate isomerase,27K, 8) myoglobin, 16K

Figure 15-Densitometric plots of eight soluble, low ionic strength proteins from a beef *semimembranosus* muscle extract cooked to instantaneous EPTs of 25 (Raw) to 73 °C (Figure 14)[a].

[a] Preliminary data Texas A&M University, Department Animal Science, Dr. J.T. Keeton



64 Kd = serum albumin, 50 Kd = enolase, and 16 Kd = myoglobin

Figure 16-densitometric plots of three soluble, low ionic strength proteins from a pork *semimembranosus* muscle extract cooked to instantaneous EPTs of 25 (Raw) to 79 °C prepared, identified, and quantified as the beef proteins in Figure 14[a].

[a] Preliminary data Texas A&M University, Department Animal Science, Dr. J.T. Keeton

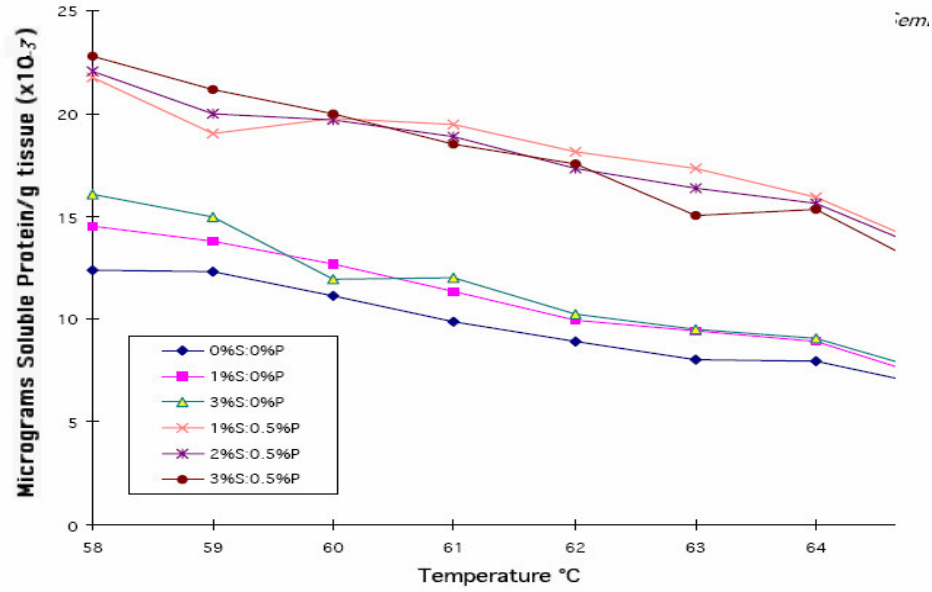


Figure 17-Comparison of the declines of total soluble protein present in beef *semimembranosus* extracts prepared with NaCl (0 to 3%) or salt plus sodium tripolyphosphate (0.5%) (%S:%P). (Miller and others 2003b)

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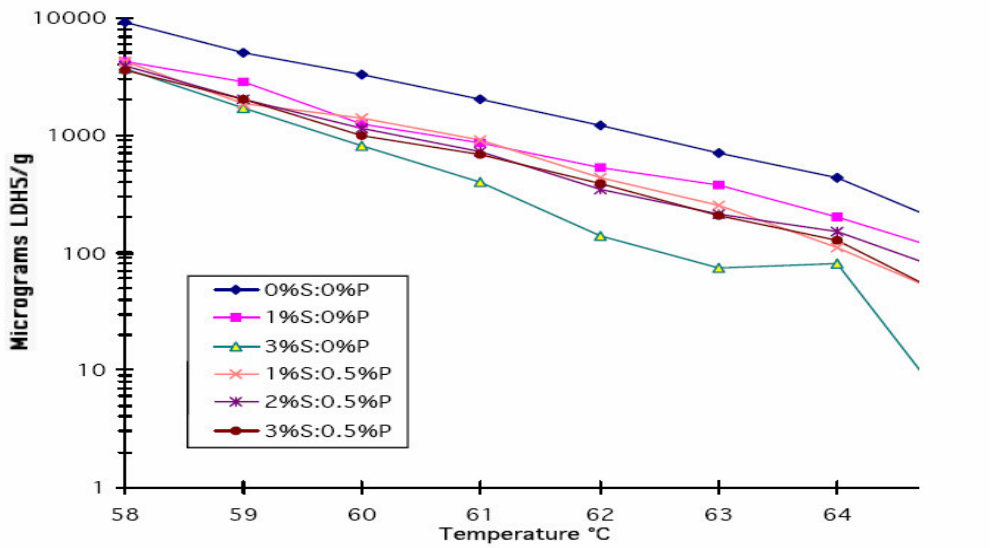


Figure 18-Comparison of the declines of immunologically measured LDH5 with endpoint heating temperature measured in beef samples prepared with NaCl (0 to 3%) or salt plus sodium tripolyphosphate (0.5%)(%S:%P). (Miller and others 2003b)

*Reprinted with permission from "BRINE INGREDIENTS ALTER THE IMMUNOASSAY OF LACATE DEHYDROGENASE ISOZYME 5 IN BEEF" by D.R. MILLER, J.T. KEETON, AND J.F. PROCHASKA, VOL. 68, p. 2072-2075, 2003 by INSTITURE OF FOOD TECHNOLOGISTS (JOURNAL OF FOOD SCIENCE).

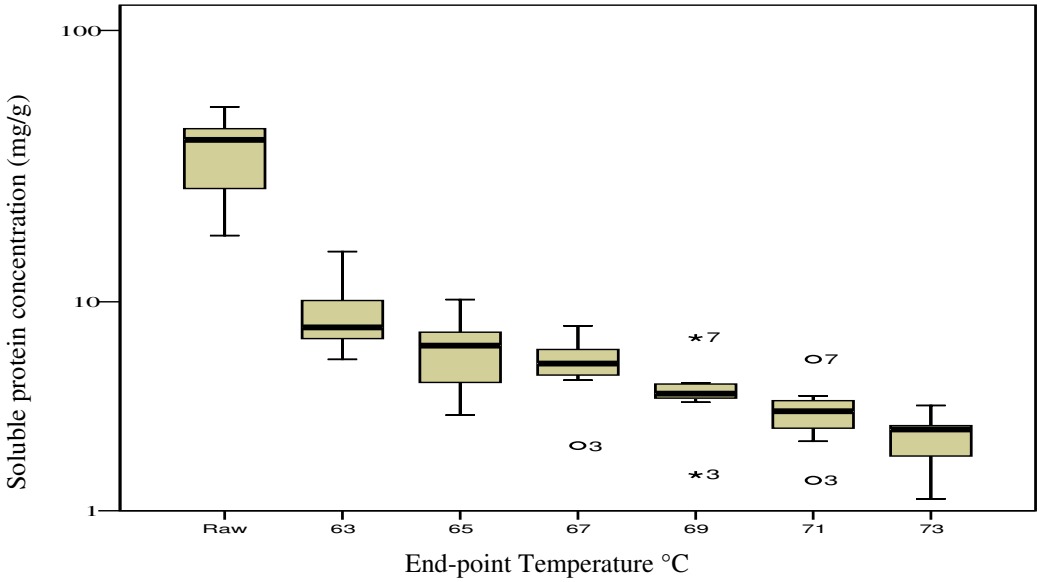


Figure 19-Box plots of Bradford protein concentrations (from Table 1) of seven beef *semimembranosus* muscle extracts heated to instantaneous end-point temperatures ranging from 25 (Raw) to 73 °C.

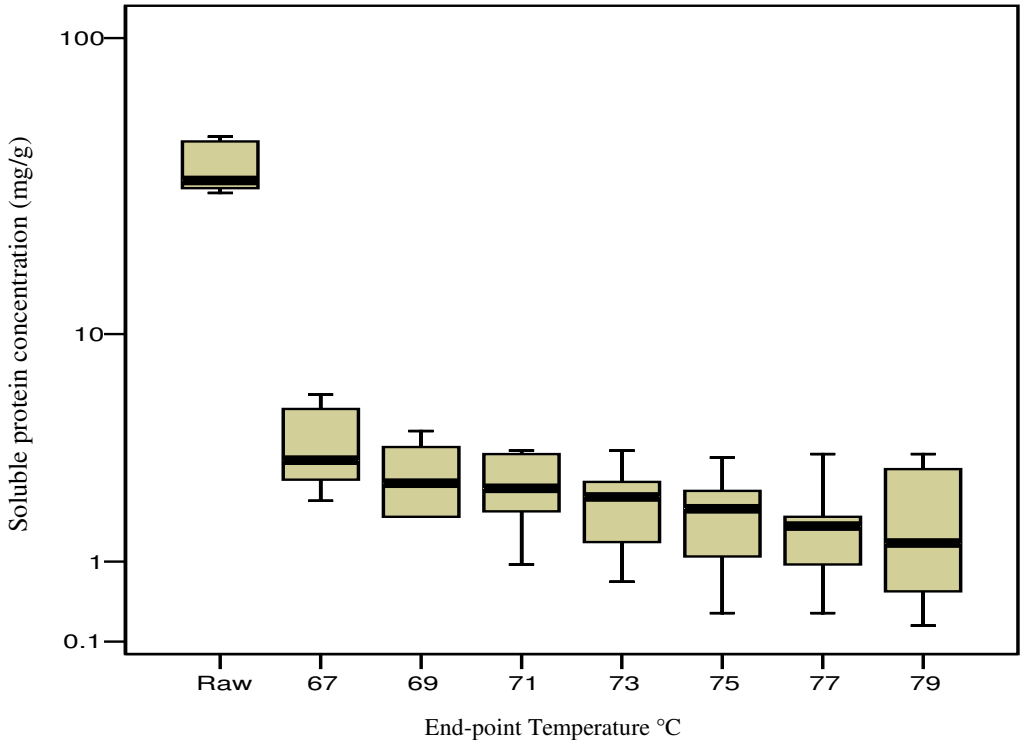


Figure 20-Box plots of Bradford protein concentrations (from Table 2) of six pork *longissimus dorsi* muscle extracts heated to instantaneous end-point temperatures ranging from 25 (Raw) to 79 °C.

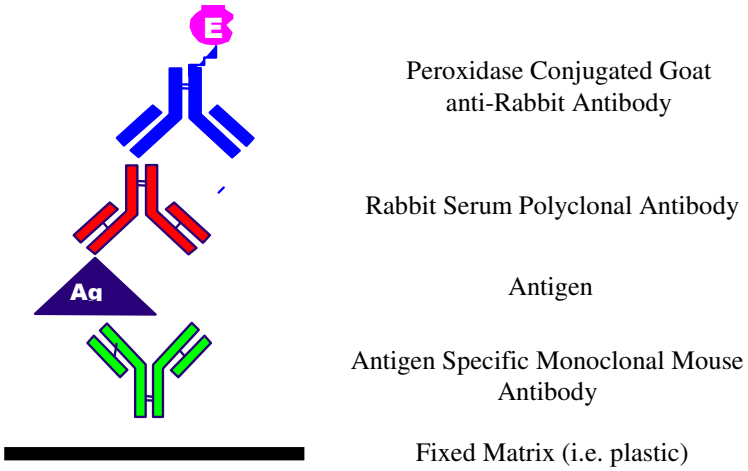
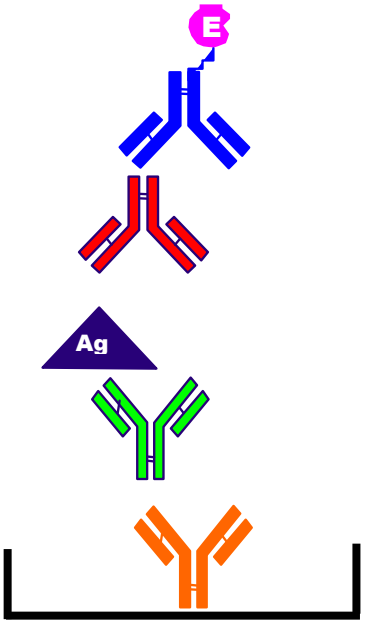


Figure 21-Typical sandwich ELISA assay structure.



Peroxidase Conjugated Goat anti-Rabbit Antibody

Rabbit Serum Polyclonal Antibody

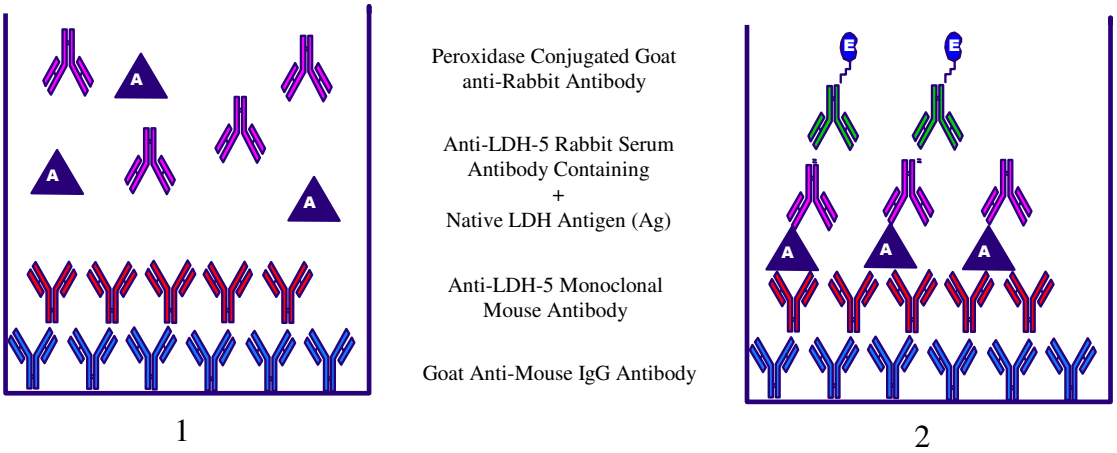
Antigen

Antigen Specific Monoclonal Mouse Antibody

Goat Anti-Mouse IgG Antibody

Plastic Microtiter Plate Well

Figure 22-Sandwich ELISA assay structure for multiple antigen EPT assay.



Peroxidase Conjugated Goat anti-Rabbit Antibody

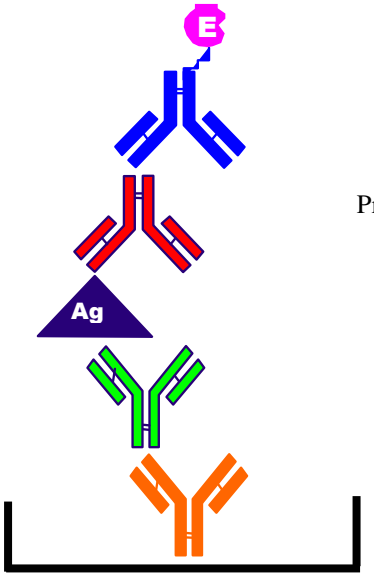
Anti-LDH-5 Rabbit Serum Antibody Containing + Native LDH Antigen (Ag)

Anti-LDH-5 Monoclonal Mouse Antibody

Goat Anti-Mouse IgG Antibody

1 2

Figure 23-Non-specific binding of an extraneous source of LDH-5 leading to increased background signal.



Peroxidase Conjugated Goat anti-Rabbit Antibody

Protein A/G Purified Anti-Bovine LDH-5 Rabbit Polyclonal Antibody

LDH-5

Anti-Bovine LDH-5 Monoclonal Mouse Antibody

Goat Anti-Mouse IgG Antibody

Plastic Microtiter Plate Well

Figure 24-Sandwich ELISA assay for determining LDH-5 concentration in beef and pork extracts cooked to various end-point temperatures.

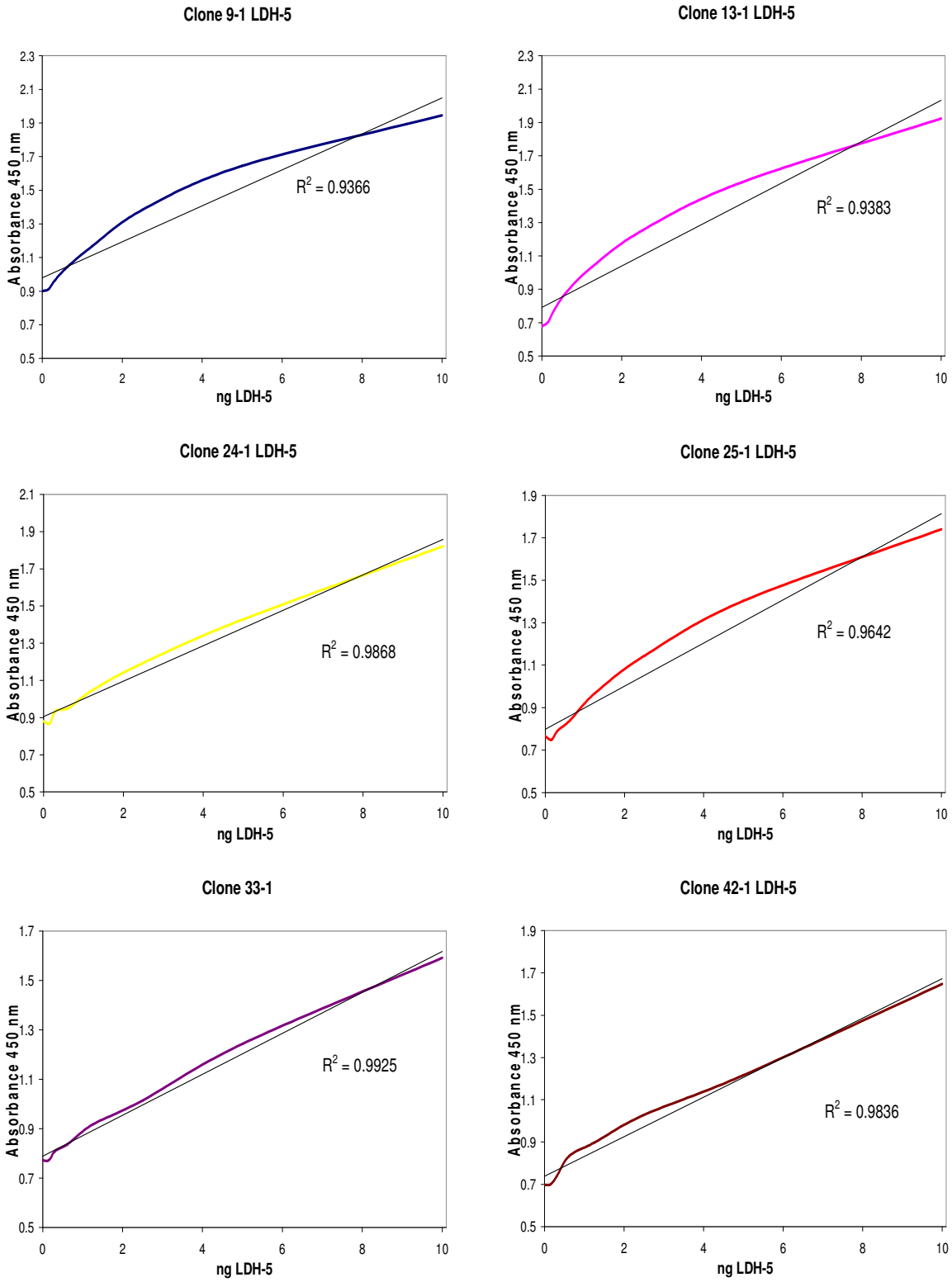


Figure 25-Standard curves of bovine LDH-5 from 0 to 10 ng using sandwich ELISA with six monoclonal antibodies developed against bovine LDH-5.

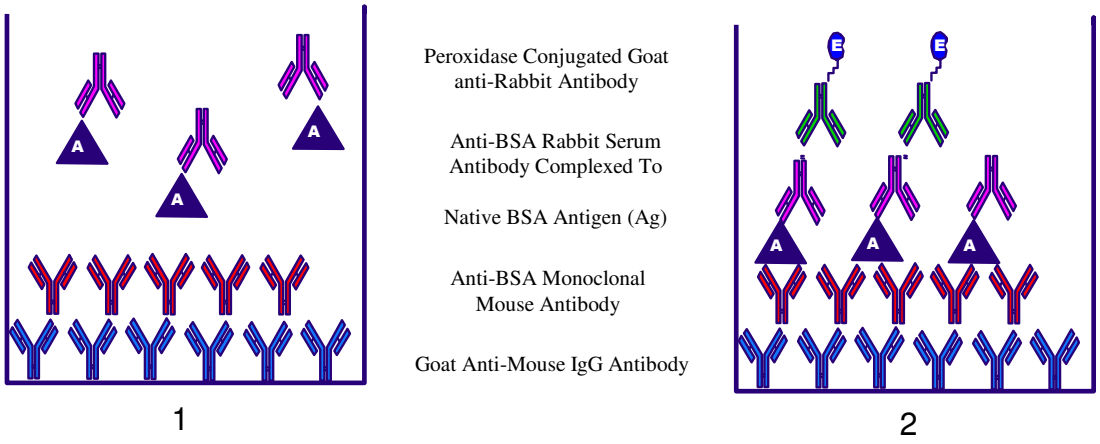


Figure 26-Possible binding of BSA anti-BSA polyclonal antibodies from purified rabbit serum leading to increased background signal.

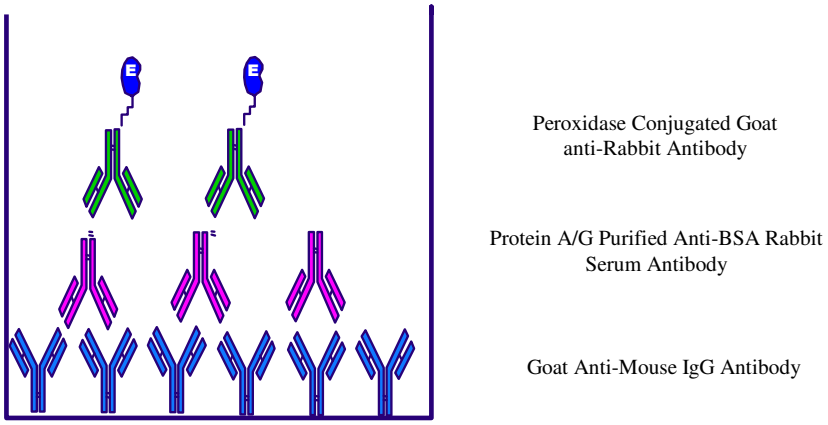


Figure 27-Cross-reactivity of goat anti-mouse IgG coating antibodies with polyclonal antibody from protein A/G purified rabbit serum leading to increased background signal.

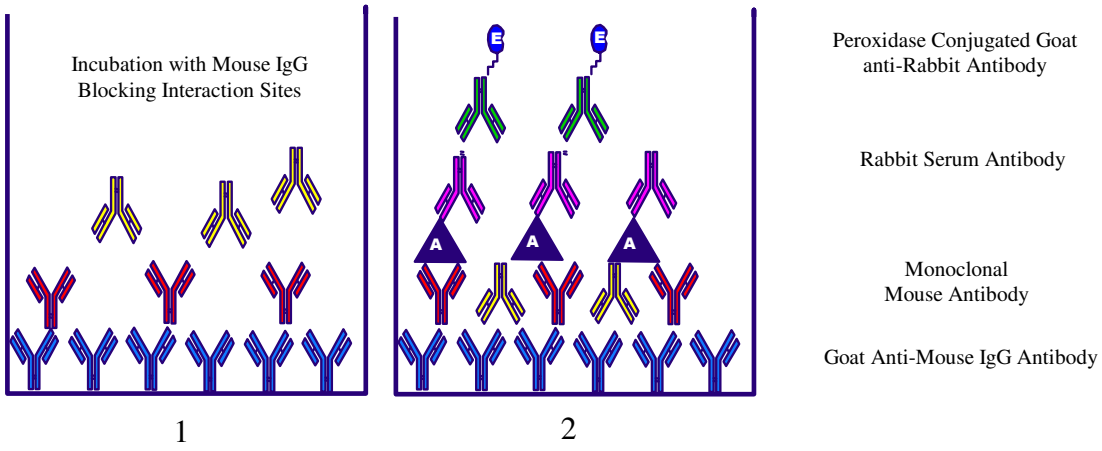


Figure 28-Blocking of excess binding sites to minimize cross-reactivity between goat anti-mouse and rabbit polyclonal antibodies.

APPENDIX B

TABLES

Table 1-Soluble protein concentration means (mg/g) of seven beef *semimembranosus* muscle extracts heated to instantaneous end-point temperatures from 25 (Raw) to 73 °C as measured by Bradford protein assay.

Temperature (°C)	Minimum	Max	Mean	Std. Error
Raw	17.90	53.04	36.25	4.81
63	5.88	15.60	9.16	1.27
65	3.36	10.20	6.43	0.90
67	2.40	8.04	5.59	0.67
69	1.68	7.20	4.34	0.61
71	1.56	5.88	3.52	0.51
73	1.20	3.72	2.59	0.33

Table 2-Soluble protein concentration means (mg/g) of six pork *longissimus dorsi* muscle extracts heated to instantaneous end-point temperatures from 25 (Raw) to 79 °C as measured by Bradford protein assay.

Temperature (°C)	Minimum	Maximum	Mean	Std. Error
Raw	30.70	47.28	37.16	3.00
67	2.16	6.00	3.79	0.63
69	1.80	4.32	2.84	0.46
71	0.96	3.60	2.49	0.41
73	0.72	3.60	2.13	0.42
75	0.36	3.36	1.86	0.43
77	0.36	3.48	1.64	0.43
79	0.24	3.48	1.66	0.54

Table 3-Total soluble protein, myoglobin, serum albumin, and LDH-5 contents of beef *semimembranosus* muscle extracts heated to instantaneous end-point temperatures from 25 (Raw) to 73 °C as measured densitometrically from standard dot blots[a].

Temperature °C		63	65	67	69	71	73
Beef 1	Protein	5550	3695	2750	1460	1030	810
	Myo	223	170	103	50	32	24
	BSA	3705	945	201	10	4	1
	LDH	823	230	90	37	10	3
Beef 2	Protein	6855	4170	4080	3060	3050	1410
	Myo	415	144	85	43	35	23
	BSA	3192	711	231	32	10	1
	LDH	1417	678	240	33	25	4
Beef 1	M/B/L*	0.073	0.782	5.69	135	800	8000
Beef 2	M/B/L*	0.092	0.300	1.53	41	140	5750

[a] Preliminary data Texas A&M University, Department Animal Science, Dr. J.T. Keeton

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