IDENTIFICATION AND VERIFICATION OF AN ENDOGENOUS TIME-TEMPERATURE INTEGRATOR TO DETERMINE PROCESSING ADEQUACY OF ROAST BEEF

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ABSTRACT

IDENTIFICATION AND VERIFICATION OF AN ENDOGENOUS TIME-TEMPERATURE INDICATOR TO DETERMINE PROCESSING ADEQUACY OF ROAST BEEF

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The USDA-FSIS requires specific thermal processing schedules for roast beef products to ensure the destruction of pathogens. A thermal process to achieve a 7-D reduction in *Salmonella* was recently proposed by the FSIS as the lethality performance standard for roast beef and cooked beef products. Triose phosphate isomerase (TPI) had a z value similar to that of *Salmonella* and was identified as a potential endogenous time-temperature integrator (TTI) to verify adequacy of roast beef processing based on previous studies. The overall goal of this study was to determine if TPI could be used as a TTI to verify compliance to USDA processing schedules. The ability of TPI to indicate thermal processing adequacy was investigated in a ground beef water bath model system and roast beef pilot studies at MSU. Three adequate temperature schedules, [62.2 °C/5 min (high), 58.3 °C/24 min (medium), and 54.4 °C/121 min (low)] and corresponding inadequate processing schedules (0.5 and 1 log cycle reduction in holding time from each adequate process) were used. In the ground beef water bath model system, TPI activity averaged 2.6 U/kg in adequately processed ground beef and increased when processing time was inadequate. In the MSU pilot plant study, TPI activities were similar when roasts were adequately processed at low and high temperatures, but TPI activity increased as processing time was decreased in the high temperature process only. Bovine muscle

TPI was purified and polyclonal antisera (PAb) were raised in rabbits. A sandwich ELISA was developed and cross reactivities of antibodies with TPI from different animal species, muscle protein concentrates, and common meat starter cultures were examined. Ground beef was heated in tubes to internal temperatures of 48.9 °C to 76.7 °C in 5.5 °C increments and extracted with PBS buffer. TPI concentration and activity in extracts of cooked ground beef decreased as temperature of water bath was increased. Both the ELISA and enzyme assay were able to detect differences in TPI due to cooking temperature of beef. In a commercial pilot plant study, no differences in TPI concentration and activity were found between adequately and inadequately processed roast beef. This may be due to inconsistent processing conditions within the smokehouse facilities or to variations in size and shape of roasts. Further research is needed to determine if TPI can be used as a TTI under commercial processing conditions.
KEYWORDS-- beef, endpoint temperature, processing

This work is dedicated to my beloved parents,

Mr. Ling-Yun Hsu and Mrs. Jean-Da Lee,

Mr. John Chang, who supported this "dreamer"

and the One I believe in.

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CHAPTER 1

INTRODUCTION

Since the January 1993 *E. coli* O157:H7 outbreak of foodborne disease caused by consumption of undercooked ground beef patties at a fast food restaurant chain in several Western states, the general public has become highly concerned about the safety of meat and poultry products (USDA-FSIS, 1996a). *Salmonella*, hemorrhagic *Escherichia coli*, *Campylobacter* and *Staphylococcus* are the major pathogenic bacteria found to cause foodborne disease outbreaks associated with domestic and imported precooked meats. A recent study estimated that foodborne outbreaks result in 5.5 to 6.2 million cases costing 5.8 to 8.6 billion U.S. dollars each year (Todd, 1994). Enteric pathogens from animal food products cause from 6.5 to 81 million outbreaks of illness each year in the U.S. as reported by the Centers for Disease Control and Prevention (CDC) (Bean and Griffin, 1990). *Salmonella* is one of the most frequent causes of foodborne disease in the U. S. Although 45,000 cases of *Salmonella* outbreaks are reported every year in the United States, foodborne cases of salmonellosis are estimated to range from 790,000 to 3.69 million annually, with a median number of 1.92 million cases (Todd, 1994). Most cases of foodborne disease attributed to *E. coli* O157:H7 were caused by consumption of undercooked and contaminated hamburger. About 10,000 to 20,000 cases of *E. coli* O157:H7 induced illness and 200 to 500 deaths occur in the U. S. every year (CDC,

1996). In fact, foodborne outbreaks associated with meat products continue to occur frequently in the U. S., thus effective methods to prevent and control foodborne pathogens are needed.

On October 1, 1996, a collaborative project to develop food safety strategies to reduce foodborne illness was initiated (USDA-FSIS, 1996a). The purpose of this project was to improve and monitor the implementation of food safety programs to decrease the number of pathogenic microorganisms, especially *Salmonella* and *E. coli* O157:H7, in meat, poultry, seafood, dairy, fruit and vegetable products. Several agencies, including the U.S. Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS), the Food and Drug Administration (FDA), CDC and public health departments, state health departments and local investigators at five locations in U. S., will collaborate to develop better methods to track the incidence of foodborne illness.

Adequate cooking is the simplest means of eliminating pathogenic bacteria from meat and poultry products. The USDA-FSIS (United States Department of Agriculture-Food Safety Inspection Services) has established specific cooking requirements for different types of precooked meat and poultry products. The purpose of these requirements is to ensure the destruction of harmful microorganisms and viruses that could cause foodborne illness in humans and livestock (Townsend and Blankenship, 1989). The USDA-FSIS (1995) has required that cooked beef and roast corned beef be heated to 62.8 ^{o}C (145 ^{o}F) instantaneously or to one of sixteen different time-temperature combinations. These processing treatments were designed to ensure a 7-D destruction of

Salmonella (a 7-D reduction of *Salmonella* is to reduce the number of *Salmonella* from 1 x 10^7 to 1 in 1 gram of meat) (Goodfellow and Brown, 1978).

On May 2 1996, USDA-FSIS proposed several rules to amend the current federal meat and poultry inspection regulations to establish "safety margins" for thermally processed meat and poultry products (USDA-FSIS, 1996b). The new regulations for meat and poultry will be applied to the following products: roast beef, cooked beef and cooked corned beef, uncured meat patties (fully cooked, partially cooked and char-marked patties), and some poultry products (fully and partially cooked products). The purpose of the new proposed performance standards for meat and poultry is to ensure the safety of meat products by eliminating pathogenic microorganisms from the products. Three major steps to achieve this objective were lethality, stabilization, and handling. *Salmonella* is a common pathogen in roast and cooked beef products and is more thermally resistant than *E. coli* O157:H7. Thus, instead of specific approved processing schedules, a 7-D reduction in *Salmonella* was proposed by FSIS as the lethality performance standard for processing of roast beef, cooked beef and cooked corned beef products. A 5-D reduction in *Salmonella* (a 5-D reduction of *Salmonella* is to reduce the number of *Salmonella* from 1 x 10^5 to 1 in 1 gram of meat) was proposed as the lethality performance standard for cooked, uncured meat patties. Also, the USDA-FSIS announced a new series of time-temperature processing requirements based on a 7-D reduction in *Salmonella* for poultry, instead of the current single temperature requirement for uncured (71.1 °C, no holding time) and cured (68.3 °C, no holding time) products. Meanwhile, FSIS is evaluating these lethality standards using different processing

conditions to accommodate more flexibility and to provide more realistic processing schedule combinations. This proposed regulation should allow for the use of more sophisticated and flexible thermal processing treatments and provide a higher standard of safety and wholesomeness in meat and poultry products.

A time-temperature integrator (TTI) is needed to verify that meat products receive adequate thermal treatment to eliminate pathogenic microorganisms. A TTI is "a measuring device that shows a time-temperature dependent, easily, accurately and precisely measurable irreversible change that mimics the changes of a target attribute undergoing the same variable temperature exposure" (Hendrickx et al., 1995). The z value or activation energy of a TTI in the revelant range of a time-temperature processing schedule should have a z-value or activation energy (kinetics of rate constant) similar to that of the target index in the food system. A TTI can be prepared from enzymes, proteins, microorganisms or chemicals. Time-temperature integrators can exist in either extrinsic or intrinsic forms. Extrinsic TTIs are added to the food system and are retrieved back from the food after thermal processing is completed. Extrinsic TTIs can be encapsulated to prevent changes in inactivation kinetics of the indicator due to reactions with the food material. However, intrinsic TTIs are prepared from compounds in the target food. If the TTI is an endogenous component of the food system, extraction or recovery procedures are necessary to analyze its concentration after processing.

An endogenous indicator protein used as a TTI to verify roast beef processing should meet two criteria. First, its concentration or activity should differ between optimal and sub-optimal processing treatments. Second, the indicator should have the same

concentration or activity in all optimal processes. For these criteria to be met, the marker protein must have a z value similar to that of the pathogen used by the USDA-FSIS to establish roast beef processing schedules.

Currently, the FSIS is using the "Bovine Catalase Test" developed by Eye (1982) for the detection of underprocessing of rare beef and cooked beef. A protein "Coagulation Test" is used for monitoring the maximum internal temperature of both beef and pork products heat processed to temperatures lower than 65 ^{o}C (149 ^{o}F) (USDA-FSIS, 1986a). These methods for verification of heating endpoint temperatures in precooked meat products have been shown to be subjective and inaccurate for predicting the actual heating endpoint temperature achieved during processing (Townsend and Blankenship, 1989). The greatest disadvantage of these tests is that none uses an indicator with a z value similar to the z value of *Salmonella* used by the USDA to define roast beef processing schedules.

The overall goal of this study was to examine the thermal inactivation kinetics of selected endogenous enzymes present in bovine *semimembranosus* muscle and then to identify a candidate marker protein as a TTI to monitor thermal processing adequacy of roast beef. A marker protein, triose phosphate isomerase (TPI), was identified as an enzymatic TTI in this study since it had a z value similar to that of *Salmonella* used to establish the USDA roast beef processing schedules. Therefore, TPI was purified and used to produce anti-TPI polyclonal antibodies. The final objective of this study was to establish an endogenous enzymatic TTI using an immunochemical assay (enzyme - linked immunosorbent assay; ELISA) to verify adherence to safe processing schedules.

Theoretically, the ELISA should provide an accurate, rapid, inexpensive and convenient method for thermal monitoring by regulatory agencies and processors for determining the adequacy of thermal processing of precooked roast beef products.

Four specific objectives of this study are listed below:

Study I - To compare the thermal inactivation kinetics of acid phosphatase and peroxidase to the D and z values of *E. coli* O157:H7 and *Salmonella senftenberg* in ground beef at four different temperatures.

Study II - To screen several endogenous bovine enzymes as time-temperature integrators for precooked roast beef using optimal and sub-optimal time-temperature processing conditions.

Study III - To purify the marker protein, develop polyclonal antibodies and devise an ELISA to quantify the marker protein in extracts of roast beef.

Study IV - To verify the ability of the selected marker protein by ELISA and enzyme assay to determine adequacy of thermal processing of beef cooked in model systems and commercial meat processing plants.

Four experiments were designed to evaluate the objectives of this study:

Experiment I - The first experiment was designed to compare the thermal inactivation kinetics of acid phosphatase and peroxidase to the D and z values of *E. coli* O157:H7 and *Salmonella senftenberg* in ground beef at four different temperatures. This research was done in cooperation with other researchers who also evaluated the thermal inactivation kinetics of triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, *E. coli* O157:H7 and *Salmonella senftenberg*.

Enzymes with a z value similar to that of *Salmonella* could be used as TTIs to verify that roast beef was adequately processed.

Experiment II - The second experiment was designed to screen several endogenous bovine enzymes as TTI indicators for precooked roast beef using different optimal and sub-optimal time-temperature processing conditions. Beef top round roasts were processed using two or three time temperatures combinations in a smokehouse used as a heat processing oven. Each combination included one or two sub-optimal processes and one optimally cooked treatment. Low salt soluble proteins were extracted and enzyme activities or concentrations of peroxidase, TPI, acid phosphatase, and lactate dehydrogenase were determined.

Experiment III - The third experiment was to purify the marker protein, TPI, and to develop polyclonal antibodies against the marker protein. Polyclonal antibodies were made against purified bovine TPI using rabbits. A TPI sandwich ELISA was developed and optimized to quantify the marker protein in extracts of roast beef in a model system.

Experiment IV - The fourth experiment was to verify the ability of TPI, measured by ELISA and enzyme assay, to determine processing adequacy of beef cooked in a commercial meat processing plant. This final experiment was designed to determine if the TPI sandwich ELISA could be used to differentiate between optimal and sub-optimal time-temperature combinations of commercially processed roast beef to confirm its applicability.

CHAPTER 2

LITERATURE REVIEW

2.1 USDA-FSIS THERMAL REGULATIONS FOR MEAT PRODUCTS

To ensure the destruction of pathogenic bacteria and viruses, *Title 9 of the Code of Federal Regulations* lists required thermal treatments for a variety of precooked meat and poultry products. Regulations also exist for imported precooked products to prevent foodborne diseases. These regulations were implemented to ensure the destruction of harmful microorganisms (e.g., *Salmonella*, hemorrhagic *Escherichia coli*, *Campylobacter*, *Clostridium*, and *Staphylococcus*) and viruses (e.g., Velogenic-Viscerotropic Newcastle Disease Virus) that cause illness in humans and animals (Townsend and Blankenship, 1989).

The United States Department of Agriculture - Food Safety and Inspection Service (USDA-FSIS) (1995) requires that cooked beef and rare roast beef be heated in accordance with one of 16 time-temperature combinations (Table 2.1). For instance, beef can be heat processed to 62.8 ^{o}C (145 ^{o}F) with no holding time or processed to 54.4 ^{o}C (130 ^{o}F) with 121 min holding time. Cured/smoked and ready-to-eat pork products must be heated to at least 58.3 ^{o}C (137 ^{o}F) or frozen to destroy *Trichinae*. For poultry products, minimum internal temperatures of 71.1 ^{o}C (160 ^{o}F) and 68.3 ^{o}C (155 ^{o}F) are required for uncured and cured poultry products, respectively.

Table 2.1 Minimum USDA thermal processing schedules for cooked beef and roast beef

Temperature (^{O}C (^{O}F))	Time (min)
54.4 (130)	121
55.0 (131)	97
55.6 (132)	77
56.1 (133)	62
56.7 (134)	47
57.2 (135)	37
57.8 (136)	32
58.4 (137)	24
59.5 (139)	15
60.0 (140)	12
60.6 (141)	10
61.1 (142)	8
61.7 (143)	6
62.2 (144)	5
62.8 (145)	0

Code of Federal Regulations, Title 9 (USDA-FSIS, 1995)

2.2 THE USDA-FSIS PROPOSED REGULATIONS TO AMEND THE CURRENT MEAT AND POULTRY INSPECTION REGULATIONS

On May 2, 1996, USDA-FSIS proposed several rules to amend the current federal meat and poultry inspection regulations to establish "safety margins" for thermally processed meat and poultry products (USDA-FSIS, 1996b). The new regulations for meat and poultry would be applied to the following products: roast beef, cooked beef and cooked corned beef, uncured meat patties (fully cooked, partially cooked and char-marked patties), and some poultry (fully and partially cooked products). The purpose of the new proposed performance standards for meat and poultry products are to ensure the safety of meat products by eliminating pathogenic microorganisms from the products. Three major steps to achieve this objective were lethality, stabilization, and handling.

A 7-D reduction in *Salmonella* was proposed by FSIS as the lethality performance standard for roast beef, cooked beef and cooked corned beef products (USDA-FSIS, 1996b). There are three reasons that *Salmonella* was chosen as the indicator strain by FSIS. First, *Salmonella* is a major and commonly found pathogen in roast and cooked beef products. Second, *Salmonella* is more thermally resistant than *E. coli* O157:H7. Third, even though *Listeria monocytogenes* is more heat resistant than *Salmonella*, it is mostly found in post-process contaminated meat and at levels much lower than typical for *Salmonella* in meat products. In general, a processing scheduled based on 7-D reduction in *Salmonella* for roast and cooked beef was designed to produce pathogen-free and safe cooked beef products, however, it may cause over-processed beef products. Since *Salmonella* is usually found in raw beef at concentration below 10^3 to 10^4

microorganisms/gram of meat, a 3-D or 4-D reduction in *Salmonella* should be sufficient to offer safe cooked beef products. At this time, FSIS is still evaluating this lethality standard by comparing different commercially applicable processing conditions.

In addition, a 7-D reduction in *Salmonella* was proposed for cooked poultry products. This is the first time USDA-FSIS has proposed a series of time-temperature schedules for poultry instead of a single temperature requirement for uncured (71.1 °C, no holding time) and cured poultry (68.3 °C, no holding time) products.

A 5-D reduction in *Salmonella* was proposed by FSIS as the lethality performance standard for cooked, uncured meat patties (USDA-FSIS, 1996b). A processing schedule sufficient to cause a 5-D reduction should provide for pathogen-free products and avoid dry, burned and low quality products caused by a more severe thermal process.

2.3 CURRENT USDA METHODS FOR ENDPOINT TEMPERATURE (EPT) DETERMINATION

Current procedures used by the FSIS for monitoring the adequacy of heat treatment of meat and poultry include three techniques. They are the Bovine Catalase Activity Test for cooked and roast beef, the Protein Coagulation Test for beef and pork products, and the residual Acid Phosphatase Activity Test for canned hams, picnics and luncheon meats. These USDA approved methods for EPT determination are inaccurate, subjective, impractical, and time-consuming. Since the number of foodborne illness outbreaks are increasing yearly, these EPT determination methods are crucial for the health of the general public. However, the current methods have shortcomings and

effective assays are urgently needed. Many alternative methods have been investigated to determine EPT in various meat products.

2.3.1 Bovine Catalase Activity Test

The Bovine Catalase Activity Test was developed approximately 11-15 years ago (Townsend and Blankenship, 1989) and is used for cooked and roast beef. The detection of residual endogenous catalase activity is commonly utilized at heating EPTs slightly above 60 °C (140 °F). Foam is produced as oxygen and is liberated from a reaction of hydrogen peroxide in the presence of sodium lauryl sulfate (shampoo). "Strong, medium, weak and no activity" must be interpreted from the quantity of foam produced (USDA-FSIS, 1989). Research has indicated the highly subjective nature of this test and endpoint temperatures interpretations varied by operator (Stalder et al., 1991).

2.3.2 Protein "Coagulation Test"

A protein "Coagulation Test" is used to monitor the maximum internal temperature of both beef and pork products heat processed to temperatures lower than 65 °C (149 °F) (USDA-FSIS, 1986a). The Protein Coagulation Test was developed about 35-40 years ago (Townsend and Blankenship, 1989).

The "Protein Coagulation Test" is based on measurable loss in protein solubility as product temperature is increased. The test involves extracting soluble muscle proteins and observing the temperature at which the first signs of cloudiness or turbidity (54-57 °C; 129-135 °F) appear when the filtrate is heated. This is considered to be the maximum

internal cooking temperature of the product. For products heat processed to 63-71 °C (145-160 °F), the temperature at which the filtrate becomes cloudy may differ by 8-10 °C (46.4-50 °F) from the known internal temperature of the product (USDA-FSIS, 1986a). This method is empirical, subjective, time-consuming and difficult to perform outside of an established laboratory (Townsend et al., 1984).

2.3.3 Acid Phosphatase Activity Test

The Acid Phosphatase Activity Test for determining the EPT was also developed 35-40 years ago (Townsend and Blankenship, 1989). The residual "Acid Phosphatase Activity Test" (USDA-FSIS, 1986b) is used to determine heat treatment of canned hams, picnic hams and luncheon meat.

Enzyme activity is determined on water extracts of ground meat samples utilizing disodium phenyl phosphate as substrate. The enzymatic reaction produces phenol- and mono-hydrogen phenyl phosphate. Phenol is subsequently reacted under alkaline conditions with 2, 6-dibromoquinonechlorimide to produce a blue chromophore which absorbs at 610 nm. The residual activity of the enzyme after cooking is expressed as μ mole of phenol formed per 1000 g meat after reacting with the substrate, disodium phenylphosphate, for 60 min at 37 °C (99 °F), pH 6.5. The residual activity of acid phosphatase in hams does not depend directly on temperature alone, but on time/temperature history. Results are poorly correlated with actual EPT (Cohen, 1969; Kormendy et al., 1987; Townsend and Blankenship, 1989).

2.4 ALTERNATIVE METHODS FOR EPT DETERMINATION

Several alternative testing procedures for meat and poultry have been developed. They include methods based on solubility of sarcoplasmic proteins (Davey and Gilbert, 1974), dominant spectral wavelengths of beef juice to predict the maximum internal temperatures in cooked beef (Nusimovich et al., 1979), intrinsic protein fluorescence to examine the structural changes of muscle protein during heating (Oreshkin et al., 1968), near infrared reflectance to measure protein denaturations (Osborne and Fearn, 1986), differential scanning calorimetry to monitor protein denaturation temperature (Quinn et al., 1980; Wright and Wilding, 1984), sodium dodecyl sulfate (SDS) gel electrophoresis of proteins (Caldironi and Bazan, 1980), residual enzyme activity (Townsend and Blankenship, 1987a, 1987b; Collins et al., 1991a, b; Stalder et al., 1991), and immunoassays (Wang et al., 1992; Abouzied et al. 1993; Wang et al., 1993, 1994, 1995). Based on a literature review, enzyme assays and immunoassays offer powerful techniques for selecting protein markers and verifying EPT in processed products of various meat species.

2.5 ENZYMATIC METHODS FOR EPT DETERMINATIONS IN MEAT PRODUCTS

Several candidate enzymes have been screened and identified as potential protein markers in different meat products.

2.5.1 Bovine Meat

Lactate dehydrogenase (LDH) has been evaluated as a potential protein marker in both bovine and porcine muscles (Collins et al., 1991a, b; Stalder et al., 1991). The influence of pH, salt, phosphate, cooking temperature, muscle type, carcass gender and maturity on LDH activity in water extracts of bovine muscle have been examined (Stalder et al., 1991). Cooking decreased LDH activity from greater than 1000 U/g muscle in raw meat to almost undetectable levels at 66 ^{o}C (151 ^{o}F), regardless of pH. Extracts heated at pH 5.6 showed sharper decreases in activity with increasing temperature than samples heated at pH 6.4. When different levels of salt (0 to 4.5% NaCl) and phosphate (0 to 0.3% sodium triophosphate) were added, LDH activity decreased between 57 and 63 ^{o}C (135-145 ^{o}F) at pH 5.6. Gender of carcasses did not affect LDH activity.

Further evaluation of LDH as a heating EPT indicator in whole cooked roast beef products was performed by Stalder et al. (1991). LDH activity decreased with increased cooking temperature in roasts prepared with and without brine. Colormetric and fluorescent assays were successfully used on juice squeezed from intact roasts to determine LDH activity. LDH was inactivated at about 62 ^{o}C (144 ^{o}F). No relationship of LDH complianced to time-temperature thermal processing schedules was investigated.

Townsend and Davis (1992) concluded that transaminase enzymes (glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase) retained considerable activity at 71.1 ^{o}C (160 ^{o}F), therefore, GOT could possibly be used for determining the adequacy of thermal treatment of imported cooked beef which must be heat processed to 79.4 ^{o}C (175 ^{o}F). These enzymes would not be good indicators for

ground beef which requires one of seven time-temperature combinations, ranging from 66.1 °C (151 °F) for 41 sec to 69.4 °C (157 °F) for 10 sec (USDA-FSIS, 1995).

2.5.2 Porcine Meat

Pyruvate kinase (PYK) has shown potential as EPT indicators in a model cured pork system and a commercial canned cured pork product (Davis et al., 1988). In the model system, high PYK activity was observed at 67.7 °C and activities were decreased when meat heated to 68.3 or 68.9 °C. When heated to 69.5 or 70.0 °C, PYK activity was not detectable from the cured pork model system. In a commercial canned cured pork study, high PYK activity was found at 62.9 °C internal temperature. Gradually decreased in PYK activities were found when products heated to 65.6 and 68.6 °C. No PYK activity was observed when canned products heated to 69.9 °C.

2.5.3 Poultry Meat

Bogin et al. (1992) examined 12 enzymes from turkey breast meat for use as EPT indicators. The activities of the enzymes were studied after heating 25 - 50 g meat in glass beakers to different temperatures (50 °C, 60 °C, or 70 °C) and for various times (15, 30, 45, 90 min) to identify possible EPT indicators. Then, 3 g of meat was removed, homogenized with 30 ml Tris-HCl buffer (0.02 M; pH 7.4), and centrifuged at 35,000 x g for 40 min. The supernatant was collected to assess enzyme activity and concentration of soluble protein. Asparate-oxoglutarate aminotransferase was found to be most suitable for verification of an EPT of 70 °C (158 °F). Creatine kinase (CK), malic

dehydrogenase, lactate dehydrogenase, and isocitric dehydrogenase were also found to decrease proportionally with heating temperature and time and were suggested potential markers for inactivation of Velogenic-Viscerotropic Newcastle Virus in turkey meat.

Hsu (1993) screened 26 endogenous enzymes extracted from turkey *pectoralis major* (breast) and *sartorius* (thigh) muscles. Lactate dehydrogenase and malic dehydrogenase from *pectoralis major* each showed consistent decreases in activity between 69 ^{o}C (156 ^{o}F) and 73 ^{o}C (163 ^{o}F) in three treatments (water treatment: ground muscle blended with 15% double distilled water; brine treatment: ground muscle blended with 15% NaCl brine by weight to result in a final concentration of 1.5% NaCl, 0.5% sodium tripolyphosphate (STPP) and 12.5% water in the tissues; and curing brine treatment: ground muscle mixed with 15% NaCl curing brine by weight to result in a final concentration of 2.5% NaCl, 0.5% STPP, 156 ppm sodium nitrite, 550 ppm sodium erythorbate and 12.5% water in the tissues), at three heating rates (0.25, 0.5, and 1.0 ^{o}C/min) in fresh and frozen tissues. Thus, these enzymes could potentially serve as EPT indicators in poultry for USDA-FSIS minimum temperatures of 68.3 ^{o}C (155 ^{o}F) (cured) or 71.1 ^{o}C (160 ^{o}F) (brined).

Davis and Townsend (1994) examined acid phosphatase activity in both non-frozen and frozen ground broiler, turkey breast and turkey dark meat. Meat was tightly packed in glass tubes (25 x 150 mm) and heated to five different temperatures: 62.8, 65.6, 68.3, 71.1 and 73.9 ^{o}C in a water bath system. The authors suggested that residual acid phosphatase activity may be product dependent; therefore, more research will be necessary to establish maximum residual concentrations for various products.

2.5.4 Comparison of EPT Indicators in Bovine, Porcine and Poultry Meats

Smith (1991) investigated 26 endogenous enzymes as EPT indicators in bovine, porcine, and poultry meats. In bovine muscle, CK, enolase, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, phosphoglucoisomerase, phosphoglucomutase, and pyruvate kinase underwent rapid denaturation between 60 ^{o}C (140 ^{o}F) to 72 ^{o}C (162 ^{o}F). In porcine muscle, enolase, malate dehydrogenase, fructose-6-phosphate kinase, pyruvate kinase, CK, aldolase, and TPI were rapidly inactivated near the federally regulated EPT of 68.8 ^{o}C (156 ^{o}F) for imported pork and pork products. In chicken muscle, aldolase, CK, enolase, fructose-6-phosphate kinase, glutamate-pyruvate transaminase, malate dehydrogenase, and phophoglucoisomerase from chicken muscles showed significant decreases in activity or no activity at an EPT range of 66-68 ^{o}C (151 - 154 ^{o}F) or 72-74 ^{o}C (162-165 ^{o}F).

2.6 IMMUNOASSAYS FOR EPT DETERMINATIONS IN MEAT PRODUCTS

Immunoassays provide a sophisticated approach to assure the safety and quality of foods (Kang'ethe, 1990; Fukal, 1991; Samarajeewa et al., 1991; Morgan et al., 1992). The use of immunological assays for detection of hormones, pesticides, antibiotics, mycotoxins, proteins and enzymes have been well documented (Pestka, 1988). Immunoassays have been used for determining EPT of several meat products, including ground beef (eg., hamburger patty) and poultry products (eg., turkey roll and turkey ham) (Wang et al., 1992; Abouized et al. 1993; Wang et al., 1993, 1994, 1995; Orta-Ramirez et al., 1996). Enzyme-linked immunosorbent assays (ELISA) are the most widely used

immunological assays in the food and agricultural sciences. ELISAs are simple, sensitive, highly specific, more accurate and less time-consuming than many other detection methods. Immunoassays may be more sensitive and less expensive than enzymatic methods.

2.6.1 Bovine Meat

Wang et al. (1995) developed an LDH sandwich ELISA to determine the EPT of ground beef. Ground beef was packed in 10 x 75 mm test tubes and heated from 62 to 74 ^{o}C at 2 ^{o}C increments in a water bath. Polyclonal antibodies against LDH were used for capture and biotinylated polyclonal antibodies were used for detection in the sandwich ELISA. Less than 4 ug LDH/g meat was detected at 69 ^{o}C and decreases ($p < 0.05$) in LDH were found when ground beef was cooked between 66 and 74 ^{o}C at 2 ^{o}C increments. Commercially cooked processed beef patties heated from 68.3 to 71.1 ^{o}C contained 2.78 μg LDH/g meat. The authors suggested that the LDH sandwich ELISA was able to detect the EPT of commercially cooked beef patties; however, thermal processing variations and formulations would affect the residual amount of LDH in processed patties.

A sandwich ELISA, using both anti-LDH monoclonal and polyclonal antibodies, was developed to verify EPT of ground beef patties (Orta-Ramirez et al., 1996). The ELISA could detect differences ($p < 0.05$) in LDH concentration between patties cooked to EPT of 62.8 ^{o}C (145 ^{o}F) and 65.6 ^{o}C (150 ^{o}F) or 62.8 ^{o}C (145 ^{o}F) and uncooked patties. No difference in LDH concentration was found among patties cooked to internal

temperatures of 65.6 °C (150 °F), 68.3 °C (155 °F) and 71.1 °C (160 °F). At different fat contents (10.7, 13.6 and 19.0%), no differences were found among patties at EPT of 68.3 °C (155 °F). Orta-Ramirez et al. (1996) also suggested that the LDH sandwich ELISA detected about 3 μg LDH/g meat in processed ground beef cooked to 70 °C in both a model study and commercially cooked beef patties.

2.6.2 Poultry Meat

Wang et al. (1992) developed an indirect competitive ELISA to determine EPT of uncured turkey breast rolls using LDH as the marker protein. Turkey rolls were processed to internal temperatures of 68.3 °C (155 °F), 69.7 °C (157 °F), 70.9 °C (160 °F) and 72.1 °C (162 °F). LDH was not observed in extracts separated by native polyacrylamide gel electrophoresis (PAGE) at 70.9 °C (160 °F) and was chosen as the EPT marker protein for immunoassay development. LDH was identified on the basis of molecular weight (35 kd) using SDS-PAGE and LDH-specific stain on native PAGE. Polyclonal antibodies (PAbs) were made against purified turkey muscle LDH and commercially available chicken muscle LDH. The LDH content in turkey breast roll extracts as determined by sandwich ELISA decreased as the cooking temperature was increased.

Abouzied et al. (1993) developed a sandwich ELISA to quantify LDH in uncured poultry products. Four monoclonal antibodies (MAbs) against chicken muscle LDH were produced. In this LDH sandwich ELISA, monoclonal antibodies were used for capture and polyclonal antibodies were used as the detecting antibodies. The ELISA could detect 1 ng LDH/mL in turkey or chicken meat extracts. The LDH sandwich ELISA was tested

on extracts of turkey rolls processed to 68.3, 69.7, 70.9 and 72.1 °C. LDH concentration was 10 times lower in rolls processed to 70.9 °C as compared to those cooked to 69.7 °C. The antibodies cross reacted with LDH from chicken and turkey, but not beef or pork. The authors suggested that the sandwich ELISA should be able to verify the EPT of turkey breast rolls previously cooked to the required USDA minimum internal temperature of 71.1 °C.

Wang et al. (1993) further examined the effects of formulation, storage and processing conditions on LDH concentration of turkey breast rolls as measured by sandwich ELISA. LDH concentration differed at processing temperatures of 70.0 °C (158 °F) and 71.1 °C (160 °F), however extractable protein and LDH activity did not differ at these temperatures. They concluded that salt concentration, cooking schedule and product casing diameter did not influence LDH concentration. Frozen storage decreased LDH content of uncooked rolls. However, the LDH ELISA could not differentiate EPT of turkey thigh rolls processed between 68.9 °C (156.02 °F) and 71.1 °C (159.98 °F) due to the presence of heat stable LDH isozymes (Wang et al., 1994). Desrocher (1994) also developed ELISAs to verify EPT of turkey hams. The LDH sandwich and immunoglobulin G indirect competitive ELISA both showed the potential to accurately determine EPT of turkey ham.

2.7 THERMAL DEATH TIME (TDT) STUDIES

Thermal death time (TDT) studies are used to determine the thermal resistance of microorganisms or enzymes in a food system. There are many factors that affect thermal

death time, such as water activity, pH, and food components and contents (protein, carbohydrate, lipid, salt). D value represents the time (min) required to destroy or inactivate 90% of the organisms or enzyme activity at one temperature. In general, decreasing water activity increased D value. Decreasing the pH of a food usually reduces heat resistance and decreases D value. Because of the protective effect provided by physical aggregation of carbohydrates, proteins, lipids and salt (up to 2-4 %), the higher the concentration of these ingredients, the higher the D value (Jay, 1992).

2.7.1 Definitions

Mathematical methods can be used to determine the effect of thermal processing on food components and microorganisms. The thermal destruction of microorganisms, enzymes, nutrients and quality factors, such as texture, color and flavor, in a food system, usually follow first order reaction kinetics (Lund, 1975).

The decimal reduction time or D value represents the time (min) needed to destroy or inactivate 90% of the organisms or food component in a system at a certain temperature. The 12D concept is applied in the canning industry to reduce the population of the most heat-resistant spore-forming pathogenic microorganism, *Clostridium botulinum,* by 1×10^{12} spores. If 1×10^{3} *Clostridium botulinum* spores (which is the estimated maximum count) were in a low-acid food; therefore, after a 12D process, there should be less than one spore in 1×10^{9} cans.

The D value is calculated from the equation:

$$D = \frac{T}{\log a - \log b}$$

where

D = decimal reduction time or death rate (min)

T = heating time (min)

a = initial number of microorganisms or initial activity of an enzyme

b = final number of microorganisms or residual enzyme activity

The thermal death time curve can be graphed on a semi-log scale. The X-axis is the temperature which is the linear scale and the Y-axis is the D value on a logarithmic scale. The best straight line through these points is the thermal death time (TDT) curve. The z value is calculated from the reciprocal of the slope of the TDT curve and is defined as the temperature change needed to transverse one logarithmic cycle of the TDT curve.

The F value is the time (min) required to destroy a certain number of microorganisms at a specific temperature. This value can be used to compare different thermal processes. The Fo value is the time required to destroy a certain number of microbial spores at 250 ^{o}F when the z value is 18 ^{o}F.

D and z values can be calculated based on microbial destruction or enzyme inactivation. For instance, catalase and peroxidase were selected as marker proteins for the blanching process for vegetables (Lund, 1975). Alkaline phosphatase was chosen as the indicator protein for milk pasteurization, since *Mycobacterium tuberculosis*, the most thermal-stable pathogen in milk, and alkaline phosphatase have the same rate of thermal

inactivation (Kay and Graham, 1933). *Clostridium botulinum* is used as an indicator of lethality in thermal process calculations system, because it is the most heat-resistant spore-forming pathogenic microorganism found in canned foods. These indicator enzymes and microorganisms can be categorized as endogenous time temperature integrators (TTIs) with thermal inactivation constants (z value or activation energy) similar to the target attributes.

2.7.2 TDT Studies in Various Meat Systems

2.7.2.1 Ground Beef

Goodfellow and Brown (1978) initiated a study to determine the D values of *Salmonella* serotypes, including *Salmonella typhimurium* strain TMI (as a reference strain, less heat resistant strain), *Salmonella newport, Salmonella agona, Salmonella bovis-morbificians* and *Salmonella muenchen* strains (the last 4 strains were isolated from food poisoning outbreaks related to meat sources) in ground beef. A mixed strain inoculum of six *Salmonella* serotypes was added to ground beef (fat content not stated). Plate Count Agar (PCA) and PCA with xylose lysine deoxycholate agar overlay were used to recover *Salmonella* spp. D values for *Salmonella* in ground beef were determined and *Salmonella* survival was also evaluated in both a water cooking and dry roasting beef system. D and z values are shown in Table 2.2. This study was the first to determine a series of industrial processing schedules which could reduce *Salmonella* by 7 D in ground beef.

Ng et al. (1969) heated individual *Salmonella* strains in broth (Trypticase Soy Broth with 2% yeast extract, TSB-YE). The D values at 57 °C of *Salmonella typhimurium* Tm-1 was 1.2 min, *Salmonella blockley* 2004 was 5.8 min, and *Salmonella senftenberg* 775W was 31 min. The authors suggested that *Salmonella senftenberg* 775W strain was more heat resistant than other *Salmonella* strains. Since Goodfellow and Brown (1978) used mixed serotype strains of *Salmonella*, the D values at 57.2 °C were 3.8 - 4.2 min, indicating that mixed strains were less heat resistant than *Salmonella senftenberg* 775W but more heat resistant than *Salmonella typhimurium* Tm-1.

Table 2.2 Thermal death time curve data and z values for *Salmonella* serotypes in a ground beef system

Fat content	D value (min)			z value (°C)
	51.6 °C	57.2 °C	62.7 °C	
Not specified	61-62	3.8-4.2	0.6-0.7	5.56

From Goodfellow and Brown (1978).

Goodfellow and Brown (1978) also recommended time-temperature schedules ranging from 53.3 to 62.2 °C to reduce *Salmonella* by 7-D in roast beef (Table 2.3). In addition, other cooking quality factors were also affected by temperature, such as degree

Table 2.3 Time-temperature schedules for a 7-D reduction of *Salmonella* in cooked beef

Internal temperature (°C (°F))	Processing time (min)
53.3 (128)	195
53.9 (129)	153
54.4 (130)	121
55.0 (131)	97
55.6 (132)	77
56.1 (133)	62
56.7 (134)	47
57.2 (135)	37
57.8 (136)	32
58.3 (137)	24
58.9 (138)	19
59.4 (139)	15
60.0 (140)	12
60.6 (141)	10
61.1 (142)	8
61.7 (143)	6
62.2 (144)	5

From Goodfellow and Brown (1978).

of doneness and color of beef roasts. The USDA established thermal processing schedules for cooked beef and roast beef based on this study, and food processors have implemented these schedules in their roast beef manufacturing.

Fain et al. (1991) determined the D and z values for *Listeria monocytogenes* strain Scott A over the same range of temperatures (from 53.3 to 62.2 °C) as Goodfellow and Brown (1978) in lean (2% fat) and fatty (30.5% fat) ground beef (Table 2.4). Two recovery mediums were used; Columbia CNA (Columbia Colistin Nalidixic Acid Agar) agar containing sodium pyruvate with horse blood overlay (CBNA) and *Listeria* Plating Medium (LPM). z Values were higher in higher fat beef than lean meat. Also, *Listeria monocytogenes* had a higher z value in LPM recovery medium than in CBNA recovery medium, suggesting that different recovery techniques for *Listeria monocytogenes* would affect D and z values in ground beef. Since the z value of *Listeria monocytogenes* (11.4 °F) was higher than that of *Salmonella* (5.56 °C) (Goodfellow and Brown, 1978), the authors suggested that *L. monocytogenes* was a potential food safety problem.

Table 2.4 Thermal death time curve data and z values for *L. monocytogenes Scott A* in lean (2% fat) and fatty (30.5% fat) ground beef systems

Fat content	Culture medium	D value (min)			z value (°C)
		51.7 °C (125 °F)	57.2 °C (135 °F)	62.8 °C (145 °F)	
2.0%	CBNA [1]	81.3	2.6	0.6	5.2
30.5%	CBNA	71.1	5.8	1.2	6.3
2.0%	LPM [2]	56.1	2.4	0.5	5.4
30.5%	LPM	34.5	4.6	1.1	7.3

[1] Columbia CNA (Columbia Colistin Nalidixic Acid Agar) agar containing sodium pyruvate with horse blood overlay (CBNA).

[2] *Listeria* Plating Medium (LPM).

From Fain et al. (1991).

Escherichia coli O157:H7 has caused serious foodborne disease outbreaks since 1982 and was first found in Oregon and Michigan. A majority of these outbreaks were associcated with hamburger (ground beef) consumption at fast food restaurants. The symptoms include hemorrhagic colitis, hemolytic uremic syndrome and death in serious cases (Dorn, 1993). Doyle and Schoeni (1984) investigated the survival characteristics of

E. coli O157:H7 in heated and frozen (-20 ^{o}C for 0 to 9 months) ground beef (17-20% fat). There was no difference in the survival of *E. coli* O157:H7 in the inoculated beef patties after 0, 3, 6 and 9 months frozen storage. They found that the D values were lower for *E. coli* O157:H7 than for *Salmonella* (Goodfellow and Brown, 1978). The D values for *E. coli* O157:H7 were 2390, 270, 70, 45, 24 and 9.6 min at 54.4, 57.2, 58.9, 60, 62.8 and 64.3 ^{o}C, respectively. The z value was 4.1 ^{o}C. By comparing the z value of *Salmonella* (5.56 ^{o}C) (Goodfellow and Brown, 1978) to that of *E. coli* O157:H7 (4.1 ^{o}C), it was suggested that *E. coli* O157:H7 is more thermally sensitive to changes in temperature than *Salmonella.*

Line et al. (1991) conducted similar research to determine the D and z values for *E. coli* O157:H7 at 51.7, 57.2 and 62.8 ^{o}C in lean (2% fat) and fatty (30.5%) ground beef (Table 2.5). They used PCA containing 1% sodium pyruvate media and the 2 hr indole test to recover *E. coli* O157:H7. Using PCA recovery, higher D values were found in fatty meat than lean meat, however fat content did not affect the z values *of E. coli* in beef. Also, different recovery techniques affected the z values in lean beef because the PCA recovery method yielded a higher z value (8.3 ^{o}C) than the 2 hr Indole recovery method. The PCA recovery procedure could recover more heat shocked *Listeria monocytogenes* after cooking.

Table 2.5 Thermal death time curve data and z values for *Escherichia coli* O157:H7 in lean (2% fat) and fatty (30.5% fat) ground beef systems.

Fat content	Culture medium	D value (min)			z value (°C)
		51.7 °C (125 °F)	57.2 °C (135 °F)	62.8 °C (145 °F)	
2.0%	PCA [1]	78.2	4.1	0.30	4.6
30.5%	PCA	115.5	5.3	0.47	4.7
2.0%	2-h Indole	80.1	4.0	0.22	4.3
30.5%	2-h Indole	121.0	7.4	ND [2]	ND

[1] PCA: Plate count agar (PCA) + 1% sodium pyruvate.

[2] ND: Not determined due to insufficient data.

From Line et al. (1991).

Ahmed et al. (1995) examined the D and z values for *E. coli* O157:H7 in ground beef, pork sausage, turkey meat and chicken breast muscle. *E. coli* O157:H7 strain 204P was inoculated into ground beef containing 7%, 10% and 20% fat and was recovered after heating on TSA (tryptic soy agar) (Table 2.6). In general, the higher the fat content, and the lower the moisture content, the higher the D values. However, the z values were similar. The 20% fat ground beef had less moisture (61.9%) than that of 10% fat (69.1%)

and 7% fat (72.6%) beef, and the D values were higher. Fat levels affect thermal lethality of *E. coli* O157:H7. In addition to fat content, different recovery techniques, including the type of media and plating procedures, can cause differences in D and z values (Line et al., 1991).

2.7.2.2 Water-cooked Beef

Goodfellow and Brown (1978) also conducted a thermal death time study of *Salmonella* in a water-cooked beef system. Beef rounds were inoculated internally with *Salmonella* using dialysis tubing (Willardson et al., 1977). Commercial time-temperature processing schedules were used. The cooling rate of the 7.3-8.2 kg roasts after cooking was slower than meat in TDT tubes. Results from the ground beef study (Goodfellow and Brown, 1978) suggested that a minimum of 100 min was necessary to reduce *Salmonlla* by 7-D at 54.4 ^{o}C (130 ^{o}F). Due to the slower cooling rate of the water cooked beef roasts compared to that of a ground beef tube system, water cooking reduced *Salmonella* from 1 x 10^{7} CFU/g roast beef to less than 0.3 CFU/g after cooking and holding for 30 min. No *Salmonella* were detected after 60 min cooking at 54.4 ^{o}C (130 ^{o}F).

Table 2.6 Thermal death time curve data and z values for *Escherichia coli* O157:H7 strain 204P in ground beef, pork sausage, turkey breast and chicken meat

Products	Fat content	D value (min)			z value (°C)
		50 °C	55 °C	60 °C	
ground beef	7%	55.34	11.40	0.45	4.8
	10%	80.66	15.30	0.46	4.4
	20%	92.67	19.26	0.47	4.4
pork sausage	7%	49.50	6.37	0.37	4.7
	10%	62.90	7.83	0.46	4.7
	30%	80.64	11.28	0.55	4.6
turkey breast	3%	70.41	6.37	0.55	4.7
	11%	115.00	9.69	0.58	4.4
chicken meat	3%	65.24	8.76	0.38	4.5
	11%	105.50	9.74	0.55	4.4

Form Ahmed et al. (1995).

2.7.2.3 Dry Roasted Beef

Goodfellow and Brown (1978) also inoculated the surface of 7.3 - 8.2 kg oven-roasted roasts with *Salmonella*. The surface of these round roasts were found to be very dry (the relative humidity was lower than 0.02). Oven temperatures of 93.2 ^{o}C (200 ^{o}F), 107.1 ^{o}C (225 ^{o}F), 121.0 ^{o}C (250 ^{o}F) and 134.9 ^{o}C (275 ^{o}F) were used. *Salmonella* was eliminated from the surface of the roasts at oven temperature of 121.0 ^{o}C (250 ^{o}F) which corresponded to an internal temperature of 54.4 ^{o}C (130 ^{o}F) with 121 min holding time. No *Salmonella* was found on roasts at internal temperatures of 54.4 ^{o}C (130 ^{o}F) or above and extremely low numbers of viable *Salmonella* were found on roasts at internal temperatures of 51.6 ^{o}C (125 ^{o}F). The authors concluded that the shape and size of roasts were the major requirement for processing round roasts to reach a 7-D reduction of *Salmonella* when roasts ranging from 2.27 - 4.55 kg and the temperature was a less important factor compared to the shape and size of roasts.

2.7.2.4 Canned Ham

The USDA method (1986b) for determining the internal temperature of canned ham measures residual acid phosphatase activity and was adapted from the method by Kormendy and Gantner (1960, 1967). However, Kormendy et al. (1987) reported that residual phosphatase activity could vary with can size. The larger the can size, the longer the time to reach the target internal temperature. The hams were cooked in bags in a water bath to constant temperatures of 60, 65, 70 and 75 ^{o}C for different times. The D and z values were determined. They suggested that the thermal death time relationship

could offer a more accurate approach to determine thermal processing equivalence. D values were 3351, 445, 70, and 9 min at 60, 65, 70, and 75 °C, respectively, and a z value of 5.85 °C was found determined using residual acid phosphatase activity in canned ham.

2.7.2.5 Pork Sausage

Ahmed et al. (1995) evaluated the D and z values of *E. coli* O157:H7 strain 204P in pork sausages containing 7, 10 and 30% fat (Table 2.6). In general, as fat content was increased from 7 to 30%, the moisture content decreased and D values increased. The authors suggested that microorganisms tended to be more thermally labile at higher water activities. The authors suggested that 30% fat sausage contained less moisture (51.1%) than that of 10% fat (70.9%) and 7% fat (73.3%) sausage. The authors suggested that fat played a protective role for the bacterial cells, so that D values were higher. The authors concluded that fat content affected heat lethality of *E. coli* O157:H7 in pork sausage products.

2.7.2.6 Ground Turkey Breast Muscle

Ahmed et al. (1995) also investigated the D and z values of *E. coli* O157:H7 strain 204P in turkey meat containing 3 and 11% fat (Table 2.6). The 3% fat turkey breast contained higher moisture than that of 11% fat (the exact moisture content was not reported). The D values were higher in meat products with higher fat composition and lower water content.

2.7.2.7 Chicken Muscle

Ahmed et al. (1995) examined the D and z values of *E. coli* O157:H7 strain 204P in chicken meat containing 3 and 11% fat (Table 2.6). As previously reported, fat and water contents influenced the thermal lethality of *E. coli* O157:H7. Chicken meat with 3% fat had higher moisture than that with 11% fat, and the D values were higher in 11% fat chicken meat at the same temperature (the exact moisture content was not reported in the publication).

To summarize the study of Ahmed et al. (1995), meat products from different species (ground beef, pork sausage, turkey meat, chicken meat) all had higher D values at higher fat contents. The z values of *E. coli* in different products were similar, ranging from 4.4 - 4.8 ^{o}C. Product composition and different recovery media were the major factors affecting D values among different meat or poultry products. However, in this study, meat from various species (Ahmed et al., 1995) did not show specific differences in D and z values at different temperatures.

2.8 TIME -TEMPERATURE INTEGRATORS (TTI) AS THERMAL PROCESSING INDICATORS

Three common methods are used to verify that thermal processes have been properly conducted. They include *in situ* methods, physical-mathematical methods, and time-temperature indicators (TTIs) (Hendrickx et al., 1995; Van Loey et al., 1996). Both the *in situ* and physical-mathematical methods are well-established and widely used.

The *in situ* method is used to measure changes in a safety or quality attribute before and after thermal treatment such as microbial counts, nutrient content, color or texture. This approach is relatively conventional and laborious because data must be collected before and after the process to verify compliance to thermal processing schedules. This method may be relatively time consuming and needs trained analysts. It may be difficult to conduct the experiment due to the detection limit of the analytical method and or sampling problems.

The physical-mathematical method is used to determine the effect of a thermal process on a certain attribute based on the time-temperature history of a processed food. A safety or quality parameter in a food can be used to develop a mathematical model and to calculate the change after the thermal treatment. To improve the accuracy and applicability of the model system, a sophisticated physical-mathematical model is established using the actual time-temperature data.

The third method, the TTI method, was designed to avoid the limitations associated with the *in situ* and physical-mathematical methods, such as collecting detailed time-temperature data during processing. The definition of a TTI is "a small measuring device that shows a time-temperature dependent, easily, accurately and precisely measurable irreversible change that mimics the changes of a target attribute undergoing the same variable temperature exposure" (Hendrickx et al., 1995). A TTI should be easily recovered from a food, inexpensive and not affect heat transfer within the food. Theoretically, the z value of a TTI (in the computed range of the time-temperature profile) should have a z value similar to that of the target attribute. A TTI has to

demonstrate the same time-temperature dependent response as that of the target attribute in a food and the temperature should be the only rate-limiting factor in the system. TTIs can be either intrinsic or extrinsic. Intrinsic TTIs are prepared from compounds already in the target food, so extraction or recovery procedures are necessary to analyze the concentration of the intrinsic TTI after processing. Extrinsic TTIs are added to the food system and are retrieved back from the food after thermal processing is completed. Extrinsic TTIs can be encapsulated to prevent changes in inactivation kinetics due to reactions with the food material.

TTIs can be classified into three categories: biological, chemical and physical. Biological TTIs, include microbial and enzymatic TTIs. The z value of an enzymatic TTIs should be similar to the z value of the target index in a food when processed in the same temperature range. Endogenous enzymatic TTIs are generally dispersed throughout a food system. Enzymatic TTIs are usually less expensive, less time consuming and give more accurate determinations than microbial TTIs (Hendrickx et al., 1995).

Currently, USDA methods to verify processing adequacy are only used to detect compliance to a single endpoint temperature in roast beef products. No offical method is available to verify thermal adequacy when roasts are processed using different USDA approved time-temperature schedules. Accurate and rapid methods are urgently needed to verify that proper thermal treatment has been achieved in beef roasts. A TTI is needed to monitor processing adequacy, if the recently proposed USDA-FSIS lethality standard (1996b) requiring a thermal process that results in a 7-D reduction in *Salmonella* is implemented. Orta-Ramirez et al. (1997) investigated the thermal inactivation kinetics of

six endogenous enzymes in bovine *semitendinosus* muscle: acid phosphatase, lactate dehydrogenase, phosphoglycerate mutase, peroxidase, glyceraldehyde-3-phosphate dehydrogenase, and triose phosphate isomerase. TPI had a z value of 5.71 °C and was similar to that *of S. senftenberg* (5.56 °C) which was used to establish the USDA roast beef processing schedules (Goodfellow and Brown, 1978; USDA-FSIS, 1995). The authors suggested that TPI might be used as an intrinsic TTI to monitor thermal adequacy of roast beef processes.

2.9 BIOCHEMICAL PROPERTIES OF TRIOSE PHOSPHATE ISOMERASE (TPI)

Triose phosphate isomerase (TPI, D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) converts dihydroxyacetone phosphate to D-glyceraldehyde-3-phosphate. This enzyme is active as a dimer with a molecular mass of 53 kd from porcine muscle and exists in multiple forms in most higher organisms (Darnall and Klotz, 1975). The molecular mass of TPI from human liver was 44 kd by gel filtration method or 49.1 kd by analytical ultracentrifugation. The molecular mass of TPI from rabbit muscle was 49.1 kd by gel filtration method or 45.7 kd by analytical ultracentrifugation (Lee et al., 1971).

TPI is present as isoforms. Five isoforms in rabbit muscle, three isoforms in horse liver and three isoforms in human liver were observed by gel electrophoresis (Lee et al., 1971). Sawyer et al. (1972) also studied human TPI from erythrocytes. They found three variants (I, II and III) and TPI was a dimer with a molecular mass of about 56 kd. The isoelectric points for variants I, II and III were 6.7, 6.5 and 6.1, respectively.

Variants I, II and III accounted for 1-5%, 70-75% and 20-25% of total TPI, respectively. Variants I and III were dimers, AA and BB. Variant II was a heterodimer, AB, and dissociated and reassociated to AA and BB forms. The amino acid composition of variant I and III differed in histidine, serine, glycine, valine and leucine. Due to its higher content of histidine, variant I was assumed to be more basic than III.

Dabrowska et al. (1978) reported that human skeletal muscle TPI, with a molecular mass of 57.4 kd, existed as a dimer. Eber and Krietsch (1980) isolated TPI isoforms from human skeletal muscle and compared their immunological properties. Three isoforms (A,B,C) were isolated by diethylaminoethyl (DEAE) cellulose chromatography and identified on gel electrophoresis. The A and C isoforms were homodimers (AA) and the B isoform was a heterodimer (AB) agreeing with the conclusions of Sawyer et al. (1972). These three isoforms were examined by immunodiffusion method and found to have the same immunological properties. They suggested that A and B polypeptide chains had the same amino acid composition, molecular weight and antigenicity.

Beisenherz (1995) reported that TPI activity may be reduced to 25% of initial activity in 0.05 M phosphate solution. Therefore, phosphate ions are inhibitors of TPI. The optimum pH of TPI is between 7 and 8 and TPI activity decreased by half at pH 6.3 compared to its activity at pH 7-8. Turnover rate at 26 ^{o}C was 945,000 moles substrate/min and the turnover rate was doubled at 38 ^{o}C.

2.9.1 Determination of TPI Activity in Muscle Tissues

TPI activity can be determined by coupling the following two reactions:

$$\text{glyceraldehyde-3-phosphate} \xrightarrow{\text{TPI}} \text{dihydroxyacetone phosphate (DHAP)}$$

$$\text{DHAP} + \text{NADH} \xrightarrow{\alpha\text{-glycerophosphate dehydrogenase}} \alpha\text{-glycerophosphate} + \text{NAD}$$

An unit (U) was defined as 1 μmole of substrate converted per minute. TPI from horse and liver tissues had specific activities of 3183 and 2397 U/mg (Lee et al., 1971). Porcine muscle contained 2 mg TPI/g tissue and 1,500U/g muscle (Scopes, 1973); however, TPI is unstable at pH 5.5 and 55 ^{o}C. Smith (1991) reported TPI activity in different species. TPI had high activity in chicken *pectoralis major* or white muscle (1260.0 ± 104.0 U/kg muscle) and *sartorius* or red muscle (957.0 ± 65.0 U/kg muscle) TPI activity decreased when heated to 60, 70 and 80 ^{o}C. TPI also had high activity in the following muscles: 984.0 ± 163.0 U/kg muscle in bovine *infraspinatus*; 1210.0 ± 156.0 U/kg muscle in bovine *semimembranosus*; 1720.0 ± 47.0 U/Kg muscle in porcine *longissimus dorsi*; and 1420.0 ± 81.4 U/Kg muscle in porcine *psoas major*. Hsu (1993) reported that TPI activity of turkey *pectoralis major* muscle with either 15% water, 15% brine or 15% curing brine decreased by 99% of its initial activity when heated to 67 ^{o}C.

2.9.2 Purification of TPI from Muscle Tissues

TPI purification procedures have been reported for calf muscle (Beisenherz, 1955), horse and human liver (Lee et al., 1971), human skeletal muscle (Dabrowska et al., 1978), rabbit muscle (Scopes and Stoter, 1982), frozen chicken breast muscle (Petell et al. 1982) and chicken breast muscle (Reiss and Schwartz, 1987).

Beisenherz (1955) purified TPI from calf muscle. Muscles were extracted with 0.05% disodium ethylenediaminetetraacetate (EDTA) solution (pH of solution was not stated) for 30 min at 3 ^{o}C. The homogenate was then centrifuged at 2,200 x g for 30 min and the supernatant was decanted and saved. The pellet was re-extracted. TPI was fractionated in 30% and 50% acetone. Then, TPI was precipitated in 60% acetone. The pellet was collected and further precipitated using 70 and 85% ammonium sulfate. After washing and recrystallization, the authors reported that the purity was increased from 2.5% (initial crude extract) to 100% (purified TPI) with a recovery of 24%.

Lee et al. (1971) purified TPI from horse and human liver tissues. Tissues were extracted with 0.05% EDTA for 4 hr (pH 6.8), followed by fractionation in 35% and 50% acetone. Proteins in the supernatant were precipitated in 60% acetone. The pellet was dialysed against 0.05% EDTA to eliminate acetone and heated at 40 ^{o}C for 15 min, then 50 ^{o}C for 30 min. TPI was eluted with 0.1 M NaCl using a Sephadex QAE [diethyl(2-hydroxypropyl)aminoethyl] A-50 (anion exchange) column. The protein was crystallized in 3.5 M ammonium sulfate. The crystals were dissolved in phosphate buffer (0.002 M, pH 7.8) and filtered through a Sephadex G-75 (superfine) column. The specific activities of purified TPI were 3,183 I.U./mg for horse liver and 2,397 I.U./mg for human liver.

The specific activities of purified TPI from horse and human tissues were increased 999 and 361 fold, respectively.

Dabrowska et al. (1978) purified TPI from human skeletal muscle. The major steps included: (1) extraction in EDTA solution (1.5 mM, pH 5.6). (2) acetone fractionation (35%, 45% and 60%), (3) heating to 50 ^{o}C for 30 min, then centrifugation to remove the denatured protein, (4) precipitation in 54% ammonium sulfate. The pellet was dissolved in a 0.05 M Tris (Tris[hydroxymethyl]-aminomethane hydrochloride) buffer containing 3 mM EDTA and 1.0 mM 2-mercaptoethanol, pH 7.5, (5) fractionation on a Sephadex G-100 gel filtration column, (6) fractionation on a DEAE (Diethylaminoethyl)-cellulose column, and (7) precipitation with ammonium sulfate. Crystals were formed after 7 days. Crystallized TPI had a specific activity of 7,200 U/mg.

Scopes and Stoter (1982) developed a scheme for purification of several glycolytic enzymes from rabbit muscle. Rabbit muscle was homogenized and extracted in a buffer containing 30 mM K phosphate, 1 mM EDTA and 10 mM 2-mercaptoethanol, pH 7.0. Several fractions were collected depending on the concentration of ammonium sulfate added to the meat extract. TPI was soluble in 2.44 M ammonium sulfate, but precipitated in 3.02 M ammonium sulfate at pH 7.0. This precipitate contained five enzymes: TPI, CK, adenylate kinase, enolase and phosphoglycerate kinase. Carboxylmethyl-cellulose column chromatography was used to absorb adenylate kinase, enolase and phosphoglycerate kinase on the column while TPI and CK were eluted. At room temperature, CK was denatured and lost enzymatic activity at pH 6.0 on a

carboxylmethyl-cellulose column allowing for the separation of TPI from CK. Finally, TPI was eluted with MES (2-[N-morpholino]ethane-sulfonic acid) buffer by increasing the pH to 6.5.

Petell et al. (1982) also reported a method to isolate TPI from chicken breast muscle. Proteins in the muscle extract were fractionated with 70% and 90% ammonium sulfate. The pellet was solubilized in phosphate buffer containing 40% ammonium sulfate. CK was precipitated by adjusting the pH to 5.4 with 1 M acetic acid. The supernatant was then dialysed against MES buffer (1 mM MES, 1 mM magnesium acetate, 1 mM EDTA, 1 mM 2-mercaptoeathanol). TPI was eluted from a phosphocellulose column with a phosphate buffer (pH 6.0, 50 mM phosphate). TPI was 95% pure as determined using SDS-PAGE with Coomassie blue stain.

Reiss and Schwartz (1987) developed a procedure to purify certain glycolytic enzymes from chicken breast muscle. These enzymes included TPI, aldolase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase, lactate dehydrogenase and CK. Chicken *pectoralis major* muscle was homogenized in a MEMT buffer (5 mM MgSO4, 0.4 mM EDTA, 7 mM 2-mercaptoethanol and 50 mM Tris-HCl, pH 6.8). The TPI purification procedure included: ammonium sulfate fractionation and pH fractionation. Then, TPI and CK were eluted using a pH gradient in a phosphocellulose column with increasing ionic strength. Affinity chromatography with Cibacron blue-2 agarose was used as the final purification step. TPI was eluted at pH 6.8 when applied using a pH 5.0 buffer, whereas, CK was eluted at pH 6.8 when applied using a pH 5.5 buffer. The specific activity of the crude

extract was 7.0 U TPI/mg muscle and the purified TPI had a specific activity of 1200 U/mg muscle. TPI was purified 170 fold from chicken *pectoralis major* muscle. TPI was about 99% pure.

Reiss and Schwartz (1987) also prepared polyclonal antibodies for each enzyme. Antibodies were purified further using immunoadsorbent techniques to yield higher specific binding between antigens and antibodies. Anti-TPI antibodies were not as specific as anti-CK, phosphoglycerate kinase, aldolase, glyceraldehyde phosphate dehydrogenase, enolase, pyruvate kinase and lactate dehydrogenase antibodies to their own antigen. In addition to TPI, anti-TPI antibodies cross reacted with aldolase.

CHAPTER 3

THERMAL INACTIVATION OF ACID PHOSPHATASE AND PEROXIDASE IN GROUND BEEF

3.1 ABSTRACT

The USDA-FSIS requires specific thermal processing schedules for roast beef products to ensure the destruction of pathogenic bacteria, such as *Salmonella* and *E. coli*. An endogenous muscle enzyme with a thermal inactivation rate constant (z value) similar to that of *Salmonella* could be used as a time-temperature integrator (TTI) to monitor the adequacy of roast beef thermal processing. A thermal process sufficient to cause a 7-D reduction in *Salmonella* was recently proposed by FSIS as the lethality performance standard for roast beef, cooked beef and cooked corned beef products. This study investigated the thermal inactivation kinetics of acid phosphatase (AP) and peroxidase (PO) to evaluate their potential use as time-temperature indicators of processing adequacy in roast beef. The D values of AP and PO were determined from 53 to 68 °C in 5.5 °C increments and their z values were calculated from the relevant temperature range. The D values for AP were 352.9, 26.3, 5.6 and 3.3 min at 53, 58, 63 and 68 °C, respectively, with a z value of 7.41 °C. The D values for PO were 3871.0, 2678.6, 769.2 and 42.9 min at 53, 58, 63 and 68 °C, respectively, with a z value of 7.80 °C. Neither of these enzymes

had a z value similar to that of *Salmonella* which was the indicator microorganism used by the USDA to establish the time-temperature processing schedules for roast beef.

Keywords: time-temperature integrators, roast beef, thermal death time study

3.2 INTRODUCTION

Salmonella and hemorrhagic *Escherichia coli* are the major pathogenic bacteria found to cause foodborne disease outbreaks from precooked meats. Enteric pathogens from animal food products cause from 6.5 to 81 million outbreaks of illness each year in the U.S. as reported by the Centers for Disease Control and Prevention (Bean and Griffin, 1990). Although 45,000 cases of *Salmonella* outbreaks are reported every year in the United States, foodborne cases of salmonellosis are estimated to range from 790,000 to 3.69 million annually with a median number of 1.92 million cases (Todd, 1994). *E. coli* O157:H7 is an emerging pathogenic bacteria in meat products. There are about 10,000 to 20,000 cases and 200 to 500 deaths attributed to *E. coli* in the United States each year (CDC, 1996).

Proper cooking is one way to eliminate pathogenic bacteria from meat products. Title 9 of the Code of Federal Regulations (USDA-FSIS, 1995) requires cooked beef and rare roast beef be heated to one of 16 time-temperature combinations. For example, beef can be heated to 62.8 ^{o}C (145.04 ^{o}F) with no holding time or to 54.4 ^{o}C (129.92 ^{o}F) with 121 min holding time. In May 1996, USDA-FSIS proposed several rules to amend the current federal meat and poultry inspection regulations to establish "safety margins" for thermally processed meat and poultry products (USDA-FSIS, 1996b). *Salmonella* was

selected as the indicator organism as it is more thermally resistant than *E. coli* O157:H7. A 5-D reduction in *Salmonella* was also proposed by FSIS as the lethality performance standard for cooked, uncured meat patties. A thermal process sufficient to cause a 7-D reduction in *Salmonella* was recently proposed by FSIS as the lethality performance standard for roast beef, cooked beef and cooked corned beef products. The proposed regulation allows processors to use more sophisticated thermal treatments and more flexible time-temperature processing combinations.

Current endpoint temperature tests used by the USDA cannot predict processing adequacy when different approved time-temperature processing combinations are used. Accurate and rapid methods are urgently needed to verify that meat products have received proper thermal processing. The proposed USDA-FSIS lethality standard based on a 7-D reduction in *Salmonella* allows for the use of a time-temperature integrator to monitor processing adequacy. A time-temperature integrator (TTI) is "a small measuring device that shows a time-temperature dependent, easily, accurately and precisely measurable irreversible change that mimics the changes of a target attribute undergoing the same variable temperature exposure" (Hendrickx et al., 1995). A TTI should have the same z value as that of *Salmonella*, indicating similar thermal inactivation kinetics (Hendrickx et al., 1995, Van Loey et al., 1996). Therefore, the objective of this research was to investigate the thermal inactivation kinetics of acid phosphatase and peroxidase muscle enzymes to find a marker which has a z value similar to that of *Salmonella*. This endogenous protein could then be used as a TTI to verify adequacy of roast beef processing.

3.3 METHODOLOGY

3.3.1 Sample Preparation

Fresh *semitendinosus* meat (eye of round) purchased from a local store was trimmed of external fat and connective tissue. Muscles were cut into 1 cm cubes and 4 or 5 pieces (500-600 g) were randomly placed in plastic pouches and vacuum packaged in Cryovac® packaging bags (W.R. Grace Co., Duncan, SC 29681). Meat was used immediately or stored in the blast freezer (-35 ^{o}C) for two weeks or less before use. Meat was thawed overnight at 4 ^{o}C (39.2 ^{o}F) prior to use.

3.3.2 Thermal Treatments

Meat was ground twice using a 3.175 mm diameter grinder plate in a Hobart grinder (Model 84181D, Hobart Mfg. Co., Troy, OH 45374) and placed into a 60 ml syringe (Becton Dickinson and Co., Franklin Lakes, NJ 07417). Two gram of meat was extruded through a plastic tube (7 cm in length and 0.5 cm in diameter) into a 10 x 75 mm thermal death time (TDT) tube. The glass tubes were then sealed using a flame. The TDT tubes were heated at four temperatures [53 ^{o}C (127.4 ^{o}F), 58 ^{o}C (136.4 ^{o}F), 63 ^{o}C (145.4 ^{o}F) and 68 ^{o}C (154.4 ^{o}F)] for different holding times (53 ^{o}C (127.4 ^{o}F): 0, 1.5, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 hr; 58 ^{o}C (136.4 ^{o}F): 0, 2, 4, 8, 10, 15, 20, 30, 35, 45, 50, 60 min; 63 ^{o}C (145.4 ^{o}F): 0, 60, 75, 90, 105, 120, 135, 150, 165, 180, 195, 210, 240, 260 sec; 68 ^{o}C (154.4 ^{o}F): 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 sec) in a water bath (Model 1268-52, Cole-Parmer Instrument Company, Chicago, IL 60648) connected to a digital programmer (Model 1268-62, Cole-Parmer). The holding times at each

temperature were selected to achieve at least a one log cycle reduction in enzyme activity. To monitor thermal processing, a RTD (Resistance Temperature Detector) thermocouple (platinum Pt100 temperature probe, ± 0.1 °C accuracy, Solomat Partners LP, Stanford, CT 06906) was inserted into the center of a TDT tube containing 2 g meat in each replicate. The water bath temperatures was set 0.2 °C higher than the target temperature. The thermocouple was connected to a Solomat MPM 200 Modumeter. Temperature and time were recorded using a Solomat MPM Logger connected to the modumeter. Zero time was defined as when the internal temperature reached the target temperature. Tubes were held for the designated holding time and then removed from the water bath and immersed in an ice-water bath for 10 min. Tubes were held at 4 °C (39.2 °F) until assayed within 12 hr. Each experiment was conducted in triplicate.

3.3.3 Preparation of Protein Extracts and Enzyme Assays

The TDT tubes were broken and cooked meat was transferred into scintillation vials (Research Products International Corp., Mount Prospect, IL 60056). Four milliliters of cold phosphate buffer saline (PBS, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2) was added to each vial. Samples were vortexed for 1 min, then samples were stirred on a magnetic plate stirrer for 15 min in a cold room at 4°C. Samples were centrifuged at 4,500 x g for 5 min (Sorvall Superspeed Automatic Refrigerated Centrifuge, Model RC2B, Norwalk, CT 06852). The supernatant was collected and held at 4°C until used within 8 hr. Enzyme activities of acid phosphatase (AP) and peroxidase (PO) were

determined as described by Bergmeyer (1974). Three replications were performed at each temperature and enzyme activity was assayed within 12 hr of cooking.

3.3.4 Calculations of D and z Values

The D values were calculated at each temperature based on the Laboratory Manual for Food Canners and Processors (National Canner Association, 1968) except that survivor curves were calculated using linear regression analysis (LOTUS 1-2-3, Version 1.0, Lotus Development Corp., Cambridge, MA 02142). TDT curves were constructed by plotting D values (min) vs. temperature (°C). z Values were calculated as the absolute value of the reciprocal of the slope of the TDT curve using regression analysis as described above.

3.3.5 Proximate Analysis

Fat and moisture contents of ground beef were determined using AOAC (1990) methods 960.39 and 950.46B, respectively. The pH was determined by homogenizing 50 g ground beef with 50 ml of double distilled water using a Waring™ blender for 30 s.

3.4 RESULTS AND DISCUSSION

Raw ground *semitendinosus* meat contained 72.8% moisture, 3.8% fat and the pH was 6.0. Acid phosphatase had a D value of 352.9 min (5.88 hr) at 53 °C, which decreased to 3.3 min at 68 °C (Table 3.1). Peroxidase activity was stable at 53 and 58 °C with the D values of 3871.0 min (64.52 hr) and 2678.6 min (44.64 hr) (Table 3.2).

Table 3.1 D values and regression analysis for acid phosphatase activity from ground beef heated at 53, 58, 63 and 68 °C

Temperature (°C)	y-intercept	slope	R^2	D value (min)
53	1.134	-0.170	0.69	352.9
58	1.488	-0.038	0.94	26.3
63	0.791	-0.003	0.98	5.6
68	1.296	-0.005	0.81	3.3

Table 3.2 D values and regression analysis for peroxidase activity from ground beef at 53, 58, 63 and 68 °C

Temperature (°C)	y-intercept	slope	R^2	D value (min)
53	1.983	-0.016	0.88	3871.0
58	1.570	-0.022	0.70	2678.6
63	1.712	-0.001	0.60	769.2
68	1.655	-0.023	0.79	42.9

Peroxidase activity decreased rapidly at 63 and 68 °C; the D values were 769.2 and 42.9 min, respectively (Table 3.2). The D values of PO at 53 and 68 °C were at least ten times higher than that of AP, whereas, the D values of PO at 58 and 63 °C were more than one hundred times higher than that of AP (Table 3.2). Thus, PO was a more thermally stable enzyme than AP from 53 to 68 °C.

Orta-Ramirez et al. (1997) determined the D values of glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, phosphoglycerate mutase, PO, triose phosphate isomerase in ground beef. PO had higher D values at 53, 58, 63 and 68 °C than all enzymes examined by Orta-Ramirez et al. (1997) except for lactate dehydrogenase at 53 °C. Lactate dehydrogenase had a D value of 6968.6 min at 53 °C. Acid phosphatase and phosphoglycerate mutase had similar D values at 53 °C (352 min and 325 min). However, AP was less stable at 58 °C than all enzymes, except triose phosphate isomerase (Orta-Ramirez et al., 1997).

The apparent z values of AP and PO in ground beef were 7.41 and 7.80 °C (Table 3.3), respectively. Orta-Ramirez et al. (1997) reported the z values for glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, phosphoglycerate mutase, and triose phosphate isomerase of 4.71, 3.98, 5.18, and 5.56 °C, respectively. Levieux et al. (1995) reported that the z values of lactate dehydrogenase M_4 and lactate dehydrogenase H_4, (lactate dehydrogenase has five isoforms which are comprised of two polypeptide subunits, H and M: H_4, H_3M, H_2M_2, HM_3, and M_4 [Holbrook et al., 1975]) were 6.1 and 5.7 °C, respectively using immunodiffusion. In general, AP and PO had higher z values (thermal inactivation rates) than those of other enzymes. The apparent z values of

Table 3.3 z Values and regression analysis from thermal death time studies of acid phosphatase (AP) and peroxidase (PO) from ground beef

Marker protein	y-intercept	slope	R^2	z value (°C)
AP	9.477	-0.135	0.92	7.41
PO	10.636	-0.128	0.87	7.80

Salmonella senftenberg and *E. coli* O157:H7 in ground beef were 6.25 °C and 5.57 °C, respectively (Orta-Ramirez et al., 1997). The z value of *E. coli* O157:H7 was close to that of the USDA roast beef process. Goodfellow and Brown (1978) reported a z value of 5.56 °C in ground beef inoculated with *Salmonella* serotypes. The z value of *Salmonella* from Goodfellow and Brown (1978) was less than that reported by Orta-Ramirez et al. (1997). The sixteen time-temperature processing schedules allowed by the USDA were established using a 7-D reduction in *Salmonella* in roast beef (Goodfellow and Brown, 1978). A 7-D reduction in *Salmonella* was recently proposed by the USDA-FSIS to develop a new thermal processing regulation for roast beef products. Our results indicate that AP and PO could not be used as endogenous TTI to evaluate the processing adequacy of roast beef since their z values were greater than that of *Salmonella*.

CHAPTER 4

IDENTIFICATION OF MARKER PROTEINS IN ROAST BEEF TO VERIFY COMPLIANCE TO USDA PROCESSING SCHEDULES

4.1 ABSTRACT

Our objective was to identify a marker protein that could determine the adequacy of roast beef processing. Three adequate (high, medium and low temperature processes were 62.2 °C/5 min, 58.3 °C/24 min, and 54.4 °C/121 min, respectively) and corresponding inadequate processing (0.5 and 1 log cycle reduction in holding time from adequate process) schedules were used. The residual enzyme activity of peroxidase (PO), acid phosphatase (AP), and triose phosphate isomerase (TPI) were determined. Lactate dehydrogenase (LDH) content was determined using immunoassay. In the ground beef water bath model system, TPI activity averaged 2.6 U/kg in adequately processed ground beef and increased to 4.9 and 13.3 U/kg when inadequately processed by reducing the holding time 0.5 log and 1.0 log, respectively. TPI activity was similar within all adequately processed beef and TPI activity increased ($P < 0.0001$) when processing time was inadequate. In the MSU smokehouse pilot study, TPI activity in adequately processed roast beef averaged 1.6 U/kg and increased to 3.7 and 7.8 U/kg when inadequately processed by reducing the holding time 0.5 and 1.0 log, respectively. TPI was identified as the best marker protein in both ground beef and roast beef cooked using

medium to high temperature processes, as TPI activity was the same when compared within adequate cooking treatments, but increased as cooking time was decreased. The other enzymes were not suitable markers as either differences in activity were found within adequately processed beef treatments or no differences were found between adequately and inadequately processed beef.

KEYWORDS-- beef, endpoint temperature, processing

4.2 INTRODUCTION

The United States Department of Agriculture - Food Safety Inspection Service (USDA-FSIS) has established specific processing requirements for precooked meat and poultry products. The purpose of these regulations is to ensure the destruction of pathogenic bacteria and viruses that could cause foodborne illness in humans and livestock (Townsend and Blankenship, 1989). Cooked beef and roast beef are required to be heated to 62.8 °C (145 °F) with no holding time or to one of sixteen different time-temperature combinations outlined by USDA-FSIS (1995). The schedules were based on the thermal inactivation in *Salmonella*. In May 1996, USDA-FSIS proposed several rules to amend the current federal meat and poultry inspection regulations to enhance the safety of thermally processed meat and poultry products (USDA-FSIS, 1996b). A 7-D reduction in *Salmonella* was proposed by FSIS as the lethality performance standard for processing of roast beef, cooked beef and cooked corned beef products.

The current methods for endpoint temperature (EPT) determination used by USDA-FSIS are a Protein Coagulation Test (USDA-FSIS, 1986a) and a Bovine Catalase

Test (Eye, 1982; USDA-FSIS, 1989). These methods have been shown to be subjective and inaccurate in predicting the actual EPT achieved during processing (Townsend and Blankenship, 1989). In fact, the major disadvantage of the current tests is that they cannot detect adequacy of processing when roast beef is processed using current USDA time-temperature schedules.

Orta-Ramirez et al. (1996) determined the z value of six potential marker enzymes in bovine *semitendinosus* muscle. The z values of acid phosphatase, lactate dehydrogenase (LDH), phosphoglycerate mutase, peroxidase, glyceraldehyde-3-phosphate dehydrogenase and triose phosphate isomerase (TPI) were 7.41, 3.99, 4.11, 7.80, 4.56 and 5.71 °C, respectively. TPI had a z value similar to that of *Salmonella senftenberg* (5.56 °C) which was used to establish the USDA approved roast beef processing schedules. The authors suggested that TPI might be used as an endogenous time-temperature integrator (TTI) to determine the adequacy of thermal processing of roast beef and beef patties. The definition of a TTI is "a small measuring device that shows a time-temperature dependent, easily, accurately and precisely measurable irreversible change that mimics the changes of a target attribute undergoing the same variable temperature exposure" (Hendrickx et al., 1995).

The objective of this study was to identify endogenous marker enzymes that could be used to determine if roast beef has been adequately processed to meet the USDA time-temperature schedules using model and pilot studies. Adequately and inadequately processed beef roasts were used to evaluate the ability of several potential marker proteins to differentiate among the processing schedules.

4.3 MATERIALS AND METHODS

4.3.1 Pilot Studies

4.3.1.1 Roast Beef Processing

Choice top round roasts (*semimembranosus*, SM) purchased from Ada Beef Company (Ada, MI 49301) were vacuum packaged and transported in ice to the MSU Meat Laboratory. All roasts were trimmed of external fat and connective tissue. Each roast was divided into two pieces, cut into a rectangular shape (about 40 cm x 25 cm) and vacuumed packaged in Cryovac packaging bags (W.R. Grace Co., Duncan, SC, 29681). Roasts were stored at 4 °C for less than 16 hr before processing.

Three different USDA approved time-temperature schedules were used to determine the effect of processing on peroxidase activity in roast beef (Table 4.1). Adequately cooked roast beef was processed to internal temperatures of 62.2 °C (144 °F)/5 min, 58.3 °C (137 °F)/24 min, and 54.4 °C (130 °C)/121 min. Under-cooked roasts were prepared by reducing the processing time by 0.5 log cycle at each temperature. For acid phosphatase, LDH and TPI, two different processing schedules were used. Roast beef was adequately cooked to internal temperatures of 62.2 °C (144 °F)/5 min and 54.4 °C (130 °C)/121 min. Under-cooked roasts were processed by reducing the holding time (min) by 0.5 and 1.0 log cycle at each temperature.

The smokehouse conditions used for the low, medium and high temperature schedules are listed in Tables 4.2, 4.3 and 4.4, respectively. A microprocessor controlled smokehouse was used as the oven or cooking chamber. Internal temperatures were

Table 4.1 Processing times and temperatures used to prepare adequately and underprocessed beef roasts

	High temperature process	Medium temperature process	Low temperature process
Under-cooked (1.0 log reduction in time)	59.4 ^{o}C (139 ^{o}F)/1.5 min	58.3 ^{o}C (137 ^{o}F)/2.4 min	54.4 ^{o}C (130 ^{o}F)/12 min
Under-cooked (0.5 log reduction in time)	61.1 ^{o}C (142 ^{o}F)/2.5 min	58.3 ^{o}C (137 ^{o}F)/8 min	54.4 ^{o}C (130 ^{o}F)/40 min
Adequately cooked	62.2 ^{o}C (144 ^{o}F)/5 min	58.3 ^{o}C (137 ^{o}F)/24 min	54.4 ^{o}C (130 ^{o}F)/121 min

Table 4.2 Smokehouse schedule used to prepare low temperature (54.4 °C; 130 °F) processed roast beef

Stage	Time (min)	Internal temperature	Dry bulb temperature	Wet bulb temperature
1	60	37.8 °C (100 °F)	86.7 °C (188 °F)	83.9 °C (183 °F)
2	60	48.9 °C (120 °F)	71.1 °C (160 °F)	69.4 °C (157 °F)
3	100	54.4 °C (130 °F)	61.1 °C (142 °F)	58.9 °C (138 °F)
4	121[a]	54.4 °C (130 °F)	61.1 °C (142 °F)	58.9 °C (138 °F)

[a] Roasts were removed as they reached target times.

Table 4.3 Smokehouse schedule used to prepare medium temperature (58.3 °C; 137 °F) processed roast beef

Stage	Time (min)	Internal temperature	Dry bulb temperature	Wet bulb temperature
1	60	37.8 °C (100 °F)	86.7 °C (188 °F)	83.9 °C (183 °F)
2	60	48.9 °C (120 °F)	76.7 °C (170 °F)	72.8 °C (163 °F)
3	120	54.4 °C (130 °F)	69.4 °C (157 °F)	67.8 °C (154 °F)
4	24 [a]	58.3 °C (137 °F)	62.8 °C (145 °F)	61.1 °C (142 °F)

[a] Roasts were removed as they reached target times.

Table 4.4 Smokehouse schedule used to prepare high temperature (62.2 °C; 144 °F) processed roast beef

Stage	Time (min)	Internal temperature	Dry bulb temperature	Wet bulb temperature
1	60	37.8 °C (100 °F)	86.7 °C (188 °F)	83.9 °C (183 °F)
2	60	48.9 °C (120 °F)	76.7 °C (170 °F)	72.8 °C (163 °F)
3	90	60.0 °C (140 °F)	69.4 °C (157 °F)	67.8 °C (154 °F)
4	90 [a]	62.8 °C (145 °F)	73.3 °C (164 °F)	68.9 °C (156 °F)

[a] Roasts were removed as they reached target times.

monitored by platinum Resistance Temperature Detector (RTD) probes, 0.33 cm diameter, ± 0.1 °C accuracy (Omega, Stanford, CT, 06906), inserted in the geometric center of the roasts. The thermocouples were calibrated at the target temperature using manufacturer's guidelines. Two additional thermocouples of the same type were also placed in the geometric center of additional roasts to monitor internal temperatures. When processing was completed, the roasts were removed and the center core (about 150-200 g) of the roast was immediately excised, wrapped in a plastic bag and then placed in ice slush for about 30 min until the internal temperature decreased to 32.2 °C (90 °F). The cooled roasts were stored at 4 °C (39.2 °F) before being analyzed within 12 hr. Twenty-five grams of meat close to the thermocouple position was removed from each roast for extraction. Thermal processing was conducted using three separate smokehouse runs for each processing schedule.

4.3.1.2 Proximate Composition

Proximate composition of *semimembranosus* muscle was analyzed for protein, fat and moisture contents using AOAC (1990) standard methods 981.10, 960.39 and 950.46B, respectively. The pH was determined by homogenizing 10 g ground beef with 90 ml of double distilled water using a Waring™ blender for 30 s.

4.3.1.3 Preparation of Protein Extracts and Enzyme Assays

Meat (25 g) was minced and extracted in 3 volumes (75 ml) of 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2, for 90 sec in a Waring Blender™. After stirring with

a motorized propeller for 1 hr, the suspension was centrifuged at 4,500 x g for 10 min to obtain the supernatant containing the soluble sarcoplasmic proteins. Acid phosphatase and LDH activities were determined using commercial Sigma® kits (acid phosphatase, 104-AL; lactate dehydrogenase, DG1340-K). Peroxidase activity was determined as described by Bergmeyer (1974). TPI activity was determined based on Bergmeyer (1983) except that the assay mixture contained 1.0 ml triethanolamine buffer (TEA, 0.2 M, pH 8.0), 0.2 ml glyceraldehyde-3-phosphate (15 mM), 10 µl NADH, sodium salt (10 mg/ml), 10 µl glycerophosphate dehydrogenase (G-6751, Sigma®; 19 mg protein/ml, 170 units/mg protein) and 10 µl meat extract (Wang et al., 1996). The change in absorbance at 340 nm was followed for 1 min at 25 °C. Enzyme activity was expressed as units per liter of meat extract (U/L meat extract) and units per kilogram of meat (U/kg meat).

4.3.2 Model System Studies

4.3.2.1 Ground Beef Cooking

Fresh SM meat purchased from a local store was trimmed of external fat and connective tissue. Meat was cut into 1 cm cubes and ground twice through a 3.175 mm diameter plate in a Hobart grinder (Model 8418D, Hobart Mfg. Co., Troy, OH, 45374). The ground beef was placed into a 60 ml syringe (Becton Dickinson and Co., Franklin Lakes, NJ 07417). Two grams of ground meat were compressed through a 7 cm long plastic tubing into a 10 x 75 mm thermal death time (TDT) tube (60825-538, VWR, Kimax® 51, South Plainfield, NJ 07080). The glass tubes were then sealed using a flame. TDT tubes were cooked in a water bath (Model 1268-52, Cole-Parmer, Chicago, Ill,

60648) connected to a digital programmer (Model 1268-62, Cole-Parmer). The temperature of the water bath was set at the target temperature or 1 °C higher than the target temperature. An RTD (Resistance Temperature Detector) thermocouple (platinum Pt 100 temperature probe, 0.15 cm in diameter, ± 0.1 °C accuracy, Solomat Partners LP, Stanford, CT 06906) was inserted into the center of a TDT tube containing 2 g meat. Three different USDA approved time-temperature schedules were used (Table 4.1). Ground meat was adequately processed to internal temperatures of 62.2 °C (144 °F)/5 min, 58.3 °C (137 °F)/24 min, and 54.4 °C (130 °F)/121 min and under cooked by reducing the processing time by 0.5 and 1.0 log cycle. Zero time was defined as when the internal temperature reached the target temperature. After cooking, tubes were removed from the water bath, immersed in an ice bath for 10 min and held at 4 °C until assayed within 12 hr. Each experiment was conducted in triplicate.

4.3.2.2 Preparation of Protein Extracts and Enzyme Assays

TDT tubes were broken and cooked meat was transferred into scintillation vials (Research Products International Corp., Mount Prospect, IL 60056). Eight milliliters of 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2 were added to each vial. Samples were vortexed for 1 min, then stirred on magnetic plates for 15 min at 4 °C. Samples were centrifuged at 4,500 x g for 5 min. The supernatant was collected and held at 4 °C until used within 8 hr. TPI enzyme activity was determined as described previously. The LDH content was determined by enzyme-linked immunosorbent assay (ELISA) (Orta-Ramirez et al., 1996).

4.3.3 Statistical Analysis

In the pilot study, top round roasts, *semimembranosus* muscles, were smokehouse processed in triplicate using three different processes (low, medium and high temperature processes). Each process was conducted in triplicate for a total of 9 separate smokehouse processing runs. In the model system, ground bovine *semimembranosus* muscles, were thermally processed in triplicate using three different processes (low, medium and high temperatures). Cooking was conducted in triplicate for a total of 9 separate water bath processing runs. Data were analyzed using two way ANOVA (analysis of variance) (treatment x replication). The linear or curvilinear change in enzyme activity or concentration with temperature was determined using the polynomial contrast test by SAS software (SAS Institute Inc., Version 6.1, 1995, Cary, NC 27513).

4.4 RESULTS AND DISCUSSION

A marker protein must meet two criteria to function as a TTI in roast beef. First, its concentration or activity should decrease as processing time at the same temperature is increased. Second, the marker protein concentration or activity within each time-temperature cooking schedule (adequately vs. inadequately processed) should be constant. An ideal indicator should be present at the same amount or activity in adequately processed beef regardless of the time-temperature schedule used.

In the pilot smokehouse study at MSU, roast beef from *semimembranosus* meat contained 59.7% moisture, 10.1% fat and 21.8% protein. The pH of the meat was 5.8. In

the water bath model system, raw ground *semimembranosus* meat contained 68.9% moisture, 3.5% fat and 24.8% protein. The pH of the meat was 6.0.

In roast beef pilot studies at MSU, peroxidase activity was the same in adequately cooked roasts processed at medium and high temperatures, but was different in roasts adequately processed using the low temperature process (Table 4.5). Peroxidase activity was different when roasts were adequately processed and undercooked by reducing the processing time by 0.5 log cycle in the low temperature process. Peroxidase activity was the same in adequately processed and inadequately processed roasts cooked to medium and high temperatures. These results indicated that peroxidase could not be used as a time-temperature integrator for roast beef because it could not differentiate between adequately and inadequately processed roasts. These results are supported by Orta-Ramirez et al. (1997) who reported a z value of 7.80 °C for peroxidase. The z value is different from that of *Salmonella* (5.65 °C) used to establish the USDA roast beef processing schedules (Goodfellow and Brown, 1978; USDA-FSIS, 1995).

Acid phosphatase activity was similar between equivalent processing schedules for both low and high temperature processes and averaged 223.6 U/kg meat (Table 4.6). Acid phosphatase activity decreased as cooking time was increased within each process. Based on our pilot study, acid phosphatase might be a promising marker protein. However, acid phosphatase is a glycoprotein bound to the cell membrane. It is present in only small amounts in animal tissues and milk systems and is difficult to purify

Table 4.5 Peroxidase activity (U/kg meat) in adequately processed and underprocessed roast beef prepared in a smokehouse

	Low temperature (54.4 °C; 130 °F)	Medium temperature (58.3 °C; 137 °F)	High temperature (62.2 °C; 144 °F)
Under-cooked (0.5 log reduction in time)	74 ± 7^{Aa}	48 ± 7^{Ab}	51 ± 14^{Ab}
Adequately cooked	35 ± 14^{Ba}	40 ± 15^{Aa}	38 ± 5^{Aa}

[AB] Means (± standard deviation) in the same column followed by the same letter are not different ($p > 0.05$).

[ab] Means (± standard deviation) in the same row followed by the same letter are not different ($p > 0.05$).

Table 4.6 Acid phosphatase activity (U/kg meat) in adequately processed and underprocessed roast beef prepared in a smokehouse

	Low temperature [a] (54.4 °C; 130 °F)	High temperature [a] (62.2 °C; 144 °F)
Under-cooked (1.0 log reduction in time)	490.7 ± 45.07[b]	370.5 ± 37.63[b]
Under-cooked (0.5 log reduction in time)	312.6 ± 50.65[b]	258.8 ± 102.05[b]
Adequately cooked	275.7 ± 65.12[b]	171.4 ± 70.99[b]

[a] Means (± standard deviation) in the same column showed a linear response to temperature; 54.4 °C ($p < 0.0035$) and 62.2 °C ($p < 0.0017$).

[b] Means (± standard deviation) in the same row followed by the same letter are not different ($p > 0.05$).

(Bingham and Zittle, 1963; Chaimovich and Nome, 1970; Debruyne, 1983; Farrell at al., 1988; Bingham and Garver, 1990).

The z value (7.41 °C) of acid phosphatase reported by Orta-Ramirez et al. (1997) was different from the z value (5.56 °C) of *Salmonella senftenberg* used to establish the USDA roast beef processing schedules. However, we cannot rule out acid phosphatase as a potential TTI based on z values alone. Further experiments are needed to verify the applicability of acid phosphatase as a TTI. Pilot studies need to be conducted to confirm the applicability of acid phosphatase as a TTI.

LDH concentration decreased as processing time was increased within low and high temperature processes (Table 4.7). However, the concentration of LDH differed when equivalent processing schedules were used at low (54.4 °C) and high (62.2 °C) temperatures. For example, the LDH concentration at the adequately cooked schedule of low temperature was 1098 μg/g meat and of high temperature was 5.6 μg/g meat. LDH is a tetrameric protein consisting of two polypeptide subunits, H and M. LDH exists as five isoforms H_4, H_3M, H_2M_2, HM_3, and M_4 (Holbrook et al., 1975). The z value of LDH (3.98 °C) from Orta-Ramirez et al. (1997) was different from that of LDH M_4 (6.1 °C) and LDH H_4 (5.7 °C) reported by Levieux et al. (1995). Orta-Ramirez et al. (1997) used an LDH enzyme assay, whereas Levieux et al. (1995) used single radical immunodiffusion (SRID) assay to quantify changes in LDH. Levieux et al. (1995) reported that epitopes of LDH M_4 and LDH H_4 recognized by anti-LDH M_4 or anti-LDH H_4 polyclonal antibodies were both conformational and sequential. Therefore, only native forms of LDH M_4 and LDH H_4 were quantified using the SRID method. The conformation of LDH required for

Table 4.7 Lactate dehydrogenase concentration (μg/g meat) in adequately processed and underprocessed roast beef prepared in a smokehouse

	Low temperature[a] (54.4 °C; 130 °F)	High temperature[a] (62.2 °C; 144 °F)
Under-cooked (1.0 log reduction in time)	1495.0 ± 562.5[b]	616.3 ± 71.9[c]
Under-cooked (1.0 log reduction in time)	1241.0 ± 201.5[b]	37.5 ± 13.9[c]
Adequately cooked	1098.0 ± 64.6[b]	5.6 ± 5.7[c]

[a] Means (± standard deviation) in the same column showed a linear response to temperature; 54.4 °C ($p < 0.0727$) and 62.2 °C ($p < 0.0105$).

[bc] Means in the same row followed by the same letter are not different ($p > 0.05$).

enzyme activity may not be related to the epitopes recognized by the polyclonal antibodies, thus causing resulting in different z values. Since LDH concentrations were different at equivalent processing schedules in the pilot study (Table 4.7) and the z values of LDH (Orta-Ramirez et al., 1997) are different from that of *Salmonella* (z = 5.65 °C) used to establish the USDA roast beef processing schedules (Goodfellow and Brown, 1978; USDA-FSIS, 1995), this protein marker may not be able to verify the thermal adequacy of roast beef.

In pilot smokehouse study at MSU, TPI activity decreased as cooking time increased when processed using the high temperature schedule ($P < 0.0160$) (Table 4.8). However, TPI activity did not decrease as cooking time increased using the low temperature schedule ($P < 0.2076$). TPI activity was the same when equivalent processes were used at low and high temperatures. For example, TPI activity of adequately processed schedules at low temperature was 2.05 U/kg meat and at high temperature it was 1.58 U/kg meat. In the ground beef model system, TPI activity increased ($P < 0.0001$) as processing time decreased in all three processing schedules (Table 4.9). TPI activity was similar in the low, medium and high temperature processes when roasts were adequately processed and inadequately processed by reducing the processing time by 0.5 log cycle. TPI had similar activity in both medium and high temperature processes when roasts were inadequately processed by reducing the processing time by 1.0 log. Our results support those of Orta-Ramirez et al. (1997) who found that the z value (5.56 °C) of TPI was similar to the z value of *Salmonella* (5.65 °C) used by the USDA to establish processing schedules for roast beef. Based on both pilot and model studies, TPI might be

Table 4.8 Triose phosphate isomerase activity (U/g meat) in adequately processed and underprocessed roast beef prepared in a smokehouse

	Low temperature[a] (54.4 °C; 130 °F)	High temperature[a] (62.2 °C; 144 °F)
Under-cooked (1.0 log reduction in time)	5.0 ± 1.99[b]	7.8 ± 3.33[b]
Under-cooked (0.5 log reduction in time)	4.5 ± 3.89[b]	3.7 ± 3.33[b]
Adequately cooked	2.1 ± 1.26[b]	1.6 ± 1.00[b]

[a] Means (± standard deviation) in the same column showed a linear response to temperature; 54.4 °C ($p < 0.2076$) and 62.2 °C ($p < 0.0160$).

[b] Means (± standard deviation) in the same row followed by the same letter are not different ($P > 0.05$).

Table 4.9 Triose phosphate isomerase activity (U/kg meat) of beef processed using three USDA approved schedules for roast beef and underprocessed by reducing the processing time by 0.5 and 1.0 log cycle in a ground beef water bath model system[a]

	Low temperature (54.4 °C; 130 °F)	Medium temperature (58.3 °C; 137 °F)	High temperature (62.2 °C; 144 °F)
Under-cooked (1.0 log reduction in time)	18.2 ± 2.47[b]	10.3 ± 2.31[c]	11.5 ± 2.14[c]
Under-cooked (0.5 log reduction in time)	4.7 ± 0.39[b]	3.7 ± 0.98[b]	6.2 ± 0.89[b]
Adequately cooked	2.1 ± 0.18[b]	2.3 ± 0.82[b]	3.5 ± 0.56[b]

[a] Means (± standard deviation) in the same column showed a linear response to temperature ($p < 0.0001$).

[bc] Means (± standard deviation) in the same row followed by the same letter are not different ($p > 0.05$).

a potential TTI for evaluation of the thermal processing adequacy using medium (58.3 °C) and high (62.2 °C) temperature schedules for beef roasts.

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CHAPTER 5

DEVELOPMENT OF AN ELISA TO QUANTIFY TRIOSE PHOSPHATE ISOMERASE IN COOKED BEEF

5.1 ABSTRACT

Triose phosphate isomerase (TPI) was identified as a potential endogenous time-temperature integrator (TTI) in beef roasts to verify the adequacy of processing in previous studies. Our objectives were to (1) prepare an ELISA using anti-TPI antibodies and (2) verify that the ELISA can quantify TPI in cooked ground beef. TPI was purified from bovine *semimembranosus* muscle by a series of procedures including 55% acetone fractionation, 90% ammonium sulfate fractionation, and carboxylmethyl cellulose cation exchange chromatography. The activity of purified TPI was increased 112 fold by purification. A single band of TPI was observed on SDS-PAGE. Polyclonal antisera (PAb) were raised in rabbits against purified TPI and yielded titers of 10^8 after 11 weeks of immunization. A sandwich ELISA was developed using PAb for capture and biotinylated PAb for detection. Cross reactivities of antibodies with TPI originating from different animal species, muscle protein concentrates, and common meat starter cultures were examined using the ELISA and Western blots. Ground beef was heated in 10 x 75 mm tubes to temperatures between 48.9 °C and 76.7 °C in 5.5 °C increments, then proteins extracted using 0.15 M NaCl, 0.01 M Na phosphate buffer, pH 7.2. The band

representing TPI on SDS-PAGE gels and Western blots decreased in intensity as processing temperature was increased. Both TPI concentration and activity in extracts of cooked ground beef decreased ($P < 0.0001$) as temperature was increased. Taken together, the results indicated that both the ELISA and enzyme assay were able to detect differences in TPI due to cooking temperature of beef.

Keywords: cooking, beef, triose phosphate isomerase, immunoassay

5.2 INTRODUCTION

Proper cooking is an easy method to effectively eliminate pathogens from meat products. Currently, the USDA-FSIS requires that roast beef be processed to one of 16 time-temperature combinations. For instance, beef can be heat processed to 62.8 ^{o}C (145.04 ^{o}F) with no holding time or processed to 54.4 ^{o}C (129.92 ^{o}F) with 121 minutes holding time. A 7-D reduction in *Salmonella,* the major enteric pathogen causing foodborne illness in meat, was proposed as the lethality standard for thermal processing of roast beef, and corned beef products (Goodfellow and Brown, 1978; USDA-FSIS, 1996b). The ultimate purpose of the 7-D reduction in *Salmonella* is to destroy pathogenic bacteria in the roast beef.

A time-temperature integrator (TTI) is "a small measuring device that shows a time-temperature dependent, easily, accurately and precisely measurable irreversible change that mimics the changes of a target attribute undergoing the same variable temperature exposure" (Hendrickx et al., 1995). A TTI can be used to verify roast beef processes. A TTI should have a z value or activation energy similar to that of the

microorganism used to establish the roast beef processing schedules required by the USDA.

Orta-Ramirez et al. (1996) investigated the thermal inactivation kinetics of six endogenous enzymes, acid phosphatase, lactate dehydrogenase, phosphoglycerate mutase, peroxidase, glyceraldehyde-3-phosphate dehydrogenase and triose phosphate isomerase (TPI), from bovine *semitendinosus* muscle. TPI had a z value (5.71 °C) similar to that of *S. senftenberg* (z = 5.56 °C) which was used to establish the USDA approved roast beef processing schedules (Goodfellow and Brown, 1978; USDA-FSIS, 1996). The authors suggested that TPI might be used as an endogenous TTI to determine the adequacy of thermal processing of roast beef and beef patties. Thus, TPI was suggested as an intrinsic TTI to verify compliance to the USDA roast beef processing schedules.

TPI was identified by Hsu et al. (Chapter 4) as the best marker protein in both ground beef and roast beef processed from 58.3 to 62.2 °C as TPI activities were the same when compared within cooking treatments, but increased as cooking time was decreased. One adequate cooking (high, medium and low temperature processes of 62.2 °C/5 min, 58.3 °C/24 min, and 54.4 °C/121 min, respectively) and two inadequate cooking schedules were evaluated at each temperature. In a ground beef water bath model system, TPI activity was similar when the meat was adequately processed using both medium and high temperature processes and TPI activity decreased ($P < 0.0001$) as processing time was increased within each process. In a roast beef pilot smokehouse study at MSU, TPI activity was similar when the meat was adequately processed using low and high

temperatures, but TPI activity only decreased ($P < 0.05$) as processing time was increased in the high temperature process.

Immunoassays have been used for determining endpoint temperatures of several meat products, including ground beef (Wang et al., 1995; Orta-Ramirez et al., 1996; Smith and Desrocher, 1996) and poultry products (Wang et al., 1992; Abouzied et al., 1993; Wang et al., 1993; Desrocher 1994; Wang et al., 1994; Smith et al., 1996; Smith and Desrocher, 1996). Immunoassays are more sensitive and less expensive than enzymatic methods and highly specific. The objectives of this study were to (1) develop an ELISA using anti-TPI antibodies, and (2) verify the applicability of the sandwich ELISA to quantify TPI in a cooked ground beef model system.

5.3 MATERIALS AND METHODS

5.3.1 Purification of TPI

Triose phosphate isomerase was purified from bovine *semimembranosus* muscle (top round choice muscle) purchased from a local meat retailer, after removing the cap muscle. All procedures were performed in a cold room at 4 °C unless otherwise indicated. Meat was diced into 1 cm cubes and visible connective tissue was removed. Meat was homogenized with 2.5 volumes of cold extraction buffer (30 mM potassium phosphate, pH 7.0, containing 1 mM EDTA and 10 mM 2- mercaptoethanol) in a Waring blender™ at high speed for two intervals of 45 sec. The homogenate was stirred at room temperature for 30 min and then centrifuged at 10,400 x g for 15 min. Fat was removed from meat extracts by filtering through cheesecloth.

Acetone (-15 °C) was added to the meat extract to reach a final acetone concentration of 40 % and the mixture was stirred on a magnetic plate for 30 min. Then, meat extracts were centrifuged at 10,400 x g for 10 min and the precipitate discarded. The supernatant was adjusted to a final acetone concentration of 55 %. The solution was stirred on a magnetic plate for 30 min at -15 °C. The solution was centrifuged for 10 min at 10,400 x g at 4 °C. The pellet was solubilized in 30 mM MES buffer (pH 6.5, including 30 mM (2-[N-morpholino]ethanesulfonic acid), 10 mM KOH, 0.5 mM magnesium acetate, 0.1 mM EDTA) and dialyzed against MES buffer overnight.

Saturated ammonium sulfate solution (pH 7.0) was added dropwise to this solution to achieve 40% saturation. The pH of the solution was adjusted to pH 5.1 with 1 M HCl to precipitate creatine kinase from the supernatant. The supernatant was stirred for 30 min at 25 °C and centrifuged at 38,000 x g for 10 min. Saturated ammonium sulfate solution (pH 7.0) was added to the supernatant to achieve 70 % saturation over a 30 min period. The solution was stirred for an additional 30 min and centrifuged at 12,000 x g for 10 min. The supernatant was collected and saturated ammonium sulfate solution (pH 7.0) was added to 90% saturation while stirring slowly for 30 min, and then centrifuged at 12,000 x g for 10 min. The pellet was collected and dissolved in a minimum amount of MES buffer (pH 6.5) and dialyzed for 48 hr against MES buffer. The buffer was replaced every 4 - 6 hr.

After dialysis, the protein solution was loaded onto a carboxylmethyl cellulose (CMC)) (156-0070, Bio-Rad®, Hercules, CA) ion exchange column (100 mL of CMC resin, 21 cm bed height and 2.5 cm diameter of column). Protein was eluted at a rate of

1.0 mL/min and 1 mL fractions were collected. Adenylate kinase, enolase, and phosphoglycerate kinase were all positively charged and were bound to the CMC column at pH 6.5 (Scopes and Stoter, 1982). TPI was negatively charged at pH 6.5 and did not bind to the CMC column. Also, it was reported that creatine kinase was denatured at room temperature when eluted from the CMC column at pH 6.5 (Scopes and Stoter, 1982). TPI activity and protein concentration were determined during each stage of the purification process. Purity of TPI was determined by SDS-PAGE as described below. TPI concentration was determined at 280 nm using an extinction coefficient, $E^{1\%} = 13.1$ (Norton et al., 1970).

5.3.2 TPI Enzyme Assay

Triose phosphate isomerase activity was assayed as described by Bergmeyer (1974) with modifications (Wang et al., 1996). The assay components of Bergmeyer (1974) contained 2.5 mL triethanolamine buffer (0.243 mol/L; pH 7.6), 0.5 mL glyceraldehyde-3-phosphate (3.8 mmol/L), 50 µl NADH, Na salt (10 mg/mL), 10 µl glycerophosphate dehydrogenase (1.5 mg/mL) and 10 µl of meat extract. The modified enzyme assay included 1 mL triethanolamine buffer (0.2 mol/L; pH 7.6), 0.2 mL glyceraldehyde-3-phosphate (15 mmol/L), 10 µl NADH, Na salt (10 mg/mL), 10 µl glycerophosphate dehydrogenase (1.5 mg/mL, 170 units/mg protein, cat. no. G-6751) and 10 µl of meat extract. The initial velocity was read at 340 nm for 1 min at 25°C. Samples were diluted in PBS (pH 7.2) so that the optical density change was less than 0.2 units per minute (Beisenherz, 1955). TPI activity was determined using an extinction

coefficient of NADH (E_{340} = 6.317 x 10^{2} l x mol^{-1} x mm^{-1}). One unit (U) of activity was defined as 1 μmol substrate converted/min (Bergmeyer, 1983). TPI enzyme activity was calculated based on the following formula:

$$\text{Activity (U/L)} = \frac{A \times 1000}{t \times \varepsilon \times d \times \varphi} \ \mu\text{mol} \times \text{min}^{-1} \times \text{L}^{-1}$$

A = absorbance

t = time (min)

ε = absorption coefficient (6.317), L x $mmol^{-1}$ x mm^{-1}

d = the distance of light path, 10 mm

φ = volume fraction of sample in assay (incubation) mixture, v/V (no units)

v = volume of sample used in assay, μl

V = assay volume, μl

5.3.3 Bradford Soluble Protein Concentration Determination

Protein concentration of meat extracts was determined following the method of Bradford (1976). Bovine serum albumin (BSA) was used to prepare a standard curve ranging from 10 to 913 μg protein/mL. Ten microliters of meat extract and 200 μl Coomassie blue G-250 dye reagent (500-0006, Bio-Rad®) were added to each microwell (Immulon® 1, Dynatech, Chantilly, VA 22021), and incubated for 5 min at room temperature. Absorbance was read at 590 nm using a Minireader II (Molecular Devices,

Menlo Park, CA 94025). Protein concentration was determined using the lowest dilution in PBS (pH 7.2) which fell on the BSA standard curve.

5.3.4 Immunization and Polyclonal Antibody Production

Three white New Zealand rabbits (3 months old; code no. 56851, 56881 and 56883) were immunized subcutaneously at 10 different locations with 500 μg of purified bovine TPI (in 0.5 mL MES buffer) in Freund's complete adjuvant (Difco®, Detroit, MI 48232). A 1.0 mL total injection volume contained 0.5 mL of TPI in MES buffer and 0.5 mL Freund's complete adjuvant. After five weeks, rabbits were boosted by subcutaneous injection with 500 μg TPI protein emulsified with Freund's incomplete adjuvant (Difco®) as described above. Ten days after each boost, rabbits were bled via marginal ear veins. Total volume of blood obtained was based on the body weight of each rabbit. Four boosts at 4, 8, 11, 18, and 23 wk were used for each rabbit. The polyclonal antibodies were purified from sera by ammonium sulfate precipitation as described by Harlow and Lane (1988) except that polyclonal antibodies were precipitated in a solution of 33% saturated ammonium sulfate solution instead of 40% saturation at pH 7.2.

5.3.5 Indirect ELISA (Titer Determination)

An indirect ELISA was used to determine the titer of anti-bovine TPI polyclonal antibodies. Titers were determined using sera collected from each rabbit after each boost. Microtiter wells (Immulon® 2, Dynatech) were coated with 100 μl bovine TPI (1 μg/mL) in carbonate buffer (0.1 M, pH 9.6) overnight at 4 °C. Microtiter plates were washed 3

times with PBS-Tween (0.01 M PBS with 0.05% Tween 20, pH 7.2). Three hundred microliters 0.5% casein (wt/vol) in PBS was added to each well as the blocking reagent to decrease nonspecific binding and incubated for 30 min at 37 °C. Wells were washed 4 times with PBS-Tween, and 50 µl of serum diluted from 10^1 to 10^8 times was added to each well and incubated at 37 °C for 30 min. Those polyclonal antibodies not bound to the TPI antigen on the plates were removed by washing 4 times with PBS-Tween (pH 7.2). Then, 100 µl of goat anti-rabbit (GAR) IgG peroxidase conjugate from Sigma® (1:500 in 0.5% casein-PBS) was added to the wells and incubated at 37 °C for 30 min. Each plate was washed 8 times to remove unbound GAR IgG peroxidase. The bound peroxidase was reacted with ABTS substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) to develop color and absorbance was read at 405 nm using a ThermoMax (Molecular Devices, Menlo Park, CA 94025) (Pestka et al., 1982). Titers were reported as the highest dilution that showed twice the absorbance of the same dilution of preimmune serum.

5.3.6 Electrophoresis

Both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Wang et al., 1992; 1995) and native PAGE (Wang et al., 1992; 1995) were performed using a Mini-Protein Gel assembly (Bio-Rad®) with a 4 % acrylamide stacking and 12 % acrylamide resolving gel. For SDS-PAGE, protein extracts were combined 9 : 1 (v/v) with sample buffer (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol), placed in boiling water and held for 15 min. For native PAGE, the

protein extracts were combined with sample buffer (0.5 M Tris-HCl, pH 6.8) at a 9 : 1 (v/v) ratio, and no heat was applied. Samples were held at -10 ^{o}C and thawed at room temperature before SDS-PAGE or native PAGE was performed. For both SDS-PAGE and native PAGE, 15 µl prepared meat extracts were loaded per well. Broad range molecular weight markers (161-0318, Sigma®) were used to determine the molecular masses of the unknown protein bands on SDS-PAGE. Purified porcine TPI (type XII, T-7526, Sigma®) was used (4µg TPI/well) as a marker protein for both native PAGE and SDS-PAGE. Electrophoresis was performed using Tris-glycine electrode buffer (0.025 M Tris, 0.192 M Glycine, pH 8.3) at a current of 55 milliamps and a voltage of 200 V for 45 min. The gels were stained with 0.2% Coomassie Brilliant Blue R-250 (161-0400, Sigma®) which has a detection limit of 0.1 to 1 µg protein per band (Wilson, 1983).

5.3.7 Western Blotting

Relative antibody specificity to purified bovine TPI and meat extracts were evaluated by the western blot method (Wang et al., 1993). Purified bovine TPI, raw meat extracts, heated meat extracts, and commercially available TPI from different species, (rabbit (type IIIS), porcine (type XII), and dog (type VI) TPI purchased from Sigma®) were electrophoretically transferred (1 hr at 100 V) from either native or SDS-PAGE gels to nitrocellulose membranes. The membranes were washed thoroughly with PBS-Tween (Tween 0.05% (v/v)). Ten milliliters of 0.5% casein-PBS solution was used as a blocking agent for 30 min at room temperature. The membranes were washed with PBS-Tween again, then 10 mL of bovine TPI PAb (diluted 1:1,000 in 0.5% casein-PBS) was added

and incubated at room temperature for another 10 min. Unbound antibodies were removed by washing with PBS-Tween and 10 mL of goat anti-rabbit IgG (GAR-IgG) peroxidase conjugate (1:2,000 in 0.5% casein-PBS) was added to the membrane, followed by incubation for 10 min at ambient temperature. Several washes with PBS-Tween were used to remove unbound GAR-IgG peroxidase conjugate. To prepare the color developing substrate, 24 mg of 3, 3', 5, 5' tetramethyl benzidine (T-2885, Sigma®) and 80 mg of dioctyl sulfosuccinate (D-0885, Sigma®) were dissolved in 10 mL of ethanol. Then, the above solution was added to 30 mL of 0.1 M citrate-phosphate buffer (pH 5.0) and 20 µl of 30% H_2O_2 was added. This substrate was used to determine bound peroxidase (Wang et al., 1992). Twenty milliliters of this substrate solution was used to stain one nitrocellulose membrane. The staining reaction was stopped using double distilled water when sufficient color was developed.

5.3.8 Biotinylation of Polyclonal Antibodies

Polyclonal antibodies were purified using a protein A/G column following instructions provided by the manufacturer (Pierce, Rockford, IL 61105). Polyclonal antibodies were biotinylated as described by Harlow and Lane (1988). The IgG concentration was determined at 280 nm using an extinction coefficient ($E_{mg/mL} = 1.4$). Three milliliters of antibody solution (1 mg/mL) was diluted in 0.1 M sodium borate buffer (pH 8.8). The antibody solution (1 mg/mL) was concentrated using a Centriprep membrane (membrane size of 10 kd; Amicon, Inc., Beverly, MA 01915). Five mg of biotin-amidocaproate N-hydroxysuccinimide ester was dissolved in 1 mL dimethyl

sulfoxide. This biotin ester solution (0.1 mg of biotin ester per gram of antibody) was added slowly to the antibody solution, followed by stirring for 4 hr at 25 °C. Twenty microliters of 1 M NH_4Cl solution (based on 0.08 µl of 1 M NH_4Cl per µg of biotin ester) was added and the solution incubated for 10 min at 25 °C. The antibody solution was dialyzed against PBS (0.01 M, pH 7.2) for 48 hr. The buffer was changed every 6 hr. After dialysis, biotinylated antibodies were aliquoted and stored at -18 °C.

5.3.9 Sandwich ELISA

The sandwich ELISA was performed by coating microtiter wells (Immulon® 2, Dynatech) with 100 µl of polyclonal antibody #56851 diluted (1:500) in 0.1M carbonate buffer (pH 9.6) and drying overnight at 37 °C (98.6 °F) in an oven. Wells were washed 4 times with PBS containing 0.05% Tween 20 (PBS-Tween), and 300 µl of 0.5% casein-PBS was added to each well to block remaining protein binding sites and incubated for 30 min at 37 °C. After washing 4 times with PBS-Tween, meat extracts or standard bovine TPI diluted in 0.5% casin-PBS (50 µl) were added to each well and incubated for 30 min at 37 °C. Plates were washed another 4 times with PBS-Tween, and 50 µl of biotinylated polyclonal antibodies diluted (1:100) in 0.5% casin PBS was added. After incubation for 30 min at 37 °C and washing 4 times with PBS-Tween, 100 µl avidin-horseradish peroxidase (HRP) from Sigma® diluted 1:8000 in PBS-casein was added to each well and incubated for 30 min. Plates were then washed 8 times with PBS-Tween. Bound peroxidase activity was determined with ABTS substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) (Pestka et al., 1982) and absorbance was read at 405

nm using a ThermoMax (Molecular Devices, Menlo Park, CA 94025). Bovine TPI ranging from 0 to 40 μg TPI/mL was used to construct the standard curve. Results were expressed as μg TPI/mL buffer solution and also expressed as μg TPI/kg meat. The coefficient of variation was used to evaluate between-run and within-run precision. The cofficient of variation (Deshpande, 1996) for between-run precision was based on 8 replicates using 5, 10, 20, and 40 μg TPI/mL. The cofficient of variation of within-run precision was based on 16 replicates at three TPI concentrations (16, 18, and 20 μg/mL).

5.3.10 Cross Reactivities of TPI Antibodies with TPI from Different Animal Species, Commercial Whey Protein Concentrates and Plasma Protein Concentrates

Rabbit (type IIIS, T-2391), porcine (type XII, T-7526) and dog (type VI, T-6635) TPI were purchased from Sigma® (muscle types were not specified). Protein concentration of TPI protein from each species or meat extracts was determined using Bradford method (1976). Cross-reactivities of bovine TPI polyclonal antibodies with rabbit, porcine, and dog TPI were examined at TPI concentrations ranging from 0.001 to 100 μg TPI protein/mL using the sandwich ELISA. The cross reactivity of a raw extract of ground beef extracted with PBS (0.1 M NaCl; pH 7.2) was also examined.

Five spray dried protein concentrates of Beef Stock, Pork Stock, Chicken Broth, AMP 800® (whey protein concentrate), and AMP 600N® (hydrolyzed protein from beef and plasma) were donated by AMPC, Inc. (Ames, IA 50010). Whey protein isolate (type A) was obtained from Davisco International® (Le Sueur, MN 56058). Protein concentration of each protein concentrate solubilized in PBS (0.1 M NaCl; pH 7.2) was

determined following the method of Bradford (1976). Cross reactivities against bovine TPI polyclonal antibodies were examined by sandwich ELISA at total soluble protein concentrations ranging from 0.24 to 250 μg/mL.

5.3.11 Cross Reactivity of TPI from Starter Cultures

Three common meat starter cultures, *Lactobacillus plantarum, Pediococcus pentosaceus,* and *Pediococcus acidilactici* were donated by ABC Research Corporation (Gainesville, FL 32602). Starter cultures were inoculated separately in a medium of 80% lactose broth and 20% APT (all purpose tween) agar (for cultivating heterofermentative *lactobacilli* (Difco®) and incubated for about 72 hr at 37 °C. Total cell counts reached about 10^{12} CFU per gram for each strain. Cells were harvested (about 1 g) after centrifugation of the bacterial suspension at 1,000 x g for 5 min. Ten volumes (about 10 mL) of cold peptone buffer (0.1%) was added to the pellet and mixed thoroughly. Then, cold acetone (-18 °C, 250 mL) was added slowly to the bacterial suspension, to avoid protein denaturation, and stirred on a magnetic plate at -18 °C for 10 min. The bacterial solution was then centrifuged at 5,000 x g for 10 min. Bacterial masses were precipitated in the pellet. The pellet was vacuum filtered through a 0.2 μm membrane to obtain the mass. A small amount of ether was added to the filter membrane to remove residual moisture from the pellet. The bacterial pellet was lyophilized for storage. Total cell counts were about 5 x 10^{11} cells/2 mL of PBS (0.01 M, pH 7.2). The TPI enzyme activity and soluble protein concentration were determined using TPI enzyme assay described in 5.3.2 and Bradford method (1976), respectively.

5.3.12 Ground Beef Water Bath Model Study

Protein, fat and moisture contents of *semimembranosus* muscle was determined using AOAC (1990) standard methods 981.10, 960.39 and 950.46B, respectively. The pH was determined by homogenizing 10 g ground beef with 90 mL of double distilled water using a Waring™ blender for 30 s.

Fresh *semimembranosus* meat (top round choice roast), purchased from a local store, was cut into 1 cm cubes and ground twice through a 3.175 mm diameter grinder plate in a Hobart grinder (Model 8418D, Hobart Mfg. Co., Troy, OH, 45374). The ground beef was placed into a 60 mL syringe (Becton Dickinson and Co., Franklin Lakes, NJ 07417). Two grams of ground meat was compressed through a 7 cm plastic tubing into a 10 x 75 mm TDT borosilicate glass tubes (60825-538, Kimax® 51, VWR, West Chester, PA 19380). Glass tubes were then sealed using telfon tape. Meat was cooked to target endpoint temperatures in a Polystat circulator water bath (Model 1268-52, Cole-Parmer) set at 82.2 °C. The water bath was connected to a digital programmer (Model 1268-62, Cole-Parmer). A Resistance Temperature Detector thermocouple (platinum Pt 100 temperature probe, Solomat Partners LP, Stanford, CT 06906) was inserted into the center of a TDT tube containing 2 g meat. Internal temperatures were determined by a thermocouple using a Solomat MPM 200 Modumeter (Solomat Partners LP) inserted into the meat. After tubes reached the target temperatures, 48.9 °C (120 °F), 54.4 °C (130 °F), 60.0 °C (140 °F), 65.6 °C (150 °F), 71.1 °C (160 °F), and 76.7 °C (170 °F), meat was placed in a ice bath for 10 min.

5.3.13 Preparation of Protein Extracts and Enzyme Assays

Glass tubes (10 x 75 mm) were broken and cooked meat was transferred into scintillation vials. Six milliliters 0.01 M, 0.05 M, 0.1 M PBS, pH 7.2 containing 0.1 M NaCl or 0.01 M MOPS, pH 7.2, 0.1 M NaCl, was added to each vial. Samples were vortexed for 1 min, then stirred on magnetic plates for 30 min at 4 °C. Samples were centrifuged at 4,500 x g for 10 min at 4 °C (Micro-centrifuge, Model 59A, Fischer Scientific). The supernatant was collected and held at 4 °C until used. TPI activity and concentration of extracts were determined within 24 hr. Preliminary experiments performed to devise this extraction protocol are described in the Appendix section.

5.3.14 Statistical Analysis

Ground *semimembranosus* muscles were heated in water bath to six different cooking temperatures. Each cooking schedule was conducted in triplicate. Data were analyzed using two way ANOVA (analysis of variance) (treatment x replication). The linear or curvilinear change in enzyme activity or ELISA with temperature was determined using orthogonal polynomial contrast test by SAS software (SAS Institute, Inc., Version 6.1, 1995, Cary, NC 27513).

5.4 RESULTS AND DISCUSSION

5.4.1 Purification of TPI

TPI was purified from bovine *semimembranosus* muscle using 40% and 55% acetone fractionations, 40%, 70% and 90% ammonium sulfate fractionations and

carboxylmethyl cellulose (CMC) chromatography. Total protein, total activity, specific activity, yield, and purification factor of selected fractions during purification were reported in Table 5.1. Selected purification fractions can be observed on a representative SDS-PAGE electrophoretogram (Figure 5.1). Two major contaminant proteins, creatine kinase and bovine serum albumin (BSA), were eliminated using specific procedures described in the following section.

Creatine kinase was the major contaminant during TPI purification since creatine kinase and TPI were both negatively charged at pH 6.5 and were not separated by CMC chromatography. Adjusting the pH to 5.1 at 40% ammonium sulfate saturation was necessary to precipitate creatine kinase from the supernatant. About 90% of the contaminating creatine kinase precipitated at this stage. The TPI specific activity increased 111.8 fold after CMC column chromatography. A single band in lane 6 (Figure 5.1) is the purified bovine TPI with a molecular mass of 23 kd, and TPI existed as dimer forms. In preliminary experiments, TPI and creatine kinase co-eluted when chromatographed using size exclusion chromatography (50-100 kd size exclusion beads) (151-1030, Bio-Gel-15mL-A, Bio-Rad®). Less than 50% of TPI and CK were separated using the size exclusion column since the shapes of the two proteins were similar, even though the molecular masses of TPI and creatine kinase were different (46 and 86 kd, respectively).

BSA was another major contaminant during TPI purification. BSA was eliminated using a series of procedures. The concentration of BSA was gradually

Table 5.1 Purification of triose phosphate isomerase from bovine *semimembranosus* muscle

Fraction	Protein Concentration (g/L)	Volume Activity (U/L)	Specific Activity (U/g)	Purification Factor
Homogenate	4.91	102.7	20.9	1
55% acetone precipitate	9.25	280.7	30.4	1.5
90% ammonium sulfate precipitate	8.47	9595.3	1133.0	54.2
CMC column [a]	0.98	2290.4	2337.7	111.8

[a] Carboxylmethyl cellulose (CMC) chromatography column.

[b] Estimated volumes for homogenate, 55% acetone precipitate (solubilized in minimum amount of 30 mM MES buffer and dialyzed), 90% ammonium sulfate precipitate (dissolved in minimum amount of MES buffer and dialyzed) and the solution before loaded into CMC column were 750, 30, 25, and 8 mL.

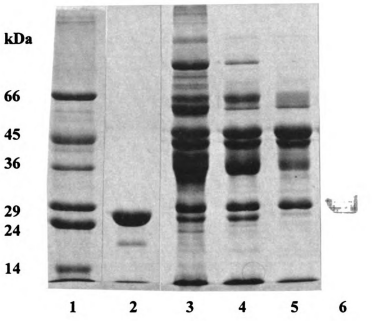


Figure 5.1 Representative sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of muscle extracts from the triose phosphate isomerase (TPI) purification procedures. Proteins were stained with Coomassie Blue. (lane 1) molecular weight standard; (lane 2) porcine muscle TPI (from Sigma); (lane 3) bovine muscle homogenate; (lane 4) 55% acetone precipitate fraction; (lane 5) 90% ammonium sulfate precipitate fraction; (lane 6) after carboxylmethyl cellulose (CMC) column chromatography.

decreased in 55% acetone pellet (Figure 5.1; lane 4), 70% ammonium sulfate supernatant and 90% ammonium sulfate pellet (Figure 5.1; lane 5) fractions.

5.4.2 Production of Polyclonal Antibody and ELISA Development

Anti-bovine TPI polyclonal antibodies were produced in rabbits to devise a TPI sandwich ELISA. Antibody titers to bovine TPI reached 10^7-10^8 in all three rabbits after the first boost (Table 5.2). Antibody titers decreased to 10^6-10^7 after the second boost and increased to 10^8 after the third boost. However, after 18 weeks, titers decreased to 10^4 which might be due to the immuno-tolerance built up against TPI (Kuby, 1994). Serum of rabbit C was used for further studies since it had the highest titer after three injections.

A sandwich ELISA was developed and optimized. Purified bovine TPI from *semimembranosus* muscle was used to prepare a standard curve from 5 to 40 μg TPI/mL. The limit of detection of the ELISA was 5 μg TPI/mL. The coefficients of variation between-runs and within-runs were determined for the ELISA. In the between-run test, TPI concentrations of 5, 10, 20, and 40 μg/mL had coefficients of variation of 8.42, 9.42, 12.77, and 6.39 %, respectively. At TPI concentrations of 5, 10, 20, and 40 μg/mL, the respective coefficients of variation were 8.06, 8.35, 3.62, and 6.94 % when precision was tested within a run.

Table 5.2 Polyclonal antibody titers [a] (serum dilution) against bovine triose phosphate isomerase from *semimembranosus* muscle

Weeks after initial immunization [b]	Antibody titer		
	Rabbit A	Rabbit B	Rabbit C
4	7.5×10^7	3.2×10^7	1.0×10^8
8	9.0×10^6	8.3×10^6	9.1×10^7
11	1.0×10^8	1.1×10^8	1.1×10^8
18	1.0×10^4	1.0×10^4	1.0×10^4
23	9.4×10^4	9.0×10^4	9.0×10^4

[a] Titer is defined as the serum dilution at which the absorbance is twice that of the pre-immune serum.

[b] Booster injections were given at 4, 8, 11, 18 and 23 wk. Sera were analyzed by indirect enzyme-linked immunosorbent assay.

5.4.3 Cross Reactivity of TPI Polyclonal Antibodies with TPI from Different Species

A sandwich ELISA was used to investigate the cross reactivity of the antibodies with TPI from the muscles of different animal species (Figure 5.2). Bovine TPI had highest cross reactivity with bovine anti-TPI polyclonal antibodies when compared to TPI from other animal species. Bovine TPI was about 0.3 absorbance units higher than porcine TPI at 25 μg TPI/mL. Porcine TPI had higher cross reactivity when compared to rabbit and dog TPI ranging from 10 to 25 μg TPI/mL; rabbit TPI and dog TPI had similar cross reactivities with bovine anti-TPI polyclonal antibodies.

Bovine anti-TPI polyclonal antibodies were highly specific to native and denatured TPI as shown on western blots (Figures 5.3 and 5.4) of raw bovine muscle extracts. Only one hybridized band was observed on the membranes, although many muscle proteins are observed on native and SDS-PAGE gels (Figure 5.1, lane 3). The substrate intensity increased as volume of meat extracts was increased on the native and SDS-PAGE gels (Figure 5.3, lanes 5 and 6; Figure 5.4, lanes 6 to 9). Cross reactivity of raw beef extracts with TPI polyclonal antibodies was also examined using TPI sandwich ELISA (Figure 5.5). A bovine raw extract of ground beef extracted with PBS (pH 7.2) cross reacted with TPI polyclonal antibodies from 15 to 250 μg total soluble protein/mL meat extract.

Western blotting was also conducted to determine the specificity of bovine anti-TPI polyclonal antibodies with native and denatured TPI from different animal species.

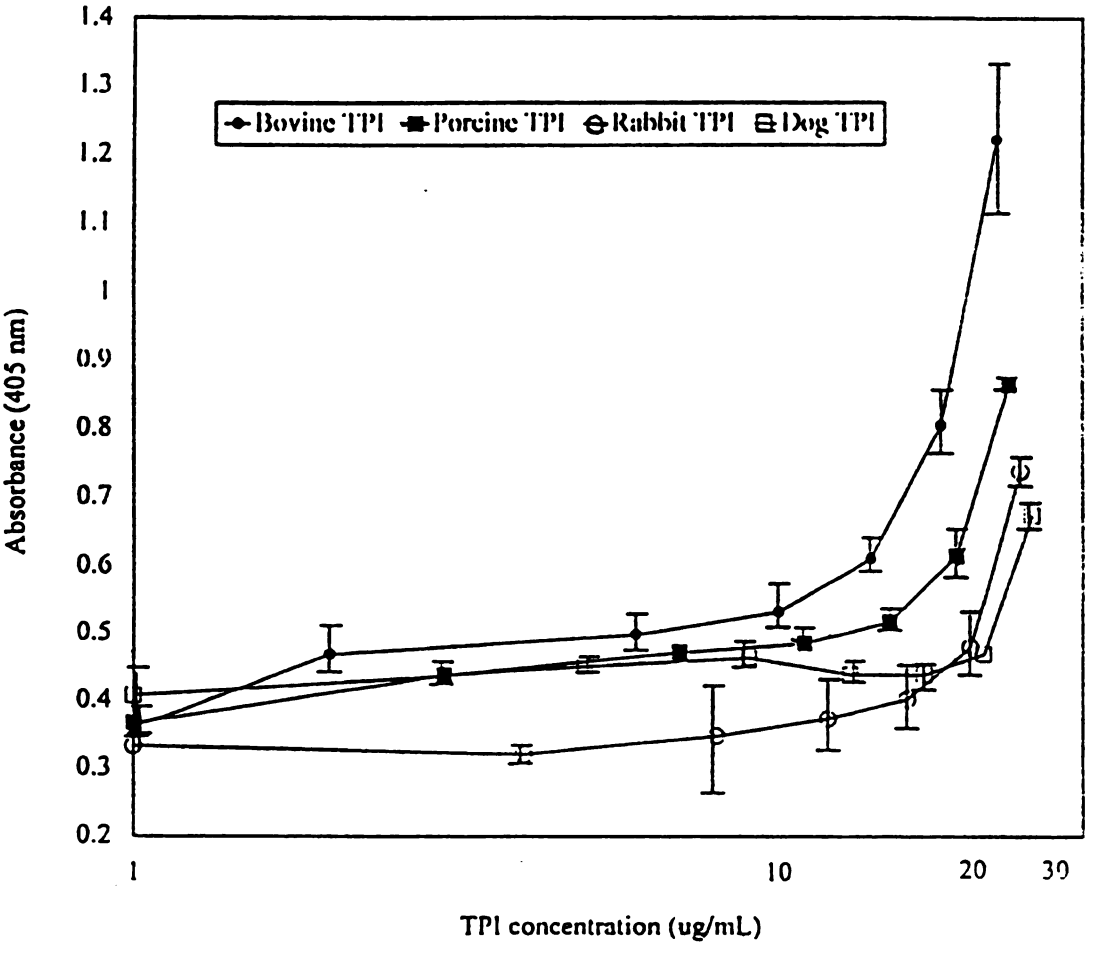


Figure 5.2 Cross-reactivity of bovine anti-triose phosphate isomerase (TPI) polyclonal antibodies with TPI from different animal species determined by sandwich ELISA. The standard deviation values that were less than 0.02 absorbance units were not plotted on the figure.

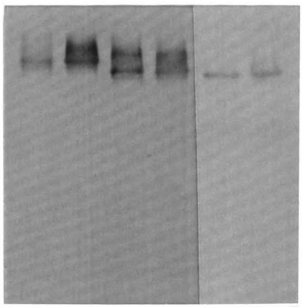
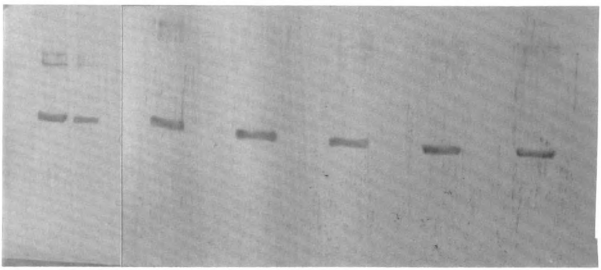


Figure 5.3 Western blot of bovine, dog, rabbit, and porcine muscle triose phosphate isomerase (TPI) and bovine muscle extract electrophoretically transferred from a native polyacrylamide gel to a nitrocelluolse membrane hybridized with anti-bovine TPI polyclonal antibodies; (lane 1; 5 μg protein) dog muscle TPI; (lane 2; 5 μg protein) rabbit muscle TPI; (lane 3; 5 μg protein) porcine muscle TPI; (lane 4; 5 μg protein) bovine muscle TPI; (lane 5; 100 μg protein and lane 6; 200 μg protein) bovine raw muscle extracts using MEMT buffer (50 mM Tris-HCl, pH 6.8 containing 0.4 mM EDTA, 7 mM α-mercaptoethanol and 5 mM $MgSO_4$).



1 2 3 4 5 6 7

Figure 5.4 Western blot of bovine, dog, rabbit, and porcine muscle triose phosphate isomerase (TPI) electrophoretically transferred from a sodium dodecyl sulfate-polyacrylamide gel to a nitrocellulose membrane hybridized with anti-bovine TPI polyclonal antibodies; (lane 1; 100 μg protein; lane 2; 50 μg protein) raw bovine muscle extracts using phosphate buffer saline (0.14 M NaCl, 0.01 Na phosphate, pH 7.2); (lane 3; 50 μg protein) raw bovine muscle extracts using MEMT buffer (50 mM Tris-HCl, pH 6.8, containing 0.4 mM EDTA, 7 mM α-mercaptoethanol and 5 mM $MgSO_4$); (lane 4; 5 μg protein) bovine muscle TPI; (lane 5; 5 μg protein) porcine muscle TPI; (lane 6; 5 μg protein) rabbit muscle TPI; (lane 7; 5 μg protein) dog muscle TPI.

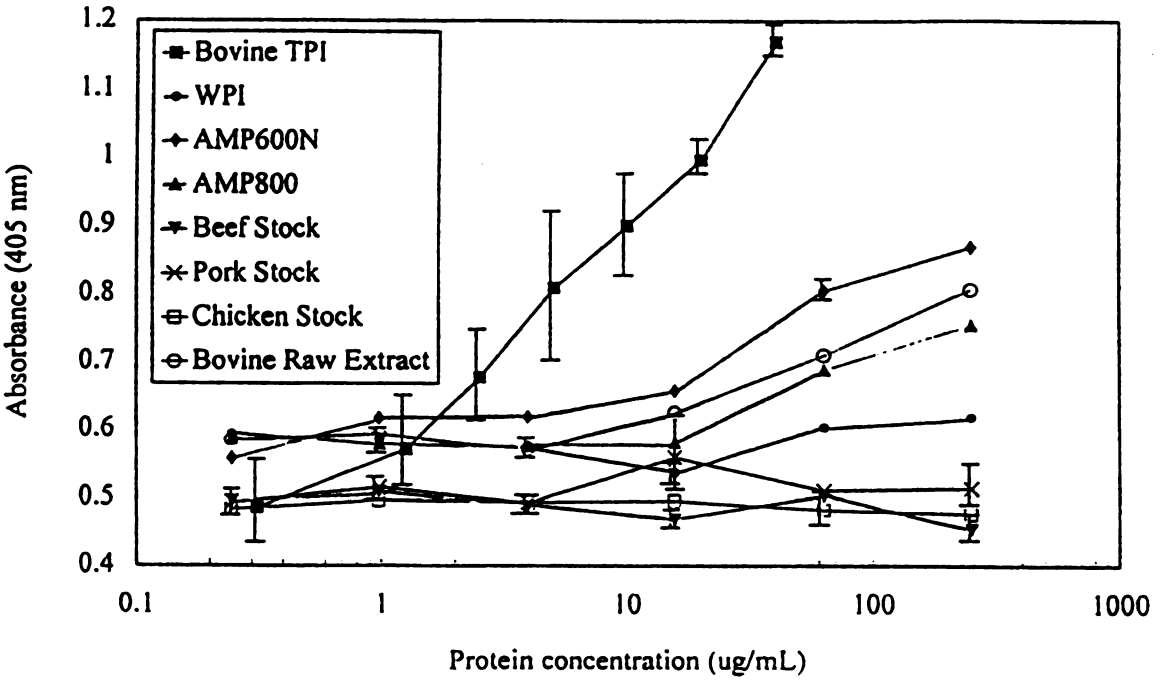


Figure 5.5 Cross-reactivity of bovine triose phosphate isomerase (TPI) polyclonal antibodies with TPI from different protein concentrates determined by sandwich ELISA. Bovine TPI = TPI purified from bovine *semimembranosus* muscle, WPI = whey protein isolate, AMP600N = hydrolyzed protein from meat and plasma, AMP800 = whey protein concentrate, bovine raw extract = ground bovine *semimembranosus* muscle extracted with phosphate buffer saline (1:3). The standard deviations less than 0.02 were not plotted on the figure.

Bovine anti-TPI polyclonal antibodies cross reacted with TPI from bovine, porcine, rabbit and dog muscles electrophoretically transferred from native gels (Figure 5.3). Single bands were observed on nitrocellulose membranes containing native TPI from different species when reacted with TPI polyclonal antibodies. Western blotting revealed that bovine, porcine and rabbit TPI had higher cross reactivities with anti-TPI polyclonal antibodies than dog TPI. The results were consistent with the results of the ELISA that showed bovine, porcine and rabbit TPI had higher cross reactivities with anti-TPI polyclonal antibodies when compared to dog TPI at 25 μg/mL.

Single bands were also observed when denatured TPI from rabbit, porcine and dog species reacted with anti-TPI polyclonal antibodies transferred from SDS-PAGE (denatured) gels (Figure 5.4). Bovine TPI (lane 4) showed higher substrate intensity compared to that of porcine (lane 3), rabbit (lane 2), and dog TPI (lane 1) when 5 μg TPI was loaded in each lane.

5.4.4 Cross Reactivity of TPI Polyclonal Antibodies with TPI from Different Sources

Some protein concentrates are widely used in commercially processed roast beef products as functional ingredients to improve sensory properties and yields. The study was designed to examine the cross reactivity of six commercial protein concentrates with bovine anti-TPI polyclonal antibodies. In general, all commercial protein concentrates had lower cross reactivities with bovine TPI polyclonal antibodies when compared to pure bovine TPI (Figure 5.5). AMP 600N® (hydrolyzed protein from beef and plasma)

and AMP 800® (whey protein concentrate) had higher cross reactivities with bovine anti-TPI polyclonal antibodies compared to whey protein isolate, beef stock, chicken stock, and pork stock at the same concentration.

AMP 600N® showed the highest cross reactivity when compared to the other protein concentrates (Figure 5.5). AMP 600N® is a hydrolyzed meat protein product (70 % protein content) containing hydrolyzed beef plasma. Beef plasma probably contains some bovine TPI which may have caused the cross reaction (Sawyer et al., 1972).

No cross reactivity was observed with whey protein isolate (95% protein) at concentrations up to 250 μg protein/mL, suggesting that whey proteins did not cross react with TPI polyclonal antibodies. Although AMP 800® is a whey protein concentrate (80 % protein content), a greater cross reactivity was observed compared to whey protein isolate in the same concentration range (15 to 250 μg protein/mL). Unknown ingredients in AMP 800®, such as contaminating plasma proteins, could lead to higher TPI cross reactivities. Plasma proteins were observed when AMP 600N was separated by SDS-PAGE (data not shown). Since ingredients in AMP 600N® and AMP 800® cross-reacted with TPI polyclonal antibodies, the use of these concentrates above concentration of 15 μg protein/mL could interfere with the accurate determination of processing adequacy.

5.4.5 Cross Reactivity of TPI Polyclonal Antibodies with TPI from Starter Cultures

Fermented meat products are produced using bacterial starter cultures to provide their characteristic sensory properties (Bacus, 1984). *Lactobacillus plantarum,*

Pediococcus pentosaceus, and *Pediococcus acidilactici* are common commercial meat starter cultures (Bacus, 1984). The cross reactivities of microbial TPI from meat starter cultures with TPI polyclonal antibodies was examined to determine if the TPI sandwich ELISA could be applied to verify the processing adequacy in the fermented meat products. TPI enzyme activity was low and averaged 5.69, 3.90, and 5.20 U/L (0.01 M, pH 7.2) in assay mixtures containing 2.5 x 10^{11} cell/mL of *Lactobacillus plantarum, Pediococcus pentosaceus,* and *Pediococcus acidilactici,* respectively. Microbial TPI was not detected in the three strains using the TPI sandwich ELISA, suggesting that TPI antibodies did not cross react with the microbial TPI produced from the starter cultures commonly used in fermented meat products.

5.4.6 Ground Beef Water Bath Model Study

TPI activities and concentrations decreased consistently ($P < 0.0001$) as cooking temperatures of ground beef were increased when determined by enzyme assay and ELISA, respectively (Table 5.3). However, the difference in TPI activity was more apparent than the difference in TPI concentration among different endpoint temperatures. For example, TPI activity was 537.53 U/kg meat at 48.9 °C and decreased to 186.96 U/kg meat at 54.4 °C. TPI concentration was 86.30 μg TPI/g meat at 48.9 °C and was decreased to 66.88 μg TPI/g meat at 54.4 °C. The differences were 350 U/kg meat and 19,000 ng TPI/g meat between 48.9 and 54.4 °C using enzyme assay and sandwich ELISA, respectively. The coefficient of variations of TPI activity and concentration were

Table 5.3 Effect of cooking temperature on triose phosphate isomerase (TPI) activity (U/kg meat) and concentration (μg TPI/g meat) of bovine meat cooked in a water bath

Internal temperature (°C)	Muscle Activity (U/kg meat)		Concentration (μg TPI/g meat)	
48.9 ± 0.1	537.5 ± 14.55 [a]	(2.71)	86.3 ± 11.55 [a]	(13.38)
54.4 ± 0.1	187.0 ± 21.88	(11.70)	66.9 ± 6.64	(9.93)
60.0 ± 0.1	72.1 ± 9.03	(12.53)	48.3 ± 6.67	(13.82)
65.6 ± 0.1	38.0 ± 3.08	(8.12)	42.6 ± 8.75	(20.56)
71.1 ± 0.1	27.4 ± 3.98	(14.54)	36.8 ± 7.17	(19.46)
76.7 ± 0.1	19.4 ± 1.01	(5.21)	34.1 ± 6.29	(18.46)

[a] Values are expressed as mean ± standard deviation of triplicate determinations and values in parentheses represent coefficient of variation. Means in the same column decreased linearly as temperature increased ($p < 0.0001$).

also used to compare method variability. In general, the coefficient of variation of TPI activity was smaller (less than 15%) when compared to that of TPI concentration.

Both SDS-PAGE and Western blotting were also used to determine the effect of heating temperature on TPI extracted from the ground beef model system. On SDS-PAGE, the intensity of Coomassie Blue dye representing TPI bands of the cooked meat extracts decreased as cooking temperatures increased when the same volume of meat extract was loaded on each lane (Figure 5.6, lanes 3-8). TPI concentration was similar in extracts of ground beef heated to 48.9 °C (120 °F) and 54.4 °C (130 °F) (lanes 3 and 4). TPI concentration decreased in extracts of ground beef heated to 60.0 °C (140 °F) (lane 5), whereas TPI was not observed at 71.1 and 76.7 °C (170 °F) (lanes 7 and 8).

A western blot was also used to examine TPI concentration using TPI polyclonal antibodies (Figure 5.7) from extracts of cooked ground beef. TPI concentration decreased as heating temperature was increased in the ground beef model system when the same volume of each meat extract was loaded in each lane, confirming the results from SDS-PAGE (Figure 5.6). Some non-specific recognition was observed between TPI polyclonal antibodies and raw extract proteins (lane 2). A higher amount of soluble protein was present in raw bovine extracts when compared to the same volume of heated extracts causing non-specific protein binding.

5.5 CONCLUSIONS

TPI could be a promising marker protein since the activity and concentration of TPI decreased as cooking temperature was increased in the ground beef model system.

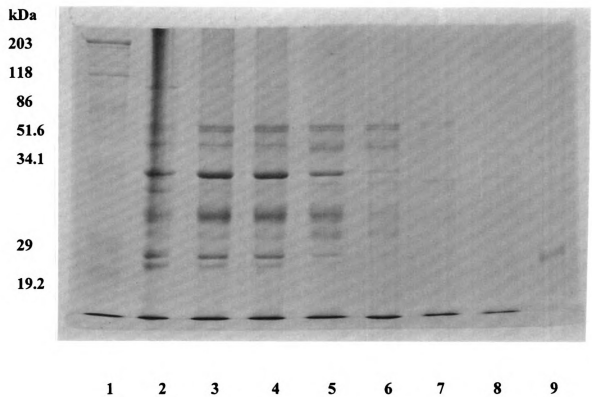


Figure 5.6 Representative sodium dodecyl sulfate-polyacrylamide gel electroetpgram of muscle extracts of bovine *semimembranosus* meat heated to different end-point temperatures. Proteins were stained with Coomassie Blue. (lane 1) molecular weight standards; (lane 2) unheated bovine muscle extracts; (lane 3) 48.9 °C; (lane 4) 54.4 °C; (lane 5) 60.0 °C; (lane 6) 65.6 °C; (lane 7) 71.1 °C; (lane 8) 76.7 °C; (lane 9) purified bovine TPI.

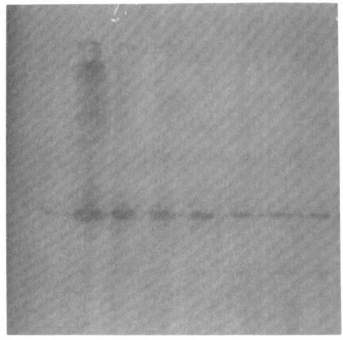


Figure 5.7 Western blot of bovine muscle extract with anti-triose phosphate isomerase polyclonal antibodies electrophoretically transferred from a sodium dodecyl sulfate-polyacrylamide gel to a nitrocellulose membrane. Bovine *semimembranosus* muscle was heated to different end-point temperature. (lane 1) bovine TPI; (lane 2) unheated bovine muscle extract; (lane 3) 48.9 °C; (lane 4) 54.4 °C; (lane 5) 60.0 °C; (lane 6) 65.6 °C; (lane 7) 71.1 °C; (lane 8) 76.7 °C.

Further study is needed to investigate the applicability of using TPI enzyme assay and sandwich ELISA to verify processing adequacy in beef roasts.

CHAPTER 6

VERIFICATION OF TRIOSE PHOSPHATE ISOMERASE ENZYME-LINKED IMMUNOSORBENT ASSAY TO DETERMINE PROCESSING ADEQUACY OF ROAST BEEF IN A PILOT STUDY

6.1 ABSTRACT

Triose phosphate isomerase was identified as a potential time-temperature integrator (TTI) to verify processing adequacy of roast beef in previous ground beef model and roast beef pilot studies. The objective of this study was to use a TPI sandwich ELISA and enzyme assay to verify processing adequacy of beef roasts processed in a commercial pilot plant. Adequate processing schedules were used to process roasts at low, medium and high temperatures [54.4 °C (130 °F)/121 min, 58.3 °C (137 °F)/24 min, and 62.2 °C (144 °F)/5 min] based on USDA schedules. Two inadequate processing schedules were used at each temperature by reducing the holding time by 0.5 and 1.0 log from the adequate processing schedules. Residual TPI was quantified in extracts from each roast using a sandwich ELISA and enzyme assay. TPI concentration and activity did not differ between adequately and inadequately processed roasts. It was difficult to consistently control processing conditions in the pilot plant due to large variations caused by smokehouse processing facilities, size and shape of roasts, types of muscles and locations

of roasts placed in the smokehouse. Future research is needed to determine if TPI can be used as a TTI under a commercial processing conditions.

KEYWORDS - ELISA, roast beef processing, TPI

6.2 INTRODUCTION

Adequate cooking is the easiest way to eliminate pathogenic bacteria and ensure the safety of meat products. Sixteen time-temperature processing schedules are allowed for cooked beef and rare roast beef (USDA-FSIS, 1995). In May 1996, the USDA-FSIS proposed several rules to regulate current federal meat and poultry inspection regulations in order to establish "safety margins" for thermally processed meat and poultry products (USDA-FSIS, 1996b). A thermal process sufficient to cause a 7-D reduction in *Salmonella* was proposed by the USDA-FSIS (1996b) as the lethality performance standard for roast beef, cooked beef and cooked corned beef products. *Salmonella* was selected as the indicator microorganism since it is more thermally resistant than E. coli O157:H7. The proposed regulation allows processors to use more sophisticated thermal treatments and more flexible time-temperature processing conditions.

A time-temperature indicator (TTI) is "a small measuring device that shows a time-temperature dependent, easily, accurately and precisely measurable irreversible change that mimics the changes of a target attribute undergoing the same variable temperature exposure" (Hendrickx et al., 1995). A TTI could be used by USDA and processors to verify processing adequacy of meat products.

Triose phosphate isomerase (TPI) was identified as a potential endogenous time-temperature integrator (TTI) in beef by Orta-Ramirez et al. (1997). TPI has a similar z value to that of *Salmonella* suggesting similar thermal inactivation kinetics (Orta-Ramirez et al., 1997). Hsu in a previous Chapter identified TPI as the best endogenous TTI to verify compliance to the USDA processing schedules in both ground beef and roast beef using medium (58.3 °C) to high (62.2 °C) temperature processes (chapter 4). In a ground beef water bath model system, TPI activity averaged 2.6 U/kg in adequately processed ground beef and increased to 4.9 and 13.3 U/kg when inadequately processed by reducing the holding time 0.5 log and1.0 log, respectively. In a MSU smokehouse pilot study, TPI activity in adequately processed roast beef 1.6 U/kg and increased to 3.7 and 7.8 U/kg when inadequately processed by reducing the holding time by 0.5 and 1.0 log at high temperature process (62.2 °C), respectively.

A sandwich ELISA was developed to quantify TPI in cooked beef (Hsu, Chapter 5). We found that both TPI activity and concentration in ground beef decreased ($P < 0.0001$) as temperature was increased, suggesting that both the TPI sandwich ELISA and enzyme assay were able to detect differences in residual TPI due to cooking temperature.

Two critical requirements must be met for TPI to function as a TTI: (1) TPI activity or concentration within equivalent adequately or inadequately time-temperature schedules should be similar, and (2) TPI activity or concentration should decrease as holding time is increased at the same temperature. Therefore, a pilot study was needed to further validate the applicability of the TPI sandwich ELISA for verifying processing adequacy of roast beef. Our objectives were to validate the applicability of TPI as an

endogenous TTI for verifying processing adequacy of roast beef. TPI activity and concentration of adequately and inadequately processed roast beef were measured by enzyme assay and ELISA to validate compliance to the USDA roast beef processing schedules.

6.3 MATERIALS AND METHODS

6.3.1 Processing Schedules

Choice top round roasts (*semimembranosus*, SM) donated by Bil-Mar Foods (Zeeland, MI) were trimmed of external fat and connective tissue. Each roast was evenly divided into two pieces (2.268 kg/each piece), cut into a rectangular shape (about 40 cm x 25 cm) and vacuumed packaged in Cryovac packaging bags (W.R. Grace Co., Duncan, SC, 29681). Roasts were stored at 4 °C for less than 4 hr before processing. The pilot study was conducted at Bil-Mar Foods (Zeeland, MI) using a H. Maurer and Sohne smokehouse (Model # ASL-3611, Ranch und Warmetechnik GmbH and Co. KG, D7752 Reichenau, Germany). Adequate processing schedules included low, medium and high temperature processes [130 °F (54.4 °C)/121 min, 137 °F (58.3 °C)/24 min, 144 °F (62.2 °C)/5 min] (Table 6.1). Inadequate processing schedules were designed using a 0.5 log and 1.0 log cycle reduction in holding time from that of the adequate processing schedules. The wet and dry bulb processing conditions in the smokehouse are listed in Tables 6.2, 6.3, and 6.4. Roasts were processed according to these schedules. After processing, roasts were chilled in an ice-slush bath and stored at 4 °C. Roasts were

Table 6.1 Processing times and temperatures used to prepare adequately and inadequately processed beef roasts

	High temperature process	Medium temperature process	Low temperature process
Under-cooked (1.0 log reduction in time)	59.4 °C (139 °F)/1.5 min	58.3 °C (137 °F)/2.4 min	54.4 °C (130 °F)/12 min
Under-cooked (0.5 log reduction in time)	61.1 °C (142 °F)/2.5 min	58.3 °C (137 °F)/8 min	54.4 °C (130 °F)/40 min
Adequately cooked [a]	62.2 °C (144 °F)/5 min	58.3 °C (137 °F)/24 min	54.4 °C (130 °F)/121 min

[a] Adequate cook schedules were based on the USDA roast beef processing schedules (USDA-FSIS, 1995).

Table 6.2 Smokehouse schedule used to prepare low temperature (54.4 °C; 130 °F) processed roast beef

Stage	Time (min)	Internal temperature	Dry bulb temperature (°C)	Wet bulb temperature (°C)
1	75 min	37.8 °C (100 °F)	132.0 °C (270 °F)	76.7 °C (170 °F)
2	40 min	48.9 °C (120 °F)	121.1 °C (250 °F)	65.6 °C (150 °F)
3	45 min	54.4 °C (130 °F)	115.6 °C (240 °F)	60.0 °C (140 °F)
4	60 min	54.4 °C (130 °F)	112.8 °C (235 °F)	57.2 °C (135 °F)
5	85 min [a]	54.4 °C (130 °F)	101.7 °C (215 °F)	54.4 °C (130 °F)

[a] Roasts were removed as they reached target times.

Table 6.3 Smokehouse schedule used to prepare medium temperature (58.3 °C; 137 °F) processed roast beef

Stage	Time (min)	Internal temperature	Dry bulb temperature (°C)	Wet bulb temperature (°C)
1	75 min	37.8 °C (100 °F)	132.0 °C (270 °F)	76.7 °C (170 °F)
2	60 min	48.9 °C (120 °F)	121.1 °C (245 °F)	65.6 °C (150 °F)
3	75 min [a]	54.4 °C (130 °F)	115.6 °C (240 °F)	60.0 °C (140 °F)

[a] Roasts were removed as they reached target times.

Table 6.4 Smokehouse schedule used to prepare high temperature (62.2 °C; 144 °F) processed roast beef

Stage	Time (min)	Internal temperature	Dry bulb temperature (°C)	Wet bulb temperature (°C)
1	60 min	37.8 °C (100 °F)	115.6 °C (240 °F)	82.2 °C (180 °F)
2	60 min	48.9 °C (120 °F)	107.2 °C (225 °F)	73.9 °C (165 °F)
3	45 min[a]	60.0 °C (140 °F)	104.4 °C (220 °F)	65.6 °C (150 °F)

[a] Roasts were removed as they reached target times.

transported at 4 °C to the the Department of Food Science and Human Nutrition, Michigan State University within 2 days of processing.

6.3.2 Extraction of TPI

A 2.54 cm slice was removed from the center portion of each roast using a meat slicer (Model 512, Hobart Mfg. Co. Troy, OH 45374). A 5 cm diameter meat core, centered around the thermocouple, was removed from this slice. Meat was minced into small pieces. Minced meat (50 g) was extracted in 3 volumes (150 ml) of 0.1 M NaCl, 0.05 Na phosphate buffer, pH 7.2, by blending twice (45 sec on, 1 min off) in a Waring Blender™. The meat homogenate was stirred on a magnetic plate for 30 min at 4 °C and centrifuged at 4,500 x g for 10 min at 4 °C to obtain the supernatant fraction containing TPI.

6.3.3 TPI Enzyme Activity and Concentration

Triose phosphate isomerase activity was assayed as described by Hsu (chapter 4). The initial velocity was read at 340 nm for 1 min at 25°C. Samples were diluted in PBS (pH 7.2) so that absorbance changed less than 0.2 units per minute (Norton et al., 1970). TPI in the extracts was quantified using a sandwich ELISA (Hsu, Chapter 5).

6.3.4 Statistical Analysis

Roasts were smokehouse processed using three different processes (low, medium and high temperature processes). Each process was conducted in triplicate for a total of 3

separate smokehouse processing runs. Data were analyzed using two way ANOVA (analysis of variance) (treatment x replication). The linear or curvilinear change in enzyme activity or concentration with temperature was determined using the polynomial contrast test by SAS software (SAS Institute Inc., Version 6.1, 1995, Cary, NC 27513).

6.4 RESULTS AND DISCUSSION

TPI activity (U/kg meat) was similar when roasts were adequately cooked at all temperatures (Table 6.5). TPI activity was similar at 54.4 and 62.2 °C when roasts were inadequately processed by reducing the holding time by 0.5 log, but different when roast beef was processed at medium temperature (58.3 °C). TPI activity was also similar when roasts were under processed by reducing the holding time by 1.0 log from the USDA schedules at all three temperatures. In general, no change in activity ($p < 0.05$) was found as processing time of roasts was increased at low, medium and high temperatures. These results suggested that changes in TPI activity could not be used to detect adequacy of thermal processing of roast beef.

TPI concentration (μg TPI/g meat) by ELISA was similar when roast beef was adequately cooked at medium (58.3 °C) and high (62.2 °C) temperature schedules (Table 6.6). TPI concentration was similar when roast beef was under processed at 0.5 log or 1.0 log reduction in holding time from the USDA schedules at medium and high temperatures. TPI concentrations did not change when the holding time was increased at medium (58.3 °C) and high (62.2 °C) temperature schedules. TPI concentration decreased ($p > 0.0121$) when the processing holding time was increased at the low processing

Table 6.5 Triose phosphate isomerase activity (U/kg meat) of roast beef processed using three USDA approved schedules for roast beef and inadequately processed by using the processing time by 0.5 and 1.0 log cycle in commercially processed roasts

	Low temperature [a] (54.4 °C; 130 °F)	Medium temperature [a] (58.3 °C; 137 °F)	High temperature [a] (62.2 °C; 144 °F)
Under-cooked (1.0 log reduction in time)	4.4 ± 2.77 [A]	7.3 ± 4.30 [A]	17.8 ± 9.81 [A]
Under-cooked (0.5 log reduction in time)	7.2 ± 3.61 [A]	23.5 ± 19.36 [B]	5.3 ± 1.74 [A]
Adequately cooked	3.4 ± 2.10 [A]	9.7 ± 5.26 [A]	9.1 ± 6.25 [A]

[a] Means (± standard deviation) in the same column showed a linear response to temperature; 54.4 °C ($p < 0.8856$), 58.3 °C ($p < 0.7151$), 62.2 °C ($p < 0.2042$).

[A B] Means in the same row followed by the same letter are not different ($p > 0.05$).

Table 6.6 Triose phosphate isomerase concentration (μg/g meat) of roast beef processed using three USDA approved schedules for roast beef and inadequately processed by decreasing the processing time by 0.5 and 1.0 log cycle in commercially processed roasts

	Low temperature [a] (54.4 °C; 130 °F)	Medium temperature [a] (58.3 °C; 137 °F)	High temperature [a] (62.2 °C; 144 °F)
Under-cooked (1.0 log reduction in time)	0.91 ± 0.177 [A]	0.33 ± 0.245 [B]	0.38 ± 0.308 [B]
Under-cooked (0.5 log reduction in time)	0.64 ± 0.424 [A]	0.13 ± 0.065 [B]	0.11 ± 0.038 [B]
Adequately cooked	0.41 ± 0.222 [A]	0.13 ± 0.025 [B]	0.11 ± 0.052 [A B]

[a] Means (± standard deviation) in the same column showed a linear response to temperature; 54.4 °C ($p < 0.0121$), 58.3 °C ($p < 0.2852$), 62.2 °C ($p < 0.1404$).

[A B] Means in the same row followed by the same letter are not different ($p > 0.05$).

temperature. Again, these results indicated that changes in TPI concentration could not be used to detect processing adequacy of commercially processed roast beef.

Thus, although, TPI was identified as a potential TTI to detect the thermal adequacy of roast beef processing in our previous pilot studies (Hsu, Chapter 4), TPI could not be used as a TTI to differentiate the thermal adequacy in roast beef prepared in a commercial pilot plant. Processing variables may have contributed to the lack of differences in TPI concentration and activity detected between adequately and inadeaquately processed roasts. For example, temperature variations within the smokehouse or differences in the size and shape of roasts may have caused uneven heating processes and differential heat transfer. Some factors were different when roasts processed in the smokehouse at the MSU pilot plant (Chapter 4) were compared to roasts processed in a commercial smokehouse at Bil-Mar Foods. For instance, processing conditions between the smokehouse were different. A 55.5 °C (100 °F) difference was found between the internal temperature (wet bulb), of the roasts and the smokehouse temperature (dry bulb) in the commercial pilot plant, whereas only a 2.8 °C (5 °F) difference was observed between the internal temperature (wet bulb) of roasts and the smokehouse temperature (dry bulb) in the MSU smokehouse. Roasts processed at MSU using a slower heating rate might undergo a more homogenous heat transfer process, allowing us to detect differences in TPI activity or concentration between adequately and inadequately processed roasts.

6.5 CONCLUSIONS

TPI concentration and activity could not be used to detect processing adequacy of beef roasts processed in a commercial processing plant using the USDA time-temperature processing schedules. Variations generated by smokehouse facilities, and product heating rate may have caused inconsistent results.

CHAPTER 7

CONCLUSIONS

Proper cooking is critical to eliminate foodborne pathogens from roast beef products. In this study, an endogenous TTI was identified and a rapid immunoassay was designed to detect this TTI for verifying that beef roasts received adequate thermal treatment. TPI was selected as an endogenous TTI based on its z value of 5.71 °C as determined in a previous study. This value was found to be similar to that of *Salmonella* (5.65 °C) which was used by the USDA to establish the roast beef process regulations.

A ground beef water bath model system and a pilot smokehouse study at MSU were conducted to further investigate the use of a TPI enzyme assay to determine the thermal processing adequacy of roast beef. TPI was identified as the best marker protein in beef cooked using medium and high temperature processes when compared to lactate dehydrogenase, peroxidase and acid phosphatase. In the ground beef water bath model system, TPI activity was lower in adequately processed ground beef compared to inadequately processed ground beef. TPI activity was similar within both medium and high temperature processes when adequately processed and TPI activity decreased as processing time was increased at these temperatures. In the pilot smokehouse study at MSU, TPI activity in adequately processed roast beef was lower compared to

inadequately processed roast beef. TPI activity remained constant within adequate cooking treatments and increased as cooking time was decreased.

This doctoral research was the first study to develop a sandwich ELISA to detect an endogenous TTI, triose phosphate isomerase (TPI), in bovine *semimembranosus* muscle to ensure compliance with the USDA roast beef processing schedules. In a time-independent ground beef system, samples were cooked from 48.9 to 76.7 °C at 5.5 °C increments and TPI activity and concentration decreased as temperature increased. This was confirmed on SDS-PAGE gels and Western blots.

TPI sandwich ELISA was not a suitable assay for verifying the thermal adequacy of commercially processed roast beef. TPI sandwich ELISA could not indicate the thermal adequacy of processed roast beef due to many factors, which may include the nature of assay, variations within the smokehouse facilities, and size and shape of roasts.

Therefore, I conclude that the TPI enzyme assay was able to determine processing adequacy for medium (58.3 °C) and high (62.2 °C) temperature USDA processing schedules in the ground beef water bath model system and in the roasts processed in the pilot plant at MSU. The TPI sandwich ELISA was not able to consistently assess thermal adequacy in the ground beef model system and at a commercial facility.

CHAPTER 8

FUTURE RESEARCH

This study was the first to use an endogenous protein as a time-temperature indicator (TTI) of the thermal adequacy of roast beef processing. Further investigations as described in the following sections should be considered.

The D and z values of TPI and acid phosphatase were determined using *semitendinosus* muscle in a previous study (Orta-Ramirez et al., 1997). D and z values should also be determined in *semimembranosus* muscle, the primarily muscle used in roast beef products to verify that the D and z values of enzymes and microorganisms in *semimembranosus* muscle are similar to those of *semitendinosus* muscle for the same temperature range.

Based on this study, TPI was identified as a TTI for medium (58.3 °C) and high (62.2 °C) temperature schedules used in roast beef processes. It would be beneficial to screen more endogenous enzymes to identify TTIs able to assess thermal adequacy of roast beef products for all USDA process schedules.

Additional research is needed to further investigate factors which may affect TPI activity and concentration in roast beef before TPI can be used as an intrinsic TTI on a practical scale. The effect of heating rate, size and shape of roasts, type of smoke house, location of roasts within the smoke house, and sampling techniques on the TPI

concentration of processed roast beef must be studied. The effect of different ingredients (e.g., salts and spices) and processing techniques (e.g., rubbing, tumbling, injection) on TPI activity and concentration in roast beef should be evaluated in both model and pilot studies.

The TPI sandwich ELISA used to assess the thermal adequacy of processed roast beef in this study utilized polyclonal antibodies and the sensitivity was low. Development of a monoclonal antibody to incorporate in a sandwich ELISA for TPI may yield a more sensitive and highly specific assay.

In addition to an intrinsic protein as a TTI, exogenous proteins or chemical markers may be useful to detect processing adequacy of roast beef. A project will be needed to identify and study how to utilize an extrinsic TTI to monitor the thermal adequacy in an actual roast beef production system.

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APPENDIX

PRELIMINARY EXPERIMENTS TO DETERMINE EXTRACTION CONDITIONS

Pure TPI Model System

Materials and Methods

Lyophilized TPI was diluted in double distilled water to achieve 0.1 mg TPI/mL MES buffer (since TPI was suspended in 30 mM MES buffer, pH 6.5, before lyophilized). The TPI solution was pipetted (0.6 mL) into 2 mL polypropylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA 15219). Tubes were heated in a Polystat circulator water bath (Model 1268-52, Cole-Parmer Instrument Co., Chicago, IL, 60714) at 82.2 °C (180 °F). Tubes were unheated or heated to 48.9 °C (120 °F), 60.0 °C (140 °F) and 76.7 °C (170 °F), vortexed, and centrifuged at 4,500 x g for 10 min. TPI activity and concentration in the supernatant was measured using enzyme assay and ELISA, respectively.

Results and Discussion

A pure TPI model system was used to understand how the denaturation and insolubilization of TPI were affected by heating and centrifugation. The effect of centrifugation on activity and concentration of TPI heated at 48.9 °C (120 °F), 60.0 °C (140 °F), and 76.7 °C (170 °F) were investigated in preliminary experiments (Table 1).

The effect of centrifugation on TPI activity and concentration in unheated and heated extracts was studied. TPI activity and concentration decreased after centrifugation

of unheated TPI extracts, suggesting that some native TPI was precipitated by centrifugation. When heated, TPI activities in centrifuged and non-centrifuged extracts were similar. Total protein in centrifuged and non-centrifuged extracts did not differ in unheated extracts. When heated, total protein decreased in centrifuged extracts as heating temperature increased. Without centrifugation, TPI activity and concentration decreased as heating temperature was increased except TPI activity at 60 °C. When centrifuged, TPI concentration or activity decreased as heating temperature was increased.

Table 1. Preliminary experiments evaluating the effect of heating and centrifugation on TPI activity and concentration in 30 mM MES buffer [a], pH 6.5

Extraction Method	TPI activity (U/L extract)	TPI concentration (μg/mL extract)	Total protein (g/L extract)
Not centrifuged			
Unheated	582.2 ± 567.55 [b] (3) [c]	60.6 ± 72.35 (3)	0.4 ± 0.03 (2)
48.9 °C	353.3 (1)	20.4 (1)	** [d]
60.0 °C	385.2 (1)	9.3 (1)	**
76.7 °C	29.4 (1)	4.7 (1)	**
Centrifuged			
Unheated	242.6 ± 105.22 (3)	12.2 ± 4.25 (3)	0.4 (1)
48.9 °C	391.7 ± 100.99 (3)	45.0 ± 63.12 (3)	0.4 ± 0.001 (2)
60.0 °C	345.6 ± 38.03 (3)	17.2 ± 22.20 (3)	0.4 ± 0.01 (2)
76.7 °C	87.4 ± 122.85 (3)	7.6 ± 9.26 (3)	0.3 ± 0.01 (2)

[a] 30 mM MES buffer: pH 6.5, including 30 mM (2-[N-Morpholino]ethanesulfonic acid), 10 mM KOH, 0.5 mM magnesium acetate, 0.1 mM EDTA.

[b] Results were expressed as mean ± standard deviation.

[c] value in parentheses indicates number of replicates.

[d] **: data not available.

Selection of Extraction Buffers

Phosphates may inhibit TPI activity (Beisenherz, 1995), therefore, a preliminary study was used to find a suitable buffer system for extracting TPI from cooked beef. Bovine TPI was extracted from ground beef with a series of phosphate buffers (PBS) of different molarities (.01, .05, 0.1 M) containing 0.1 M NaCl, pH 7.2, or a MOPS buffer (0.1 M NaCl, .01 M MOPS, pH 7.2). In general, no difference in TPI activity was found when different molarities of PBS, ranging from .01 to .1 M, were used to extract TPI from ground beef (Table 2). TPI activities were higher when meat was extracted with phosphate buffers of different molarities than with MOPS buffer.

Water and PBS (0.15 M NaCl, 0.01 M Na phosphate, pH 7.2) were used as diluents to examine their effect on TPI activities in meat extracts. When diluted in water, after extraction, but prior to determination of enzyme activity, TPI activities were about 50% lower in raw meat extracted with MOPS than with phosphate buffer; TPI activities were similar in phosphate buffer systems. When diluted in PBS, TPI activities were about 70% lower in raw meat extracted with MOPS than with phosphate buffers. Results suggested that phosphate ions increased the extractability of TPI from ground beef. Phosphates are known to cause swelling and destruction of myofibrillar fibers by enhancing electrostatic repulsion between myofilaments (Offer and Trinick[1], 1983); therefore, phosphates facilitate the release of sacroplasmic enzymes from the muscle fibers and enhanced the extractability of TPI. In PBS and MOPS extraction systems, TPI activities were similar or slightly higher in meat extracts diluted in water compared to dilution in PBS.

Table 2. Effect of extraction buffers and diluents on TPI activity (U/kg meat) extracted from *semimembranosus* muscle at pH 7.2

	0.1 NaCl 0.01 Na phosphate	0.1 M NaCl 0.05 M Na phosphate	0.1 M NaCl 0.1 M Na phosphate	0.1 M NaCl 0.01 M MOPS
diluted in water	3425.12	3680.79	3741.76	1809.48
diluted in PBS [a]	3445.24	3536.31	3342.47	1037.56

[a] 0.15 M NaCl, 0.01 M Na phosphate (pH 7.2) buffer

[b] n = 1

Methods of Meat Extract Preparation

The purpose of this study was to establish a suitable method to extract TPI from cooked ground beef. Three sample preparation methods were examined: (1) supernatant collected after centrifugation of meat extracts (treatment A), (2) filtrate collected after filtering the meat extract through # 4 Whatman filter paper (treatment B), and (3) filtrate collected after filtering the meat extract through 0.22 μm filter (19952, Millipore Products Division, Bedford, MA 01730) (treatment C).

The filtrate was collected and held at 4 °C until used. TPI activity and concentration of extracts were determined within 24 hr. The filtrate was diluted in either water or 0.15 M NaCl, 0.01 M Na phosphate (pH 7.2) buffer prior to the enzyme assay. TPI activity and concentration were compared to establish a more consistent and reliable procedure for extracting TPI from a cooked ground beef model system.

In treatments A, B, and C, TPI activities of ground beef decreased ($P < 0.0001$) as temperature was increased from 48.9 °C (120 °F) to 76.7 °C (170 °F) (Table 3). However, in treatment B, TPI activity was lower in the 60 °C extract than in the 65.6 °C extract. The coefficient of variations of treatments A, B, and C were also used to compare method variability. In general, the coefficient of variation of TPI activity was smaller (less than 15%) in treatment A when compared to treatments B and C, suggesting that the centrifugation method (treatment A) of meat extract preparation provided more consistent results.

In treatments A, B, and C, TPI concentrations by ELISA decreased in ground meat as temperature was increased from 48.9 °C (120 °F) to 76.7 °C (170 °F) (Table 4).

Table 3. Effect of cooking temperature on triose phosphate isomerase activity (U/kg meat) of bovine meat cooked in a water bath and extracted using centrifugation (treatment A), centrifuging and filtering (treatment B), and centrifuging and filtering through 0.22μm filter (treatment C) [a]

Internal temperature (°C)	Treatment A	B	C
48.9 ± 0.1	537.5 ± 14.55 [a] (2.71)	409.6 ± 13.14 (3.21)	549.5 ± 20.58 (3.75)
54.4 ± 0.1	187.0 ± 21.88 (11.70)	109.6 ± 5.37 (4.90)	179.1 ± 6.11 (3.41)
60.0 ± 0.1	72.1 ± 9.03 (12.53)	38.2 ± 10.65 (27.91)	47.0 ± 11.01 (23.45)
65.6 ± 0.1	38.0 ± 3.08 (8.12)	66.0 ± 23.63 (35.78)	21.3 ± 2.14 (10.05)
71.1 ± 0.1	27.4 ± 3.98 (14.54)	31.9 ± 2.94 (9.22)	15.4 ± 3.09 (20.12)
76.7 ± 0.1	19.4 ± 1.01 (5.21)	28.1 ± 1.06 (3.77)	12.3 ± 0.64 (5.22)

[a] Values are expressed as mean ± standard deviation of triplicate determination and values in parentheses represent coefficient of variation. Means in the same column decreased linearly as temperature increased ($P < 0.0001$).

Table 4. Effect of cooking temperature on triose phosphate isomerase content of bovine meat cooked in a water bath and extracted using centrifugation (treatment A), centrifuging and filtering (treatment B), and centrifuging and filtering through 0.22μm filter (treatment C)

Internal temperature (°C)	Treatment A	B	C
48.9 ± 0.1	86.3 ± 11.55 [a] (13.38)	68.9 ± 27.17 (39.45)	59.2 ± 30.41 (51.40)
54.4 ± 0.1	66.9 ± 6.64 (9.93)	44.8 ± 10.80 (24.12)	32.8 ± 7.07 (21.59)
60.0 ± 0.1	48.3 ± 6.67 (13.82)	26.7 ± 15.33 (57.50)	26.6 ± 9.56 (35.94)
65.6 ± 0.1	42.6 ± 8.75 (20.56)	23.6 ± 11.56 (48.98)	19.7 ± 8.31 (42.20)
71.1 ± 0.1	36.8 ± 7.17 (19.46)	28.7 ± 17.10 (59.60)	24.9 ± 10.13 (40.75)
76.7 ± 0.1	34.1 ± 6.29 (18.46)	20.7 ± 9.82 (47.39)	29.4 ± 19.53 (66.41)

[a] Values are expressed as mean ± standard deviation of triplicate and values in parentheses represent coefficient of variance. Means in the same column decreased linearly as temperature increased, Treatment A ($P < 0.0001$), Treatment B ($P < 0.0031$) and Treatment C ($P < 0.0432$).

However, in treatment B, TPI concentration was higher in meat cooked at 71.1 °C than at 60 and 65.6 °C. In treatment C, TPI concentration was lower in 65.6 °C than at 71.1 °C; TPI concentration was greater in meat cooked to 76.7 °C than at 71.1 °C. Greater coefficients of variation were also found in treatments B and C, ranging from 21 to 66%, compared to treatment A (the coefficient of variations were all less than 20%). The centrifugation method was the best method to prepare meat extracts as it produced the most consistent experimental results when TPI was measured by enzyme assay and ELISA.

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[1] Offer, G. and Trinick, J. 1983. On the mechanism of water holding in meat: the swelling and shrinking of myofibrils. Meat Sci. 8:245-281.

PROXIMATE COMPOSITION OF BIL-MAR ROAST BEEF

Beef roasts (*semimembranosus*) from Bil-Mar was analyzed for protein, fat and moisture contents using AOAC[2] (1990) standard methods 981.10, 960.39 and 950.46B, respectively. The pH was determined by homogenizing 10 g ground beef with 90 mL of double distilled water using a Waring™ blender for 30 s.

Protein, fat and moisture contents were 27.05 ± 0.95 %, 1.19 ± 0.11 % and 74.19 ± 4.05 %, respectively. The pH was 5.8.

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[2] AOAC. 1990. *Official Methods of Analysis*, 15th ed. Association of Official Analytical Chemists, Washington, DC.