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Kinetics of Tropomyosin Denaturation as a Predictive Model for Verifying Thermal Processing of Beef Products

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed to study thermal denaturation of tropomyosin (Tm) using the time-temperature requirements for cooked beef. The ELISA employed a monoclonal antibody (MAb 2C9) raised against bovine Tm for quantifying residual Tm in muscle extracts. The specificity of MAb 2C9 to bovine Tm was demonstrated by Western blot and the analytical validity of ELISA was confirmed by dot blot. Thermal denaturation of Tm, in the temperature range between 54.4 and 70.0°C, showed first-order dependency. Kinetic parameters of Tm denaturation were derived from isothermal heating of beef muscle extract at 54.4, 57.2, 60.0, and 62.8°C. Temperature dependency of the rate constant (k) was demonstrated by Arrhenius plot; the activation energy (E_a) of Tm denaturation was determined to be 484 kJ·mol^{-1}. A mathematic model describing the impact of the heating time-temperature on Tm denaturation was developed. Predicted Tm from the integrated time-temperature model agreed closely with the measured Tm in dynamically heat-processed beef samples. Percent errors between the measured and the predicted values ranged from −5.1 to 5.3%. The kinetic model provides an accurate and reproducible prediction of the impact of actual heating time-temperature on residual Tm in cooked beef. The MAb-based ELISA and kinetic model developed in this study have the potential to be adapted by the meat industry as a quality control tool.

An adequate heat process is critical for ensuring destruction of potential foodborne pathogens in precooked meat products. The U.S. Department of Agriculture, Food Safety Inspection Service (USDA-FSIS), has established time-temperature guidelines for various precooked meat products *(20, 21).* For instance, cooked beef products are required to be thermally processed to one of the prescribed time-temperature combinations, ranging from 54.4°C for 121 min to 70.0°C without holding time. However, post-processing verification of whether a precooked beef product had met the time-temperature requirement is often technically challenging *(2, 15, 16).*

For regulatory and quality control purposes, analytical methods are required to assess the sufficiency of heat treatment of precooked meat products. Principally, these methods are based on the detection of inactivation of enzyme activity or change of muscle protein solubility *(6, 12, 13, 16, 18, 23, 24)*; applications of these methods have been limited due to intrinsic variations and storage changes of enzymes and muscle proteins in the meat products. Myofibrillar proteins, the structure proteins of muscle tissues, are more consistent than enzymes and sarcoplasmic proteins *(4)*; therefore, we proposed to use a myofibrillar protein, such as tropomyosin (Tm), as an indigenous indicator of adequate heat processing.

Kinetic approaches have been demonstrated to be effective and reliable in monitoring changes of quality or safety attributes of thermal processed foods *(5, 8–11).* The impact of heat processing can be quantified by applying the concept of time-temperature integrator (e.g., enzyme activity) which exhibits a time-temperature dependency and irreversible changes that can be correlated to the changes of a target attribute. Studies have been conducted to evaluate the possibility of using phycoerythrin as a time-temperature integrator for ascertaining the degree of thermal inactivation of food pathogens in meat products *(19).* The researcher suggested that phycoerythrin has the potential to be used as an extrinsic marker to verify processing adequacy *(22).* However, one of the drawbacks associated with the application of an extrinsic component in a food system is that it needs to be incorporated into the raw materials before processing. It would be advantageous and convenient if an indigenous component of muscle tissue could be identified and utilized as an intrinsic indicator for this purpose.

We have evaluated the feasibility of using Tm as an intrinsic indicator for verifying adequacy of thermal process. By establishing an integrated kinetic model, the time-temperature impact on Tm in a cooked beef sample can be reliably quantified and applied to verifying the adequacy of a thermal process. The objectives of this study were to (i) develop an enzyme-linked immunosorbent assay (ELISA) protocol using monoclonal antibodies (MAbs) for quantification of Tm in cooked beef, (ii) study Tm denaturation kinetics using the developed ELISA protocol, and (iii) assess the reliability of the kinetics for prediction of Tm in cooked beef samples using an integrated time-temperature model.

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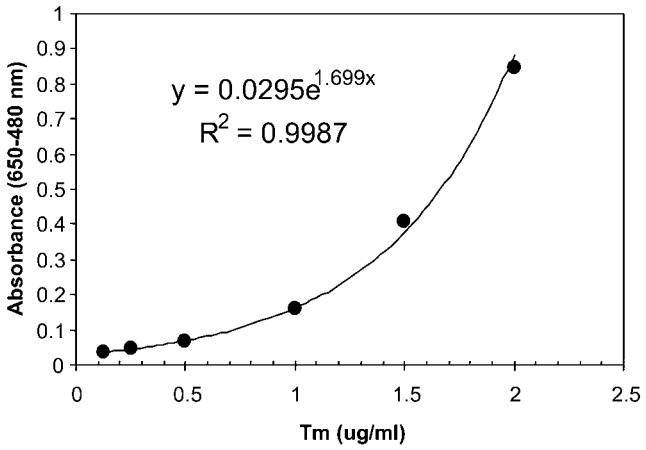

FIGURE 1. *A representative ELISA calibration curve for Tm; the detection range of the assay was between 0.125 and 2.0 μg/ml.*

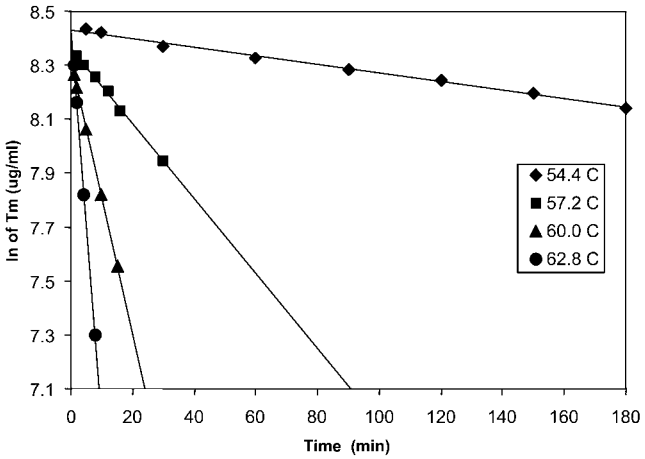

FIGURE 3. *Effects of temperature and time on residual Tm in muscle extracts under isothermal conditions (54.4, 57.2, 60.0, and 62.8°C).*

MATERIALS AND METHODS

MAb production. Tm from bovine muscle was prepared according to a standard protocol *(7).* Purified Tm (1.0 mg/ml) in phosphate-buffered saline (PBS, pH 7.2), containing 0.1% of sodium dodecyl sulfate (SDS) was used for immunization. Four BALB/c mice (7 to 10 weeks old) were injected either subcutaneously or intraperitoneally with 150 mg of Tm mixed 1:1 (vol/vol) with Freund's complete adjuvant followed by two booster injections at 4-week intervals with 100 mg per mouse of Tm mixed 1:1 with Freund's incomplete adjuvant. Test sera were collected by tail bleeding 10 days after each injection; the titer of the sera was then determined by indirect ELISA as previously described *(3).*

The mouse exhibiting the highest serum titer to bovine Tm then received a final boost of 100 mg of the antigen in PBS, 4 days before fusion. Spleen cells from the selected mice were fused with the myeloma cell line (P3363.Ag8.653, ATCC CRL1580) at a ratio of 5:1 in the presence of polyethylene glycol (molecular weight, 4,000). The cells were subsequently diluted to an appropriate density and cultured in hypoxanthine-aminopterinthymidine medium. Screening of hybridomas was performed by indirect ELISA *(3).* Cell lines with distinct reaction patterns to Tm were cloned at least twice by a limiting dilution method and subsequently propagated in culture media. A total of six hybridoma cell lines were selected during the course of screening. MAbs from each of the cell lines were produced in culture supernatants and pools of supernatant (500 ml) from each of the MAbs were collected.

ELISA development. An ELISA protocol employing the MAb was developed to quantify Tm in muscle extracts. Performance of each MAb was compared in the ELISA format; MAb 2C9 was selected to be used in the final protocol because of its superior specificity and affinity. ELISA procedures were optimized to achieve a high sensitivity and reproducibility. The finalized procedures are described below. Costar polystyrene 96-well EIA plates (Corning, Inc., Corning, N.Y.) were incubated with muscle extracts diluted 1:4,000 in carbonate buffer (pH 9.6) at 37°C for 1 h and remaining binding sites on the plates were blocked by adding 1% bovine serum albumin (BSA) in PBS. Supernatant of MAb 2C9 diluted 1:4 in antibody buffer (1% BSA in PBS with 0.05% Tween 20) was added to the wells and incubated at 37°C for 1 h, followed by addition of a goat anti-mouse immunoglobulin M peroxidase conjugate (Sigma, St. Louis, Mo.) 1:3,000 diluted in antibody buffer. The plates were washed three times with PBS containing 0.05% Tween 20 between steps using an automated microplate washer (Wellwash AC, Thermo Electron, Waltham, Mass.). The bound enzyme activity was determined by

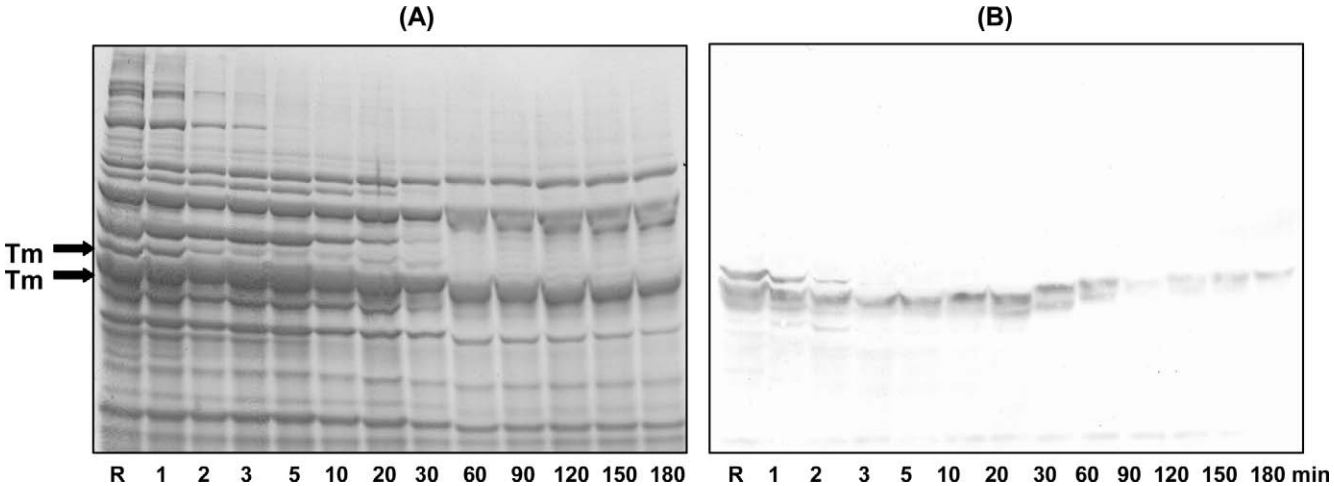

FIGURE 2. *SDS-PAGE of muscle extracts heated at 57.2°C from 1 to 180 min (A); Western blot of the same gel with Tm detected using MAb 2C9 (B). R indicates raw muscle extract.*

TABLE 1. *Kinetic parameters of Tm denaturation at different temperatures estimated from regression analysis*

Temp (°C)	k^a	$\ln[Tm_0]^b$	$R^{2\,c}$
54.4	0.0016	8.432	0.9935
57.2	0.0139	8.362	0.9979
60.0	0.0507	8.377	0.9998
62.8	0.1436	8.435	0.9962

[a] First-order rate constant (s^{-1}).
[b] ln of [Tm] at $t = 0$.
[c] Coefficient of determination.

addition of 3,3′,5,5′-tetramethyl-benzidin (TMB) liquid substrate (Sigma). Color developments were measured after 30 min incubation at room temperature by a Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, Calif.). Calibration standards of Tm (0.1 to 2 μg/ml) were analyzed concurrently on each of the plates.

Gel electrophoresis and immunoblotting. Proteins in the beef extracts were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) using a Criterion precast gel system (Bio-Rad). Gels were stained with Coomassie brilliant blue G-250. For Western blot, proteins on the gels were transferred (100 V; 30 min) to nitrocellulose membrane using a Criterion blotter with a transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). For dot blot, proteins in the beef extracts were bound to nitrocellulose membrane by using a Bio-Dot microfiltration apparatus (Bio-Rad). Colorimetric detection of Tm bound to the membrane was performed using the MAb 2C9 and an amplified Opti-4CN detection kit (Bio-Rad), following manufacturer's instruction. Color density of the immunoblot was analyzed by a Kodak gel logic 100 imaging system with 1D image analysis software (Eastman Kodak, Rochester, N.Y.).

Kinetic studies of Tm denaturation. Rates of Tm denaturation under isothermal conditions were studied using muscle extracts. Semitendinosus muscles were trimmed off excess fat and connective tissue and ground twice through a hand grinder. Ground beef muscle was then mixed with 2 volumes (wt/vol) of ice-cold PBS and homogenized using a PowerGene 125 homogenizer (Fisher, Pittsburgh, Pa.). The homogenate was held at 4°C for 1 h and then centrifuged at 15,000 × *g* for 30 min. The supernatants of the muscle extract (100 μl) were dispensed into Eppendorf thin-wall PCR tubes which were then submerged into a circulating water bath preheated to desired target temperatures (54.4, 57.2, 60.0, and 62.8°C). When a required holding time was obtained at the target temperature, the tubes were retrieved from heat and immediately chilled in ice water. After centrifugation to remove coagulum, the supernatants of muscle extract were analyzed for the presence of residual Tm by ELISA.

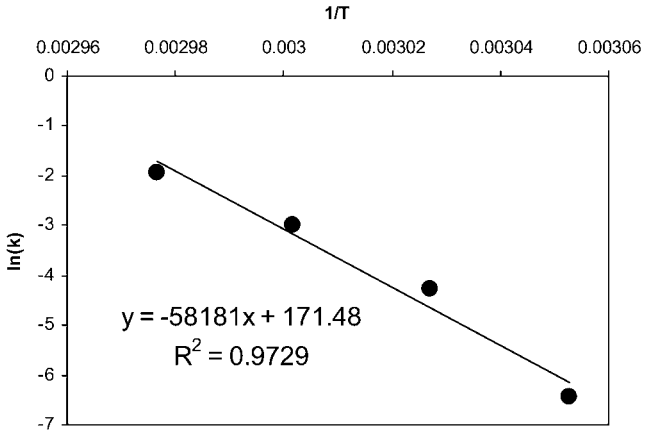

FIGURE 4. *Arrhenius plot depicting temperature dependency of rate constant;* T *is the absolute temperature in Kelvin (°K);* k *is the first-order rate constant (s^{-1}).*

Kinetic model for Tm denaturation. Tm denaturation data were modeled by first-order reaction kinetics that can be represented as:

$$-d[\mathrm{Tm}]/dt = k[\mathrm{Tm}]$$

After integration, the equation can be written as:

$$\ln([\mathrm{Tm}]/[\mathrm{Tm}_0]) = -k{\cdot}t$$

where [Tm] is the residual Tm concentration at time *t,* $[Tm_0]$ is the Tm concentration at $t = 0$, and *k* is the first-order rate constant. Kinetic parameters of Tm denaturation at 54.4, 57.2, 60.0, and 62.8°C were derived from the isothermal data. Regression analyses were performed using SPSS 12.0 for Windows. The activation energy (E_a) of Tm denaturation was derived from the Arrhenius equation $k = Ae^{(-E_a/RT)}$, where *k* is the first-order rate constant, *A* is the frequency factor, *R* is the ideal gas constant (8.314 J/mol °K), and *T* is absolute temperature in Kelvin (°K).

The prediction of [Tm] in muscle extract was achieved by the integrated equation:

$$\ln([\mathrm{Tm}]/[\mathrm{Tm}_0]) = -\int k\,dt$$

By substituting *k* from the Arrhenius equation, the equation can be written as:

TABLE 2. *Measured and predicted residual Tm after heating using constant temperature (54.4°C) with various holding times*

	Holding time (min)					
	30	60	90	120	150	180
Predicted Tm (μg/ml)[a]	4,301	4,032	3,779	3,542	3,320	3,112
Measured Tm (μg/ml)[b]	4,417	3,863	3,760	3,478	3,442	3,162
(Standard deviation)[c]	(104)	(84)	(129)	(64)	(71)	(173)
Error (%)[d]	2.7	−4.2	−0.5	−1.8	3.7	1.6

[a] Values were calculated from the integrated kinetic model.
[b] Values were determined by ELISA.
[c] $n = 6$; 2 replicated samples and 3 measures for each sample.
[d] Percent error from predicted value.

TABLE 3. *Measured and predicted residual Tm after heating using sets of USDA time-temperature combinations for cooked beef*

	Temp (°C):							
	54.4	57.2	60.0	62.8	65.6	67.2	68.3	70.0
Holding time (min)	112	36	12	4	1.12	0.57	0.37	0
Predicted Tm (μg/ml)[a]	3,604	3,231	2,743	2,195	2,027	1,706	1,496	1,251
Measured Tm (μg/ml)[b]	3,582	3,137	2,631	2,300	1,956	1,769	1,575	
(Standard deviation)[c]	(134)	(129)	(109)	(158)	(98)	(107)	(156)	ND[e]
Error (%)[d]	−0.6	−2.9	−4.1	4.8	−3.5	3.7	5.3	ND

[a] Values were calculated from the integrated kinetic model.
[b] Values were determined by ELISA.
[c] $n = 6$; 2 replicated samples and 3 measures for each sample.
[d] Percent error from predicted value.
[e] Not determined.

$$\ln([\mathrm{Tm}]/[\mathrm{Tm}_0]) = -\int_0^t Ae^{-E_a/RT(t)}\,dt$$

where $T(t)$ is the temperature profile as a function of time. [Tm] of a sample can be numerically integrated by using the Simpson rule *(1).* Integration of time-temperature data from temperature logger and calculation of [Tm] from a given sample were performed using Microsoft Excel program.

Simulation of dynamically heated beef samples. Beef samples with detailed histories of heating time-temperature were simulated in our laboratories. Ground semitendinosus muscle (3 g) was place into the bottom of glass tubes (12 by 75 mm). Temperature probes (EPT-010) connected to the Spectrum 1000 precision temperature datalogger (Veriteq, Richmond, British Columbia, Canada) were inserted into the geometric center of the samples to record time-temperature profiles during heating. Beef samples were first equilibrated in a water bath at 30°C for 5 min; the temperature was gradually increased to 54.4 or 60.0°C with various heating rates ranging from 0.4 to 1.6°C/min, and different holding times (0, 12, 60, 120, and 180 min) at the target temperatures. When the prescribed treatments were attained, samples were immediately withdrawn from heat and chilled in ice water. Temperature data were downloaded to a PC with the Spectrum data logger software and then exported to Microsoft Excel for numerical integration analysis. Residual Tm in each cooked beef sample was extracted and measured by ELISA as described in previous sections. Numbers of replicated samples and ELISA measurements are indicated in the respective tables where data are presented.

RESULTS AND DISCUSSION

Performance of ELISA. An ELISA protocol was developed using MAb 2C9 specific to Tm as the detection agent. Quantitative analysis of residual Tm in muscle extract was achieved by applying an exponential calibration curve using purified Tm (Fig. 1). The limit of detection for this assay protocol was 0.12 μg/ml. The high sensitivity of the assay allowed the application of a high dilution (1:4,000) of the muscle extract for ELISA analysis, which minimized the matrix effects due to the presence of other muscle proteins in the sample. Repeatability and reproducibility of the ELISA were determined from repetitive measurements of the control samples. The intra- and interassay coefficients of variation for ELISA measurements were 5.7 and 6.9%, respectively. The analysis time for ELISA was about 4.5 h which could be shortened to 2 to 3 h with further optimization of the protocol to reduce the incubation time. Analytical validity of ELISA was further confirmed by dot blot (data not shown). The specificity of MAb 2C9 to Tm was demonstrated by Western blot (Fig. 2), which showed two isoforms of Tm being detected by MAb 2C9. As evidenced from the fading of Tm bands on SDS-PAGE

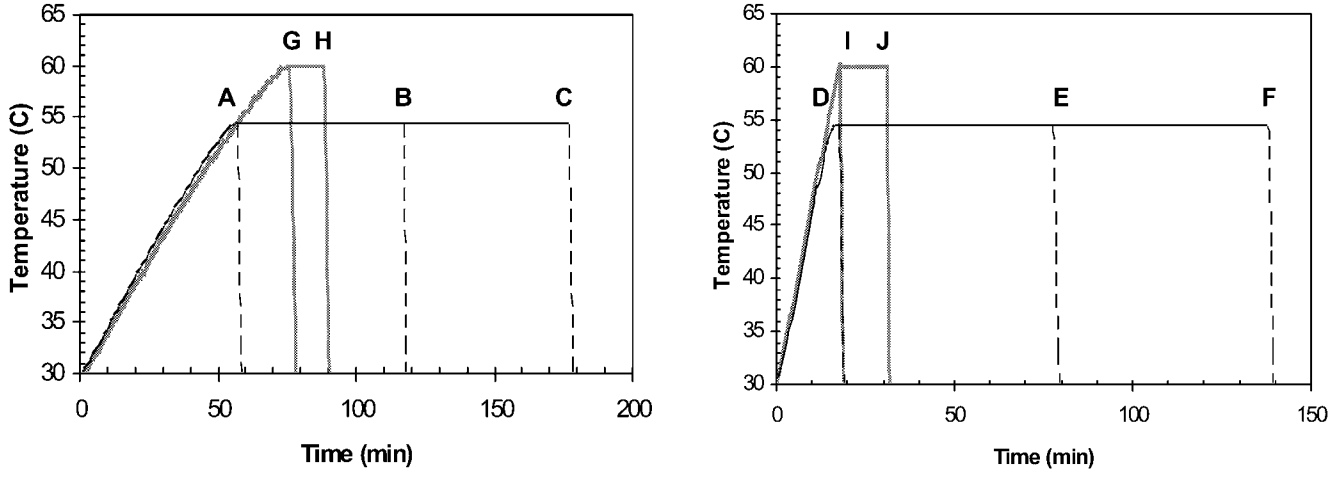

FIGURE 5. *Time-temperature profiles of dynamically heated beef samples; alphabetical labels represent the sample IDs as in Table 4.*

and Western blot, Tm was rapidly denatured as the heating time increased and consequently formed insoluble aggregates that were precipitated and removed from muscle extract by centrifugation.

Denaturation kinetics of Tm. Kinetic data were derived from studies of Tm denaturation under isothermal conditions. Residual Tm in the muscle extract after heating closely followed first-order kinetics (Fig. 3). Kinetic parameters of Tm denaturation were estimated from regression analysis and are presented in Table 1. Temperature dependency of the rate constant was demonstrated by the Arrhenius plot (Fig. 4); the E_a of Tm denaturation calculated from the Arrhenius equation was 484 kJ·mol^{-1}, which is comparable to the activation energy found for denaturation of other proteins, such as ovalbumin *(25)*, γ-lactoglobulin *(14),* actin, carboxypeptidase, and creatine kinase *(17).*

An integrated model as described in ''Materials and Methods'' was developed to predict residual Tm in the muscle extract after heating. The reliability of the model was evaluated using muscle extracts heated under various isothermal conditions. The predicted and measured values of residual Tm in muscle extracts heated at 54.4°C from 30 to 180 min are compared in Table 2. Percent errors between the values of residual Tm measured by the ELISA and the values predicted by the kinetic model were between −4.2 and 3.7%. The kinetic model was further evaluated using muscle extracts heated to the USDA time-temperature requirements for cooked beef (Table 3). As expected, the results showed an excellent consistency between the measured values and the predicted values, with minor variations between −4.1 and 5.3%. Simulation of the heating condition at 70.0°C using the current experimental protocol is not achievable when the required holding time is approaching 0 s; however, a predicted value is provided in Table 3 for comparison with other heating conditions.

Prediction of Tm in cooked beef. The kinetic approach and time-temperature integrated model have been demonstrated to be a feasible means for assessing thermal processing of milk *(5)* and canned ravioli *(9).* We have demonstrated in the current study that the kinetic model is able to accurately predict the impact of heat processing on Tm denaturation. It is our intent to apply the kinetic model and ELISA as an analytical tool for evaluation of beef products processed under typical manufacture conditions that consist of rising, holding, and declining of the temperature as reflected in various manufacturing schedules. To demonstrate the feasibility of this application, cooked beef samples subjected to ramping, dwelling, and cooling periods were simulated in our laboratories as described in ''Materials and Methods.'' Recorded time-temperature profiles for these cooked beef samples are presented in Figure 5. Predicted values of Tm were obtained by integration of the time-temperature data using the kinetic model. The results showed that the model closely predicted residual Tm in cooked beef samples, as measured by ELISA, with percent errors between −5.1 and 5.3 (Table 4).

As shown in Table 4, the effects of prolonged temperature ramping (sample A with a heating rate of 0.436°C/

TABLE 4. *Measured and predicted residual Tm after heating using two target temperatures with varied heating rates and holding times*

	Sample ID:										
	A	B	C	D	E	F	G	H	I	J	
Temperature (°C)	54.4	54.4	54.4	54.4	54.4	54.4	60	60	60	60	
Heating rate (°C/min)	0.436	0.436	0.436	1.406	1.406	1.406	0.400	0.400	1.656	1.656	
Holding time (min)	0	60	120	0	60	120	0	12	0	12	
Predicted Tm (μg/ml)[a]	4,526	3,977	3,494	4,566	4,012	3,525	3,222	1,930	4,291	2,571	
Measured Tm (μg/ml)[b]	4,295	4,140	3,424	4,598	3,960	3,659	3,135	2,032	4,244	2,658	
(Standard deviation)[c]	(223)	(267)	(94)	(193)	(150)	(211)	(113)	(194)	(224)	(155)	
Error (%)[d]	−5.1	4.2	−2.0	0.7	−1.3	3.8	−2.7	5.3	−1.1	3.4	

[a] Values were calculated from the integrated kinetic model.
[b] Values were determined by ELISA.
[c] n = 18; 3 replicated samples and 6 measures for each sample.
[d] Percent error from predicted value.

min and sample G with a heating rate of 0.40°C/min) resulted in a lower residual Tm, compared to fast ramping (sample D with a heating rate of 1.406 and sample I with a heating rate of 1.656°C/min). The effects of prolonged dwelling time were evident when comparing the residual Tm among samples A, B, and C, or among samples D, E, and F, which had the same final temperature (54.4°C), but different dwelling times (0, 60, and 120 min, respectively). Similarly, the effects of dwelling time were observed when comparing residual Tm between samples G and H, or between samples I and J, which had the same final temperature (60.0°C), but different dwelling times (0 and 12 min, respectively).

Our results indicated that the effects of the ramping and cooling period as well as temperature variation during the dwelling period need to be considered when using the kinetic model to evaluate cooked beef. In a manufacturing setting, the same types of products are continuously produced using similar processing schedules; therefore, the impact of heat on Tm in a particular type of beef product is expected to be identical. Ideally, a preset cutoff value derived from the kinetic model could be used to evaluate a particular type of beef products with similar processing conditions, such as the same products from the same processing facility. For example, a cutoff value of 1,496 μg/ml may be applied to assess cooked beef patties produced by a manufacturing facility that uses a target temperature of 68.3°C (Table 3).

Analytical tools for determining if the required time-temperature conditions had been achieved during the processing would provide further verification of the safety of precooked beef products. The kinetic model described in this article is able to accurately predict the impact of heating time-temperature on Tm denaturation in simulated cooked beef samples. The kinetic model and the MAb-based ELISA developed in this study have the potential to be adapted by the meat industry as an analytical tool for optimizing processing conditions and for postprocessing verification of various precooked beef products. Further studies are needed to evaluate the feasibility of applying ELISA and the kinetic model in a quality control–quality assurance program and the reliability of using preset cutoff values for different types of cooked beef products in manufacturing settings.

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