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Correlation between a novel calpastatin biosensor and traditional calpastatin assay techniques

C.L. Bratcher [a], S.A. Grant [b], R.C. Stringer [b], C.L. Lorenzen [c,*]

[a] *Department of Animal Sciences, University of Missouri, Columbia, MO 65211, United States*
[b] *Department of Biological Engineering, University of Missouri, Columbia, MO 65211, United States*
[c] *Department of Food Science, University of Missouri, Columbia, MO 65211, United States*

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Abstract

An optical fiber biosensor to detect calpastatin has been investigated as a preliminary step in developing tenderness detection instrumentation. *Longissimus dorsi* samples were taken from beef carcasses ($n = 21$) at 0, 24, 36 and 48 h postmortem. Muscle homogenates were assayed for calpastatin activity using traditional methods and an optical fiber biosensor. Warner–Bratzler shear force was also performed on a steak from each carcass at 14 d postmortem. Results demonstrated that the measurements with highest correlation between traditional calpastatin assays and optical biosensor readings were taken at 48 h postmortem ($r = 0.597$, $P \leq 0.01$), suggesting that this is the best time for use of this biosensor in an on-line grading system. This research further advances the development of a calpastatin biosensor and would be useful in laboratory determination of the presence of biologically active calpastatin concentrations.

Keywords: Biosensor; Calpastatin; Fluorescence; Beef; Tenderness

1. Introduction

The development of instrument grading tools has been a priority of the beef industry for a number of years. Great strides have been made through this research and the USDA Agricultural Marketing Service has adopted standards that allow graders to use on-line instrumentation to asses both yield and quality grade factors. While these technologies are precise and accurate for USDA yield and quality grades, they only evaluate predictors of tenderness (Belk et al., 2000).

Numerous research projects have led to the general belief that calpastatin, the inhibitor of the calpain proteolytic system, is a primary component responsible for regulating the tenderness response due to aging (Koohmaraie, 1992). Our research has focused on developing a biosensor that can accurately predict calpastatin and then be incorporated into an instrument that can be used at the time of grading to sort cattle based on their predicted tenderness value.

An in-solution biosensor was previously developed using a dual binding technique coupled with fluorescence resonance energy transfer (FRET; Grant et al., 2005). FRET is advantageous because it allows sensor stability due to ratiometric measurements, which are not subject to signal interferences and component variations. In the dual binding technique, an antibody that binds to calpastatin was labeled with a donor fluorescent dye and μ-calpain was labeled with an acceptor fluorescent dye. In the presence of calpastatin, both the antibody and calpain bind to calpastatin and fluorescence changes (Grant et al., 2005). The results showed that in the presence of calpastatin, the labeled μ-calpain and antibody would bind to calpastatin, eliciting a measurable change in fluorescence (Grant et al., 2005). The limit of detection for calpastatin was 120 ng/mL in a meat homogenate and a stable response time was achieved within 5 min (Grant et al., 2005). A drawback with the dual binding technique was the difficulty in immobilizing both the antibody and μ-calpain onto a waveguide at specific distances apart in order to achieve measurable energy transfers.

Geesink et al. (2005) reported on quantification of calpastatin using an optical surface plasmon resonance (SPR) biosensor.

* Corresponding author at: 256 William C Stringer Wing, University of Missouri, Columbia, MO 65211, United States. Tel.: +1 573 882 2452; fax: +1 573 884 7964.
E-mail address: lorenzenc@missouri.edu (C.L. Lorenzen).

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According to this research, biosensor results for several experiments were linearly related to calpastatin activity measurements with correlation coefficients ranging from 0.51 to 0.99. It was also reported that calpastatin content, as determined by the biosensor, at 1 d postmortem was correlated to shear force at 14 d postmortem ($r = 0.75$).

The current research had two goals. First was to utilize a noncompetitive binding sensing mechanism and immobilize the sensing elements onto optical fibers, then test in a series of complex homogenized beef samples. The second goal was to establish a relationship between the biosensor technique, calpastatin assays and Warner–Bratzler shear force (WBSF).

2. Materials and methods

2.1. Quantification of muscle calpastatin with standard assay

Standard calpastatin assays were performed according to Koohmaraie (1990) with the exception that the samples were heated before they were dialyzed. *Longissimus* muscle samples ($n = 21$) were collected at 0, 24, 36 and 48 h postmortem from beef cattle harvested at the University of Missouri abattoir and used for calpastatin activity determination. Cattle were Continental crossbred steers and heifers and one dairy-type heifer. Samples taken at 24, 36 and 48 h were selected to simulate potential grading times observed in the beef industry. Samples, 2.54 cm thick, were collected from the 13/14th rib interface at 0 h from a randomly selected side of the carcass. The rest of the samples were removed at the appropriate sampling time from the opposite side of the carcass. The samples were taken randomly from dorsal to ventral orientation of the carcass to omit any location effects. A fresh 25 g sample of *longissimus* muscle was homogenized in three 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 heated to 95 °C and cooled in an ice bath. It was then dialyzed in elution buffer (2 mM Tris, 0.025 mM EDTA) for at least 18 h. A 1 mL portion of the homogenate was removed after dialysis for sampling to determine if the biosensor could detect calpastatin in different proteinaceous environments. 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 with 200 mM NaCl.

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

The samples were then stored at 4 °C until testing could be performed with the developed biosensor.

2.2. Warner–Bratzler shear force determination

Warner–Bratzler shear force, a mechanical measure of meat tenderness, was performed on 2.54 cm *longissimus* muscle steaks 14 d postmortem. Steaks were removed from the 12/13th rib interface of the carcass during the 48 h sampling time and subsequently stored in a vacuum package until 14 d postmortem. The steaks were then frozen until WBSF could be determined according to AMSA guidelines (1995). Briefly, steaks were cooked using a Hamilton Beach Portfolio Indoor/Outdoor Grill (Southern Pines, 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 (∼22 °C). Six cores (1.27 cm diameter) were removed from each steak parallel to the muscle fibers. Cores were sheared perpendicular to the long axis of the muscle fibers (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 mm × 70 mm), test speed (250 mm/min), return speed (500 mm/min) and setup scales (CAP = 226.8). The mean WBSF value for six cores was reported for each steak. These values were correlated with calpastatin activities and pre- and post-column biosensor technique readings.

2.3. Fiber tip preparation

A noncompetitive binding sensing mechanism was utilized. The conjugation of the dyes to the proteins occurred using a procedure found in the Invitrogen labeling kits (Carlsberg, CA) where the labeling occurred via the binding of a succinimidyl ester moiety of the Alexa Fluor 546 (AF546) and Alexa Fluor 594 (AF594) to the primary amines of the proteins. Mouse anti-calpastatin IgG (Research Diagnostics, Inc., Flanders, NJ) was conjugated to the donor fluorophore, AF546, while secondary antibodies (mouse anti-calpastatin IgG2) were conjugated to the acceptor fluorophore, AF594. The tips of four silica optical fibers (600 μm core diameter) were tapered using hydrofluoric acid. Briefly, approximately 1 cm of a blunt-ended fiber tip was placed in hydrofluoric acid for 1 h. After the 1 h incubation time, the tip was rinsed in distilled water and the cladding was carefully removed with a razor blade. Due to capillary forces, the hydrofluoric acid was drawn up the fiber tip and thus tapered the ends of the fiber. Tapering increases the capture of fluorescence into the fiber tip and results in higher signals. Protein A was then immobilized to the optical fiber by way of silanization as explained below. Silanization is one of the most commonly used covalent immobilization methods of attaching proteins to silica (Bhatia et al., 1989). Approximately 0.5 cm up to 2.0 cm of the fiber tip was silanized. Protein A is first immobilized onto the fibers, because protein A specifically binds to the Fc region of the antibody, allowing the epitope sites to remain available.

The silanization procedure is as follows: briefly, a thiol-terminal silane (3-mercaptopropyl) trimethoxysilane (MTS) was attached by way of the hydroxyl groups of the silica fiber tips. A bifunctional N-gamma-maleimidobutyryloxy-succinimide ester (GMBS), a heterobifunctional crosslinker, was then attached which allows amide binding to the terminal amino group of protein A. After treatment with GMBS, the tips of the optical fibers were incubated for 1 h in protein A. The surface was then rinsed in PBS, exposed in a 1 M solution of ethanolamine to quench any unreacted residues, and rinsed with Triton X-100 to reduce nonspecific adsorption.

Each fiber was scanned to obtain a background absorption spectrum in the Fluoromax 3 spectrofluorometer (Edison, NJ) by way of a specially designed fiber optic stage. The fiber tips were immersed into PBS in a 4 mL cuvette in a completely dark environment. The scans were taken over 560–650 nm wavelength emission of the fibers when excited at 546 nm.

2.4. Biosensor testing

The following solutions were prepared in 100 μl amounts in separate 0.4 mL polyethylene microcentrifuge tubes for each optical fiber tip to be examined: 0.10 g/L AF546 – mouse anti calpastatin D10 in PBS, 0.5 g/L nonfat dry milk blocking agent in PBS and 0.10 g/L AF594 – mouse anti calpastatin D6 in PBS. The calpastatin samples from the homogenized beef samples were transferred into microcentrifuge tubes for each fiber tip. Eight solutions were analyzed from each sample set: four pre-purification and four post-purification column samples aged 0, 24, 36 and 48 h.

Each fiber tip was first exposed to AF546—mouse anti calpastatin D10 for 15 min, followed by a 5-min rinse in PBS with stirring. The fibers were then exposed to the nonfat dry milk blocking agent for 15 min. The spectrofluorometer scans were repeated with the same parameters as described above to obtain donor-only emission spectra. Next, the fiber tips were incubated in the calpastatin solutions for 15 min followed by the final incubation in the AF594—mouse anti calpastatin D6 solution for 15 min. After immersion in each solution, the fiber tips were rinsed in PBS with stirring for 5 min.

When the fiber optic biosensor was completed (as shown in Fig. 1), the fibers were scanned again to obtain the final AF546/AF594 interaction spectra for each fiber. As shown in Fig. 2, the raw spectra of before and after calpastatin exposure is displayed. Selectivity was also an important parameter to examine; therefore, the fiber optic biosensors were tested in bovine serum albumin (BSA, nonspecific analyte) as well. The donor/acceptor ratios as well as the donor/isosbestic point ratios were determined and the response of the "specific" calpastatin biosensors to "nonspecific" BSA biosensors was compared. After testing, the tips of the four fibers were cleaved and the fibers were once again prepared as stated above.

2.5. Calculations

The fiber's 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/isosbestic point (*D*/*I*) was found. The donor peak was calculated by averaging the intensity values from 570 to 575 nm, and the isosbestic point was calculated by averaging the intensity values near the point where the curves intersect, usually from 590 to 595 nm. The average donor peak intensity value was divided by the average isosbestic point intensity value to give the *D*/*I* value for the before and after analyte exposure. Finally, the percent change in *D*/*I* for the before and after analyte exposure was calculated. The result is a relative measure of calpastatin concentration in the test sample.

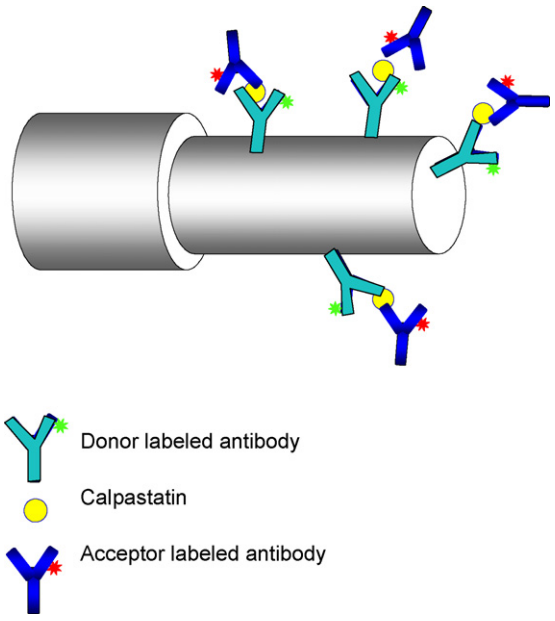

Fig. 1. Schematic of the optical fiber biosensor used for detection of calpastatin in meat extracts.

2.6. Statistical analysis

All statistical procedures were performed using SAS (SAS Inst. Inc., Cary, NC). Means, standard deviations, minimum

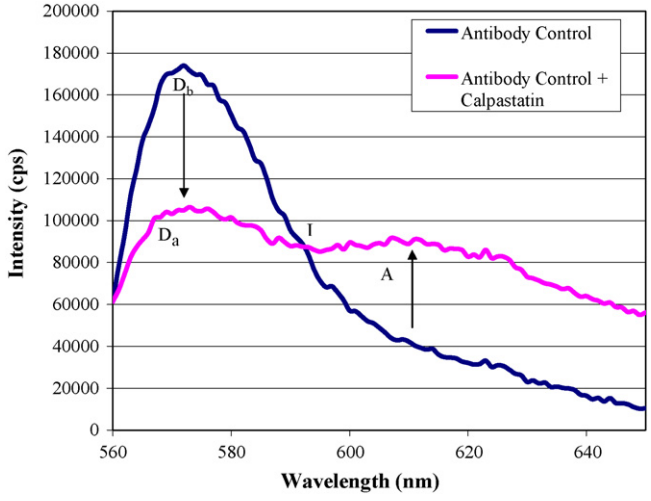

Fig. 2. Raw spectra of donor before (D_b), donor after calpastatin (D_a), isosbestic (*I*) and acceptor after calpastatin (*A*) peaks for optical fibers before and after calpastatin exposure.

and maximum values and correlations were generated using PROC CORR for pre- and post-column biosensors, traditional calpastatin assays and WBSF. Scatter plots were generated for relevant data.

3. Results

3.1. Binding of calpastatin versus BSA

In order to demonstrate that the optical fiber biosensor was able to detect calpastatin with specificity, the system was tested using a known concentration of calpastatin and compared to the same concentration of BSA. The donor fluorescence to isosbestic point (*D*/*I*) ratio of the specific analyte was compared to the *D*/*I* of the nonspecific analyte. Additionally, the donor fluorescence to acceptor fluorescence ratio (*D*/*A*) was also calculated. The ratiometric method provided a measure of energy transfer from the donor fluorophore to the acceptor fluorophore.

As shown in Fig. 3, after addition of specific (calpastatin) and nonspecific (BSA) antigens and the acceptor-labeled antibody, there was a greater decrease in *D*/*A* value (10.05% (S.D. 0.05) versus 0.55% (S.D. 0.019)) for the fibers exposed to calpastatin than the fibers exposed to BSA indicating greater binding of the calpastatin to the optical fiber. Because the binding was higher for calpastatin, these experiments illustrated that the optical fiber biosensors specifically detected calpastatin. The *D*/*A* and *D*/*I* ratios both illustrated binding of calpastatin, but due to the ease of calculating and reporting, the *D*/*I* values were utilized for the reported results.

Five experiments were performed, using four optical fiber biosensors. All of the biosensors demonstrated selectivity and sensitivity toward calpastatin; however, the experiments imparted variable results in detecting calpastatin. Each optical fiber cable appeared to have an intrinsic background signal arising from the silica core and/or polymeric cladding. This was noted in the background signals that were acquired prior to protein A and antibody immobilization. Additionally, the SMA connectors for each optical fiber cable may also have contributed to variable background signal as well as variable signal capture. Due to these inherent factors, the fiber optic cables had different attenuation profiles which resulted in different acquired sensitivities for the optical fiber biosensors.

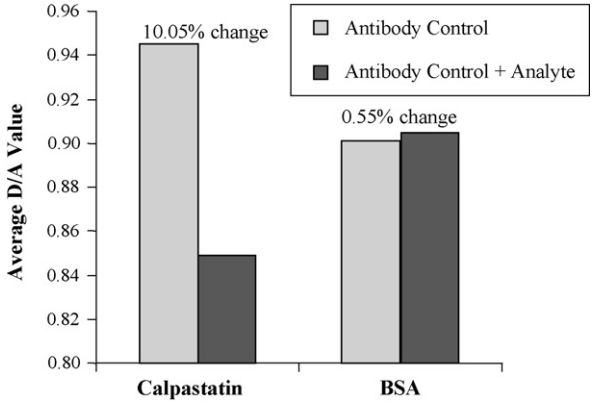

Fig. 3. Averaged donor/acceptor ratio optical fiber biosensor to determine specific (calpastatin) and nonspecific binding (bovine serum albumin).

Table 1
Means, standard deviations, minimum and maximum values ($n = 21$) for calpastatin assay, optical fiber biosensor and Warner–Bratzler shear force

	Mean	S.D.	Minimum	Maximum
Calpastatin assay[a]				
0 h	1.13	0.62	0.14	2.40
24 h	0.75	0.56	0.08	1.94
36 h	0.92	0.49	0.04	1.66
48 h	1.04	0.64	0.35	2.54
Pre-column biosensor[b]				
0 h	33.55	7.90	18.68	46.79
24 h	31.03	8.73	17.32	46.25
36 h	27.65	9.17	12.00	45.99
48 h	25.87	9.31	5.85	42.68
Post-column biosensor[b]				
0 h	25.91	6.02	14.08	35.29
24 h	26.18	10.05	9.35	46.12
36 h	27.61	8.93	12.30	43.71
48 h	29.99	10.28	8.44	49.73
WBSF[c] (N/cm^2)				
14 d	33.12	8.33	21.07	54.10

[a] Expressed as activity = the amount of calpastatin that inhibits one unit of μ-calpain (Koohmaraie, 1990).
[b] Expressed as percent change in donor/isosbestic is a quantitative measure of the amount of acceptor bound to the system relative to the amount of donor.
[c] Warner–Bratzler shear force.

3.2. Correlation between assays and optical sensor

The means, minimum, maximum and standard deviations for the current study are shown in Table 1. The minimum and maximum values for the traditional calpastatin assay are 0.04–2.54 U which are numerically less than those reported by other researchers.

There was a strong correlation between calpastatin activity and the 48 h pre-column biosensor readings ($r = 0.597$, $P \leq 0.01$) as well as 48 h post-column with the 48 h postmortem traditional calpastatin assay ($r = 0.501$, $P = 0.03$; Table 2) indicating the strongest relationship between the biosensor and amount of calpastatin activity in a beef homogenate at 48 h postmortem. The greatest correlation was elicited with 36 h pre-column and 48 h traditional assay ($r = 0.656$). There were also significant correlations with the 0 h traditional calpastatin assay and the 48 h pre-column biosensor ($P = 0.025$).

As shown in Table 2, there were no significant correlations between WBSF at 14 d postmortem and either traditional calpastatin assay or optical fiber biosensor readings. This is contradictory to research performed previously in other laboratories. The low correlations and lack of significance could be due to the storage time and degradation of calpastatin after purification, indicating that these factors need to be taken into account when detecting calpastatin.

Table 2
Correlation coefficients and *P* values of traditional calpastatin assay activity, pre-column and post-column optical fiber biosensor readings, and Warner–Bratzler shear force

	Calpastatin activity				Pre-column biosensor				Post-column biosensor				WBSF[a]
	0 h	24 h	36 h	48 h	0 h	24 h	36 h	48 h	0 h	24 h	36 h	48 h	14 d
Calpastatin activity													
0 h	1	0.459 (0.042)	0.371 (0.107)	0.491 (0.028)	0.016 (0.945)	−0.203 (0.377)	0.295 (0.207)	0.498 (0.025)	0.029 (0.902)	−0.067 (0.787)	0.263 (0.292)	0.159 (0.515)	0.101 (0.665)
24 h		1	0.482 (0.037)	0.406 (0.085)	−0.060 (0.802)	−0.252 (0.284)	0.219 (0.368)	0.329 (0.170)	−0.152 (0.522)	−0.336 (0.159)	−0.189 (0.467)	0.219 (0.382)	0.022 (0.928)
36 h			1	0.655 (0.002)	−0.209 (0.376)	0.130 (0.584)	0.348 (0.132)	0.372 (0.117)	0.263 (0.262)	0.041 (0.871)	−0.088 (0.728)	0.245 (0.328)	0.046 (0.848)
48 h				1	0.167 (0.481)	−0.276 (0.239)	0.656 (0.002)	0.597 (0.007)	0.238 (0.312)	−0.043 (0.866)	0.232 (0.355)	0.501 (0.029)	0.296 (0.205)
Pre-column biosensor													
0 h					1		0.298 (0.202)	−0.077 (0.746)	−0.057 (0.805)	0.294 (0.222)	0.167 (0.508)	−0.164 (0.501)	0.346 (0.125)
24 h						0.313 (0.167)	0.198 (0.404)	−0.356 (0.123)	−0.290 (0.203)	0.239 (0.325)	0.221 (0.378)	−0.183 (0.453)	0.095 (0.682)
36 h						1	1	0.422 (0.072)	−0.211 (0.372)	0.261 (0.295)	0.120 (0.634)	0.295 (0.235)	0.177 (0.456)
48 h								1	0.090 (0.708)	−0.031 (0.904)	0.453 (0.068)	0.182 (0.469)	0.017 (0.942)
Post-column biosensor													
0 h									1	0.123 (0.615)	0.135 (0.592)	−0.020 (0.942)	−0.025 (0.918)
24 h										1	0.331 (0.211)	0.019 (0.944)	0.225 (0.355)
36 h											1	−0.131 (0.616)	−0.001 (0.998)
48 h												1	0.238 (0.327)

[a] Warner–Bratzler shear force (N).

4. Discussion

Calpastatin activities using the method reported by Koohmaraie (1990) were lower in this trial compared to the values reported in previous papers which followed the same protocol. Wulf et al. (1996) and Shackelford et al. (1994) reported mean calpastatin activities of 2.58 and 2.80, respectively. Although the samples should have been stable after processing, it is possible that the samples could have lost activity during the time that they were stored at 4 °C prior to the assay for activity. The samples were stored at 4 °C after processing but prior to the time that the biosensors were ready for measurements. It has been shown by several researchers that calpastatin degrades over time of storage (Boehm et al., 1998; Doumit and Koohmaraie, 1999; Koohmaraie et al., 1995; Kristensen et al., 2006). Boehm et al. (1998) reported ranges of 17–67% of at-death calpastatin activity after 1 d postmortem that were reduced to 15–38% after 7 d of postmortem storage. Goll et al. (2003) stated that the calpastatin molecule is in a random coil conformation and is labile to proteolytic degradation and it is likely that the harsh conditions used to purify calpastatin cause proteolytic degradation. Therefore, it is likely that the calpastatin degraded before the assay, leading to lower values than previously reported research. It was, however, important to read the calpastatin assays and the biosensor assays around the same time frame to assure that the biosensor readings were true to the amount of calpastatin present in the sample.

Although the results of this trial did not report a high correlation between optical fiber biosensor readings or traditional calpastatin activity and WBSF, there was a small sample size that encompassed a variety of cattle types. This variation was essential to the development of the optical fiber biosensor to assure that multiple levels of calpastatin activities were being detected and so that the biosensor could be used over the variety of cattle that are harvested for the beef industry. The correlation between the 0 h pre-column optical fiber biosensor reading and WBSF was 0.346 and approached significance ($P = 0.125$) which is lower than the correlation of 0.75 between calpastatin amount and WBSF reported by Geesink et al. (2005). The greatest correlation of calpastatin activity with WBSF was 0.296 ($P = 0.205$) for this study. Our correlations were at the lower end of the range reported by other researchers who reported ranges from 0.27 to 0.66 (Whipple et al., 1990; Shackelford et al., 1991, 1994; Lonergan et al., 1995; Woodward et al., 2000). However, the WBSF range reported from the current research was 21.07–54.10 N ± 8.53 with 9.5% of the samples above or tougher than the consumer acceptable level of tenderness of 38.22 and 45.08 N for retail and foodservice steaks, respectively (Shackelford et al., 1991).

There were three sampling times within the pre-column optical fiber biosensor readings that were significantly correlated to the traditional calpastatin assays and only one from the post-column optical fiber biosensor reading. However, comparisons between the calpastatin assay and optical fiber biosensor readings taken at the same time period were considered to be the most meaningful. Based on this assumption, our results indicate that the calpastatin activity of 48 h postmortem most closely

correlated with pre-column biosensor readings at 48 h ($r = 0.597$). This result agrees with Geesink et al. (2005), who found a correlation of $r = 0.51$ using an SPR biosensor at 48 h with dialysized meat homogenates. Post-column calpastatin should have been more purified than pre-column calpastatin since the calpastatin is concentrated on the column. The optical fiber biosensors could have been detecting other proteins that were similar to calpastatin due to coelution or incomplete elution. Koohmaraie (1990) reported that if the salt concentration was not at least five column volumes, then there could be coelution of calpastatin and calpain. It is also possible that the biosensor was saturated beyond its detection limit when using the post-column samples. Previous research by Grant et al. (2005) showed a detection limit of 120 ng/mL of calpastatin in solution measurements. That research used known concentrations of calpastatin not meat extracted calpastatin. The calpastatin was added from 30 ng/mL up to an amount of 240 ng/mL with the detection limit being at 120 ng/mL, which might have been due to less proteinaceous material in solution (Grant et al., 2005). The meat extracts used in this study with more proteinaceous material may crowd the solution and prevent the binding of the anti-calpastatin antibody to calpastatin.

Additionally, the utilization of fiber optic cables showed an inherent signal variability that may be caused by (1) different background signals for inherent defects in the optical fibers, connectors and/or (2) autofluorescence of the cladding material (Thomas, 2006). This led to different sensitivities of the fiber optic sensors and variability between the calpastatin biosensors used in this study. Therefore, it is critical in future research to ensure high quality fiber optic cables in order to obtain similar background signals.

The main difference in the SPR biosensor studied by Geesink et al. (2005) and the optical fiber biosensor developed here is that the optical fiber biosensor utilizes a fluorescence intensity-based system while SPR utilizes a resonance shift-based system. It is desirable to design a user-friendly, low cost system that could be utilized by meat producers to allow meat grading. While SPR can ensure higher sensitivity that may not be needed for meat grading, the optical waveguide detection equipment is more user-friendly and less expensive.

5. Conclusions

The aim of this research was to determine if the fiber optic FRET biosensor was capable of detecting specific, biologically active levels of calpastatin in meat and to determine if those levels were correlated with tenderness measurements. The highest correlations between traditional calpastatin assays and the optical fiber biosensor were taken at 48 h postmortem, suggesting that this is the best time for use of the biosensor. The current biosensor would be useful in laboratory determination of differences in biologically active calpastatin concentrations. However, future studies will involve capillary waveguides in order to eliminate the use of the variable fiber optic cables.

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