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Enhanced efficiency of a capillary-based biosensor over an optical fiber biosensor for detecting calpastatin

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Abstract

A capillary-based optical biosensor has been developed to detect calpastatin, an indicator of meat tenderness. *Longissimus* muscle samples ($n = 11$) were extracted from beef carcasses at 0 and 48 h post-mortem. These samples were assayed for calpastatin by traditional laboratory methods and with a newly developed capillary tube biosensor as well as for Warner–Bratzler shear force (WBSF) and crude protein and the responses were compared. Additionally, the response from the capillary-based biosensor was compared to a previously developed optical fiber biosensor. When the 0 and 48 h sampling periods were combined, the capillary tube biosensor was moderately accurate in predicting calpastatin activity ($R^2 = 0.6058$). There was less variation in the 0 h capillary tube biosensor compared to the 0 h pre-column ($P = 0.006$) and post-column optical fiber biosensors ($P = 0.047$), therefore the capillary tube biosensor is a more precise system of measurement. This research further advances the development of a calpastatin biosensor and makes online assessment one step closer to reality.

Keywords: Biosensor; Calpastatin; Fluorescence; Beef; Tenderness

1. Introduction

Beef tenderness consistently ranks as one of the most important meat quality attributes by consumers (Neely et al., 1998) and studies have shown that consumers are willing to pay for guaranteed tender meat (Boleman et al., 1997; Shackelford et al., 2001). Because of the relationship between tenderness and consumer satisfaction, many researchers are working on the ways to classify beef carcasses according to their level of tenderness. The most effective method to date requires removing a steak from the carcass to perform a mechanical measure of tenderness (Koohmaraie and Geesnik, 2006). A non-destructive means of accurately predicting tenderness would be more desirable for the meat industry. Many research projects have lead to the belief that calpastatin, the inhibitor of the calpain system, is responsible for regulating the tenderness response due to aging (Koohmaraie, 1992). Calpastatin has produced correlations with mechanical measures of tenderness that ranges from 0.27 to 0.75 (Shackelford et al., 1994; Geesink et al., 2005) making calpastatin a good candidate for developing a biosensor to predict meat tenderness.

Our previously developed calpastatin biosensor utilized a dual binding technique coupled with fluorescence resonance energy transfer (FRET; Grant et al., 2005). This dual binding technique was built upon in-solution testing and tested in pure calpastatin samples from beef muscle (Grant et al., 2005). The next generation of our FRET based system immobilized calpastatin antibodies to optical fibers in the form of a sandwich immunoassay to detect the level of calpastatin present in homogenized meat samples (Bratcher et al., 2008). The results from the optical fiber research (Bratcher et al., 2008) suggested that the best time for use of the biosensor in an online grading system was at 48 h post-mortem. However, the optical fibers were highly variable both within and between fibers (Bratcher et

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al., 2008). While the optical fiber biosensor would be useful in laboratory determination of differences in calpastatin activities, variable results indicated that a new biosensor platform may increase accuracy and precision; therefore the aim of the current research was to use the same sensing mechanism, i.e., the sandwich immunoassay, coupled to a less variable waveguide system, i.e., capillary tubes.

Capillary biosensors are integrated sensors in that they serve as both a sampling device and an optical waveguide (Weigl and Wolfbeis, 1994). The capillary tubes support fluid flow through the interior and light propagation in the walls of the capillary tube (Weigl and Wolfbeis, 1994). This allows for both sampling and analysis in one location. Since capillary tubes can easily be adapted to automated devices, it is envisioned that entire capillary biosensor systems will be small, portable devices capable of sampling and analyzing with minimal human intervention (Thomas, 2006).

Capillary tubes utilized as waveguides provide advantages over other waveguide systems. These advantages include reduced sample volumes and reduced incubation times which lead to reduced sampling cost (Weigl and Wolfbeis, 1994). Capillary tubes are also less expensive compared to other optical waveguides such as optical fibers (Thomas, 2006). Low cost and mass production allow capillary tubes to be a disposable component of the biosensor system (Misiakos and Kakabakos, 1998).

The current research had two goals which built upon our previous research with the optical fiber calpastatin biosensor (Bratcher et al., 2008). The first goal was to immobilize the sensing elements into the inner walls of capillary tubes and test the calpastatin response in homogenized beef samples. The sensing mechanism, like the optical fiber biosensor's sensing mechanism, was build upon non-competitive binding immunoassay, which utilizes FRET technology. The second goal was to establish a relationship between this biosensor technique, calpastatin assays, Warner–Bratzler shear force (WBSF), and crude protein with less variability than the optical fiber biosensor presented in Bratcher et al. (2008).

2. Materials and methods

2.1. Sample preparation

Calpastatin extraction from beef samples was performed according to Lorenzen et al. (2000). *Longissimus* muscle samples ($n = 11$) were collected at 0 and 48 h post-mortem to simulate potential grading time in industry. A fresh 5 g sample of *longissimus* muscle was homogenized in 5 volumes of extraction buffer (50 mM Tris, 10 mM EDTA for pre-rigor muscle and 100 mM Tris, 10 mM EDTA for post-rigor muscle). After centrifugation, the supernatant was dialyzed in elution buffer (2 mM Tris, 0.025 mM EDTA) for at least 18 h. The dialyzed sample was then heated to 95 °C and cooled in an ice bath. The supernatant was purified on a 10 mL DEAE-Sephacel column that was equilibrated with the elution buffer. The sample was eluted from the column using the elution buffer + 200 mM NaCl. The samples were assayed at the time of biosensor testing.

2.2. Quantification of muscle calpastatin with standard assay

Calpastatin activity was determined according to the procedures of Koohmaraie (1990). Fractions that were eluted from the column were screened to determine which fractions were active for calpastatin. Active and partially active fractions were pooled. Each assay was run in triplicate. The assay reaction consisted of: the sample, elution buffer, purified *m*-calpain, assay media (100 mM Tris, 1 mM NaN_3, 7 mg/mL Casein), and 100 mM $CaCl_2$. The reaction was then incubated in 25 °C water bath and the reaction was stopped with 2 mL of 5% TCA. After centrifugation, the supernatant was read on a Beckman Spectrophotometer Model DU-640 (Fullerton, CA) at a UV wavelength of 278 nm.

2.3. Warner–Bratzler shear force determination

As an instrumental measure of tenderness, WBSF was performed on 2.54 cm *longissmus* muscle steaks 14 d post-mortem. Steaks were removed from the carcass during the 48 h sampling time and subsequently stored in a vacuum package until 14 d post-mortem. The steaks were then frozen until WBSF could be determined. WBSF were performed according to the AMSA guidelines (1995). Briefly, a copper constantan thermocouple (Omega Engineering, Inc., Stamford, CT) was placed in the geometric center of each steak and then attached to an HH-21 calibrated thermometer. Steaks were cooked using a Hamilton Beach Portfolio Indoor/Outdoor Grill (Washington, NC) to 35 °C, flipped once, and removed from the grill at 71 °C. Steaks were cooled at 4 °C for 24 h and then allowed to equilibrate to room temperature (approximately 22 °C). Six cores (1.27 cm diameter) were removed from each steak parallel to the muscle fiber. Cores were sheared perpendicular to the long axis of the cores (AMSA, 1995). Shear force measurements were completed on the United STM 'SMART-1' TEST SYSTEM SSTM-500 (United Calibration Corp., Huntington Beach, CA). Settings for the system were as follows: force units (kg), linear units (mm), cycling (1 × 70 mm), test speed (250 mm/min), return speed (500 mm/min), and setup scales (CAP = 226.8). The mean WBSF value for the six cores was reported for each steak.

2.4. Protein analysis

Crude protein was also determined on a fresh ground 5 g sample collected at harvest by the University of Missouri Agricultural Experiment Station by the Kjeldahl method (AOAC, 1984).

2.5. Biosensor preparation

Using a procedure modified from Invitrogen (Carlsbad, CA) labeling kits, mouse anti-calpastatin IgGs (Research Diagnostics, Inc. Flanders, NJ) were labeled to the donor fluorophores, Alexa Fluor 546 (AF546), while secondary antibodies (mouse anti-calpastatin IgG2) were labeled to the acceptor fluorophores, Alexa Fluor 594 (AF594). The 1.5 mm inner diameter capillaries

(Fisher Scientific, Pittsburgh, PA) were cut in half by scoring and cutting with a razor blade and cleaned by a 30 min immersion in a boiling water bath. The interior surface of the capillary was silanized in order to immobilize the proteins. Silanization is one of the most commonly used covalent immobilization methods of attaching proteins to glass (Bhatia et al., 1989). Protein A was immobilized first onto the capillaries followed by the antibodies because Protein A binds to the Fc region of the antibody so that the epitope sites remain available.

Silanization occurred on the inner surface of the capillaries. Briefly, a thiol-terminal silane methyltrichlorosilane (MTS) solution was injected and incubated in the capillaries for 1 h. After rinsing, a bifunctional *N*-gamma-maleimidobutyryloxy-succinimide ester (GMBS), a heterobifunctional crosslinker, solution was then incubated for 30 min to promote amide binding to the terminal amino group on the proteins. After treatment with GMBS, the capillaries were incubated overnight with Protein A. The Protein A solution was removed from the capillaries, which were then rinsed in a large volume of phosphate buffer solution (PBS). A background fluorescence scan was obtained for each Protein A labeled capillary as described below. Next, each capillary was exposed to 0.10 g/L AF546–M anti-calpastatin D10 in PBS for 15 min. The scans were repeated in order to obtain donor-only emission spectra. Finally, the capillaries were then exposed to the nonfat milk-blocking agent for 15 min.

Before and after the donor-labeled antibody attachment, the capillaries were placed on a platform as shown in Fig. 1. The capillaries were positioned onto a stage with one terminus coupling into a 600-μm-core diameter optical fiber and clamped into place. The capillary tube was illuminated transversely with a green laser diode module at a wavelength of 546 nm. Any fluorescent emission was propagated to the terminus of the capillary tubing and coupled into the optical fiber. The emission was then directed to an Ocean Optics USB2000 miniature spectrometer (Dunedin, FL) via an optical fiber and a real-time fluorescence emission spectrum was obtained.

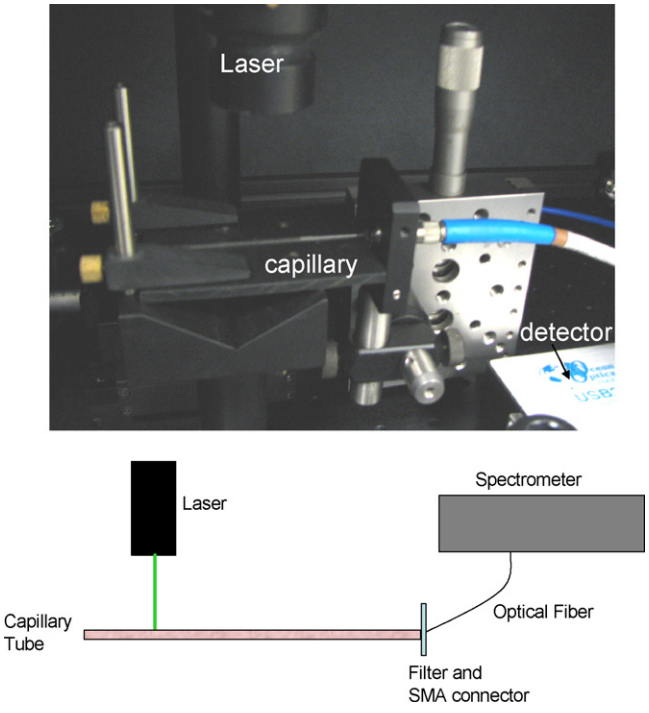

Fig. 1. Photo and schematic of the capillary biosensor used for detection of calpastatin in meat extracts.

2.6. Biosensor testing

The following solutions were prepared to fill the capillaries: 0.5 g/L nonfat milk blocking agent in PBS and 0.10 g/L AF594–M-anti calpastatin D6 in PBS. Samples, 0 and 48 h post-mortem, were analyzed from each animal set in triplicate. The capillaries were exposed to the samples for 15 min and finally to the AF594–M-anti calpastatin D6 for 15 min. After filling with each solution, the capillaries were rinsed in PBS. When the sandwich immunoassay was completed, the capillaries were scanned again to obtain the final AF546/AF594 interaction spectra for each capillary.

2.7. Calculations

The capillaries background scan was subtracted from the donor-only and final scans. To quantify the amount of binding that had taken place, a ratio of donor peak/acceptor peak (D/A) was determined. The donor peak was calculated by averaging the intensity values from 565 to 575 nm, and the acceptor peak was calculated by averaging from 605 to 615 nm. The average donor peak intensity value was divided by the average acceptor peak intensity value to give the D/A value. The result is a relative measure of calpastatin concentration in the test sample.

2.8. Statistical analysis

All statistical procedures were performed using SAS (SAS Inst. Inc., Cary, NC). Correlations were generated using PROC CORR for the biosensors, calpastatin assays, percentage protein and WBSF, as well as the means, standard deviations and minimum and maximums. Regression analysis was run using PROC REG to determine the ability of the biosensor readings to predict calpastatin activity. Scatter plots were generated for relevant data. The variability of the capillary-based and optical fiber biosensors was also compared to the standard calpastatin assays and WBSF using an *F*-test statistic.

3. Results and discussion

The means, minimum, maximum and standard deviations for calpastatin activity, biosensor readings, WBSF and crude protein are shown in Table 1. The minimum and maximum values for the traditional calpastatin assay were numerically higher for this research as compared to our optical fiber research, 0.51 and 2.73 versus 0.04–2.54 (Bratcher et al., 2008). The research from this experiment reported comparable calpastatin activity values to previous research reported by those who used the same protocol which ranged from 2.58 to 2.80 (Wulf et al., 1996; Shackelford et al., 1994). It has been shown by many researchers that calpastatin degrades over storage time (Koohmaraie et al., 1995;

Table 1
Means, standard deviations, minimum and maximum values ($n = 11$) for calpastatin assay, capillary tube biosensor and Warner–Bratzler shear force

	Mean	S.D.	Minimum	Maximum
Calpastatin assay[a]				
0 h	2.20[d]	0.46	1.51	2.73
48 h	0.82[e]	0.22	0.51	1.15
Biosensor[b]				
0 h	91.40[d]	3.53	85.46	95.68
48 h	61.40[e]	9.92	38.94	76.22
WBSF[c], kg				
14 d	3.36	0.82	2.39	5.10
Protein, %	21.86	0.51	21.01	22.60

Means with common superscripts (d,e) within the same method are not different ($P > 0.05$).
[a] Expressed as activity = the amount of calpastatin that inhibits one unit of m-calpain (Koohmaraie, 1990).
[b] Expressed as percent change in Donor/Acceptor is a quantitative measure of the amount of acceptor bound to the system relative to the amount of donor.
[c] Warner–Bratzler shear force (kg).

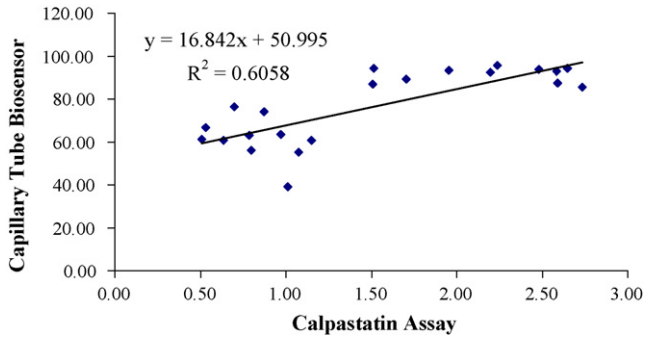

Fig. 2. Relationship between 0 and 48 h calpastatin activity and capillary tube biosensor reading.

Boehm et al., 1998; Doumit and Koohmaraie, 1999; Kristensen et al., 2006) and this may be an explanation for slightly lower calpastatin activity reported in this study at the 0 h sampling time. The degradation is also evident in the decreased ($P < 0.05$) calpastatin activities and biosensor readings at 48 h compared to the 0 h sampling time. However, it was important to run the traditional calpastatin assay at the time of the biosensor readings to assure that the readings from the biosensor were true to the actual activity of known calpastatin.

The biosensor reading values reported in Table 1 are much higher than those reported for an optical fiber biosensor (Bratcher et al., 2008). Capillaries are able to produce an enhanced signal which allows for increased sensitivity in detection. This increased sensitivity can be related to an increase in precision as well.

There was a wide range in WBSF values, 2.39–5.10 kg (Table 1). Shackelford et al. (1991) reported that a WBSF of less than 4.6 kg should have a 50% chance of being rated slightly tender or higher and with a WBSF of 3.9 kg or less there should be a 68% chance. Therefore, the steaks used for this research would range from tough to tender. Having steaks in both of these classifications is important when developing instruments to discriminate between tough and tender. The percentage protein for the samples was a narrow but normal range (21.01–22.60%) and a small standard deviation.

The correlation between crude protein and 0 h biosensor reading ($r = 0.7190$; Table 2) was the only significant correlation ($P = 0.013$) found in this study. Since calpastatin degrades over storage time, activity decreases (Koohmaraie et al., 1995; Boehm et al., 1998; Doumit and Koohmaraie, 1999; Kristensen et al., 2006) but fragments of the inhibitor still exist which are detectable and capable of inhibiting calpain activity (Koohmaraie and Geesnik, 2006). It is possible that the capillary tube biosensor is detecting these fragments of calpastatin as well as whole, intact calpastatin molecules because it was developed to detect the amount of calpastatin. Grant et al. (2005) reported a detection limit of 120 ng/mL of pure calpastatin in known concentration measurements in-solution.

The positive relationships between 0 and 48 h calpastatin activities and 0 and 48 h biosensor readings (Table 2) were expected and serve as verification that sample preparation and assay techniques were consistent. Correlations between calpastatin activity and capillary tube biosensor readings were not significant for either the 0 or 48 h measurements ($P = 0.984$ and 0.237, respectively) indicating that these two measurements are independent of each other. However, when the 0 and 48 h samples are pooled and the traditional calpastatin assay is compared to the capillary tube biosensor readings through regression (Fig. 2) the R^2 value is 0.6058, indicating that the biosensor is moderately accurate for detecting changes in calpastatin activity.

Table 2
Correlations and *P*-values of traditional calpastatin assay, biosensor, protein and Warner–Bratzler shear force

	Calpastatin assay		Biosensor		Protein	WBSF
	0 h	48 h	0 h	48 h		14 d
Calpastatin activity[a]						
0 h	1	.4381 (.178)	−.0069 (.984)	−.1106 (.746)	.2265 (.503)	.4533 (.161)
48 h		1	−.1107 (.746)	−.3890 (.237)	.2454 (.467)	−.1436 (.674)
Biosensor[b]						
0 h			1	.4502 (.165)	.7190 (.013)	−.4339 (.182)
48 h				1	.1501 (.660)	.0174 (.942)
Protein, %					1	−.0820 (.811)

[a] Expressed as activity = the amount of calpastatin that inhibits one unit of *m*-calpain (Koohmaraie, 1990).
[b] Expressed as percent change in Donor/Acceptor is a quantitative measure of the amount of acceptor bound to the system relative to the amount of donor.

Table 3
Comparison of variation between capillary tube and optical fiber biosensing methods

		Variance	Test of difference *P*-value		
			Pre-column optical fiber biosensor	Post-column optical fiber biosensor	Capillary tube biosensor
Pre-column optical fiber biosensor[a] ($n=21$)	0 h	62.4		0.116	0.006
	48 h	86.7		0.331	0.386
Post-column optical fiber biosensor[a] ($n=21$)	0 h	36.2	0.116		0.043
	48 h	105.7	0.331		0.474
Capillary tube biosensor ($n=11$)	0 h	12.5	0.006	0.043	
	48 h	98.4	0.386	0.474	
Calpastatin assay trial 1[a] ($n=21$)	0 h	0.38	<0.001	<0.001	
	48 h	0.41	<0.001	<0.001	
Calpastatin assay trial 2 ($n=11$)	0 h	0.21			<0.001
	48 h	0.05			<0.001

[a] Variance of optical fiber biosensor and calpastatin assay calculated from standard deviations in Bratcher et al. (2008).

The correlation coefficient for the pooled samples, $r=0.7783$, was an increase over those reported for the optical fiber (Bratcher et al., 2008) but was in the middle of the range reported by Geesink et al. (2005) for an optical surface plasmon resonance (SPR) biosensor. SPR is a technique that measures shifts in resonance frequency upon antibody–antigen binding.

The correlation between calpastatin activity and WBSF ($r=0.4533$; Table 2) at 0 h was within the range of those reported by previous researchers that range from 0.27 to 0.66 (Whipple et al., 1990; Shackelford et al., 1994; Lonergan et al., 1995; Woodward et al., 2000) and was approaching significance ($P=0.161$). The samples used in this study encompassed a variety of genetics that represented a large range in WBSF and calpastatin activity values (Table 1) which was expected because calpastatin levels vary among different breeds within species (Shackelford et al., 1994). The small number of samples is likely responsible for the lack of significance in the correlation between these two traits.

The optical fiber biosensor research (Bratcher et al., 2008) showed inherent signal variability within and among fibers which led to variability in the calpastatin biosensor. A comparison of the optical fiber biosensor and capillary tube biosensor are reported in Table 3. The variance was lower for the 0 h capillary tube biosensor readings compared to 0 h pre-column ($P=0.006$) and post-column ($P=0.0473$) readings for the optical fiber biosensor indicating that this is a more precise method for measuring calpastatin. The standard deviations ranged from 6.02 to 10.28 with the optical fiber biosensor (Bratcher et al., 2008), whereas with the capillary tube biosensor the standard deviations were 3.53 and 9.92 (Table 1). There was no difference ($P>0.05$) in the variance of readings for 48 h samples from either biosensor platform. This could be due to the degradation in the activity of calpastatin over time. As expected, all of the biosensor methods at all time points were more variable than the traditional assays. The calpastatin assay was the gold standard that the biosensor platforms were compared to and its methodology is well established so there would be less variation in this method than a new technique.

4. Conclusions

The capillary tube biosensor was developed to detect calpastatin. The results reported in this research indicated that the capillary tube biosensors had less variability than the optical fiber biosensors. Additionally, the capillary tube biosensors demonstrated lower variance for the 0 h measurements than the optical fiber biosensors. In conclusion, the capillary tube biosensor would be useful in laboratory determination of the differences in biologically active calpastatin concentrations. It is possible that this technology will advance the development of an online assessment device to predict meat tenderness at the time of grading.

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