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QUANTIFICATION OF CALPASTATIN USING AN OPTICAL SURFACE PLASMON RESONANCE BIOSENSOR

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Introduction

There is a large body of evidence indicating that µ-calpain is the major cause of postmortem tenderization of skeletal muscle through degradation of structural proteins (for reviews see Koohmaraie, 1996; Geesink et al., 2000). The activity of µ-calpain in postmortem muscle is controlled by calpastatin, its endogenous inhibitor. Post rigor calpastatin activity (1-2 days p.m.) accounts for a greater portion of the variation in tenderness of aged beef longissimus (~40%) than any other single measure (Whipple et al., 1990; Shackelford et al., 1994). For this reason calpastatin quantification in skeletal muscle is commonly performed in research on meat tenderness.

Most frequently, calpastatin quantity, measured as m-calpain inhibitory activity, is performed on samples subjected to ion-exchange chromatography to separate calpastatin from the calpains (Koohmaraie, 1990). To facilitate quantification of a large number of samples in a breed evaluation program, muscle extracts were heated to 95°C instead of subjected to chromatography, making use of the fact that calpastatin retains its activity under these conditions (Shackelford et al., 1994). Methods based on immunological quantification include an ELISA (Doumit et al., 1996) and Western blotting (Sensky et al., 1999). Although the ELISA method lends itself to automation, all of these methods are rather labor intensive and require at least several hours to complete.

An alternative to these methods is the use of a biosensor, which is a measurement system that uses biological ligands as a part of a biotransducer that transforms chemical information into an analytically useful signal. A number of recent publications have addressed the suitability of surface plasmon resonance (SPR) based biosensors for analyses in the food industry (see Karlsson, 2004 for a review). From these studies it appears that SPR biosensors offer a valuable alternative to other techniques because it is both rapid and real-time.

Objectives

The purpose of the present study was to explore the suitability of a SPR biosensor system (Biacore Q) for quantification of calpastatin.

Methodology

Samples

Samples were obtained in three separate experiments. The Roman L. Hruska U.S. Meat Animal Research Center Animal Care and Use Committee approved the use of animals in the first two experiments. The animals were slaughtered humanely and dressed using typical procedures. Carcasses were chilled at 0°C.

Experiment 1

Twelve sheep (12 months of age) were used. From 5 animals, a portion of the longissimus of one carcass side was dissected after dressing for determination of calpastatin activity. At 2 days p.m. the longissimus of the intact carcass side was dissected and a portion was removed for determination of calpastatin activity. After vacuum storage at 4°C, the remainder of the muscle was sampled for determination of calpastatin activity at 10 days p.m.

Experiment 2

From thirty steers (14-16 months of age), a portion of the longissimus of one carcass side was sampled at 2 days p.m. for determination of calpastatin activity.

Experiment 3

Thirty longissimus samples from female cattle (300-400 kg carcass weight) were obtained at 1 day p.m. from a commercial slaughterplant. The muscles were sampled at 1 day p.m. for determination of calpastatin activity. Samples were stored at –80°C until quantification of calpastatin. The remainder of the muscle was vacuum packed and aged at 2°C till 14 days p.m.. After the aging period a 2 cm thick steak was vacuum packaged and stored at –20°C until determination of the shear force.

Preparation of samples for quantification of calpastatin

Samples (25 g) were homogenized in three volumes of cold 50 mM Tris (pre rigor) or 100 mM Tris (post rigor), 10mM EDTA, pH 8.3, containing 0.05% ß-MCE, 100 mg/L ovomucoid, 2 mM PMSF, and 6 mg/L leupeptin. The homogenates were centrifuged at 16,000 x g for 2 hours at 4°C. The supernatant was collected, heated to 95°C in a waterbath, and maintained at this temperature for 15 min. Samples were chilled on ice and clarified by centrifugation at 37,500 x g for 1 hour at 4°C. The supernatants were dialyzed overnight against 40 mM Tris, 5 mM EDTA, 0.05% ß-MCE, pH 7.35 at 4°C. Calpastatin activity of the heated extracts was determined as described by Koohmaraie (1990).

In experiment 1 and 2, aliquots (2 mL) of the extracts were frozen at –80°C and shipped on dry ice to CCL research. Samples were stored at –80°C until use. For the beef samples, aliquots were also collected before dialysis. Extracts from the third experiment were assayed directly for calpastatin content as described below.

Preparation of the biosensor chip

This assay was developed on a BIACORE Q biosensor system (Biacore, Uppsala, Sweden) controlled by a BIACORE Q Software package version 3.01.

Human erythrocyte calpastatin (Calbiochem, San Diego, CA) was used to coat the biosensor chip and for the calpastatin standard curve.

Prior to coating the CM5 biosensor chip (Biacore, Uppsala, Sweden), calpastatin was diluted to 40 µg/mL in 10 mM sodium acetate pH 4.0. The carboxymethylated dextran layer on the sensor chip surface was activated by derivatization with N-hydroxysuccinimide (NHS) mediated by N-ethyl-N'-(3-diethylaminopropyl)carbodiimide (EDC) according to Fagerstam et al. (1992). Activation, coupling of calpastatin and blocking of reactive groups were performed at a flow rate of 10 µL/min and 25°C. Activation was carried out at through injection of 70 µL 200 mM EDC, 50 mM NHS. Covalent coupling of calpastatin was achieved through injection of 70 µL of the calpastatin solution. Remaining dextran-conjugated NHS-esters were deactivated with 70µL 1 M ethanolamine pH 8.5. Non-covalently bound proteins were washed from the surface with running buffer (HBS-EP: 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4).

Assay for quantification of calpastatin

For this assay monoclonal anti-calpastatin antibody 3B9 was used (Doumit & Koohmaraie, 1999). The antibody was purified from hybridoma cell supernatant using Immunopure immobilized protein G (Pierce, Rockford, IL), according to the manufacturers instructions. The protein concentration of the purified antibody was 4.6 mg/mL. Prior to use, the antibody was diluted 20-fold with HBS-EP.

The heated muscle extracts were filtered over 0.45 µm filters. Samples with a calpastatin content higher than covered by the standard curve were diluted with dialysis buffer. To all samples 0.3 g/L carboxymethylated dextrane from a stock solution of 3 g/L was added to prevent non-specific interaction with the dextrane matrix of the chip. Subsequently, samples were transferred to 96-wells microtitre plates and placed in the biosensor's autosampler compartment. For each series of 12 samples a standard curve of calpastatin (0.05 – 2.0 µg/mL) was included.

The system was operated at a flow rate of 20 µL/min. Before injection of the sample, HBS-EP was passed through the flow cell during 200 s. Prior to injection of the sample, the autosampler mixed 1 part of antibody solution with 9 parts of sample. This mixture was passed through the flow cell during 120 s, followed by HBS-EP during 80 sec. The chip surface was regenerated by injection of 8 mM NaOH during 120 s. followed by HBS-EP during 20 s. The difference in response 5 s before and 5 s after injection of the sample was recorded and expressed as µg calpastatin/mL using the standard curve.

Shear force

Frozen vacuum packaged steaks were tempered for 48 hours at 2°C, cooked during 1 hour in a waterbath heated at 75°C and chilled under running tapwater. Of each sample 10 rectangular subsamples of 1 cm^2 were cut parallel with the fibre direction. Shear force (N/cm^2) was determined perpendicular to the fibre direction using a draw bench (Adamel Lhomargy DY 30; Division d'Instruments S.A., Paris, France) equipped with a triangular shearing blade. The average shear force of the 10 measurements per sample is reported.

Statistical analysis

Significance of correlation coeficients between parameters was determined with Pearson's test using the SAS statistical package.

Results & Discussion

Results from the biosensor method correlated well with the activity measurements, but the intercept of the linear relationship did not pass through zero (Figures 1). The reason for this is that the immunological detectable amount of calpastatin decreases faster during postmortem storage than the amount detectable in the enzymatic assay (Figure 2). A similar observation, using Western blotting, was reported by Geesink & Koohmaraie (1999). The explanation for this is that calpastatin is degraded postmortem, but some proteolytic fragments retain their inhibitory activity (Mellgren & Carr, 1983; Imajoh et al., 1984; Doumit & Koohmaraie, 1999). Thus, the epitope recognized by the antibody is degraded faster than the inhibitory sites of calpastatin. As a consequence, the biosensor assay is not suitable to study the evolution in calpastatin activity during postmortem storage of muscle.

Since post rigor and not pre rigor calpastatin activity is related to tenderness of aged longissimus (Whipple et al., 1990) the calpastatin activity was determined in 30 beef muscle extracts (2 days p.m.) and they were shipped to CCL.

The procedure to produce the extracts includes a dialysis step. Omission of this step would save a considerable amount of time and labor. To test whether this step can be omitted without a detrimental effect on the results, samples of the beef extracts were taken before and after dialysis. Ommission of dialysis resulted in a decreased response in the biosensor assay with a mean value of 0.42 µg/mL. However, the correlation with the measurements on the dialyzed samples was good ($r = 0.82$; data not shown), and correlation with the activity measurements was significant ($p < 0.01$; Figure 3). Thus, the dialysis step can be omitted from the extraction procedure without a detrimental effect on the results.

In the third experiment we tested whether post rigor calpastatin content, as determined using the biosensor assay, is related to the shear force of aged beef. In agreement with results of Whipple et al. (1990), post rigor calpastatin content was significantly correlated ($p < 0.01$) to the shear force of aged longissimus (Figure 4).

The Biacore Q system contains four flow channels, allowing for the sequential analysis of four different compounds in a single sample. The stability of the chip surface has not been fully tested, but we have performed more than 700 assays using a single flow channel. Thus one chip should allow for the measurement of at least 2800 samples, including standard curves.

The repeatability and reproducibility were tested on twelve beef samples which were measured in duplicate on different dates. The mean intra-assay CV's were 3.2% and 5.6% on the different dates. The mean inter-assay CV was 5.8%. This performance is similar to the ELISA for calpastatin developed by Doumit et al. (1996). In comparison, the ELISA showed a stronger correlation with activity measurements than the biosensor assay. The correlation between activity measurements and biosensor measurements can probably be improved by using the same polyclonal antibodies as used in the ELISA. Another important difference between the ELISA and biosensor assays is that with the ELISA a large number of samples can be processed simultaneously whereas the biosensor assay processes them sequentially. However, Biacore has developed a parallel 8-channel system and array systems are in development. With this development, high-throughput

applications appear possible in the near future. One of these possibilities would be on-line screening for calpastatin content as a predictor of beef tenderness. Given the fact that the sample preparation procedure is simple (extraction, heating, clarification), it should also lend itself to automation with sufficient speed to process the number of samples generated at production speed in beef plants.

Conclusions

At present, the calpastatin biosensor assay appears suitable for research purposes where large amounts of samples need to be processed like breed evaluation or selection programs.

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Tables and Figures

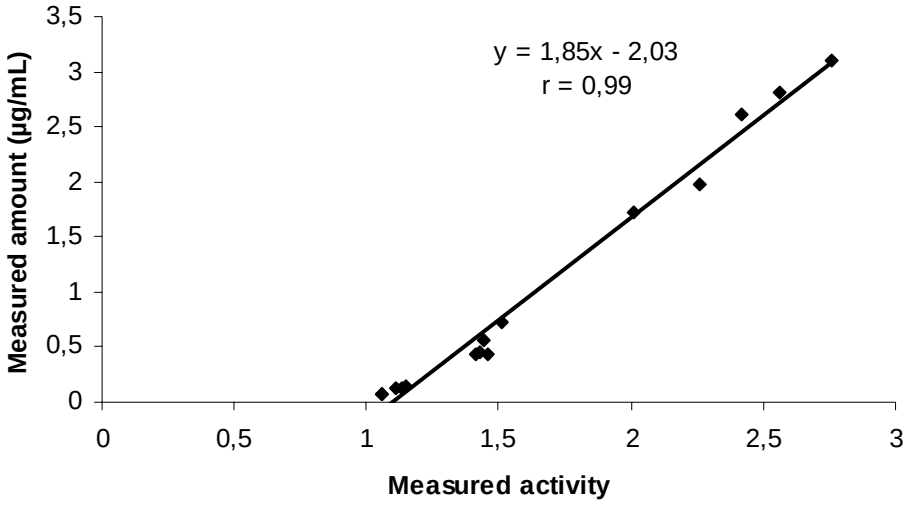

Figure 1. The relation between the activity and immunologically detectable amount of calpastatin in ovine longissimus muscle sampled at 0, 2 and 10 days postmortem.

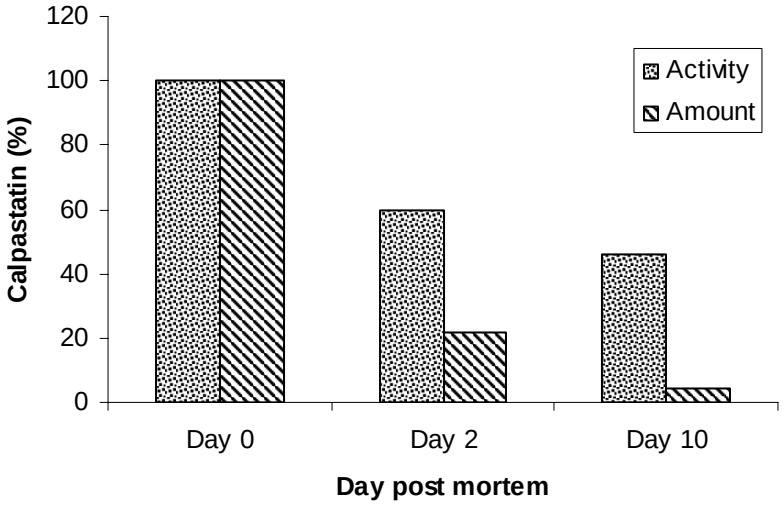

Figure 2. Relative amount of calpastatin in ovine muscle at 0, 2 and 10 days postmortem.

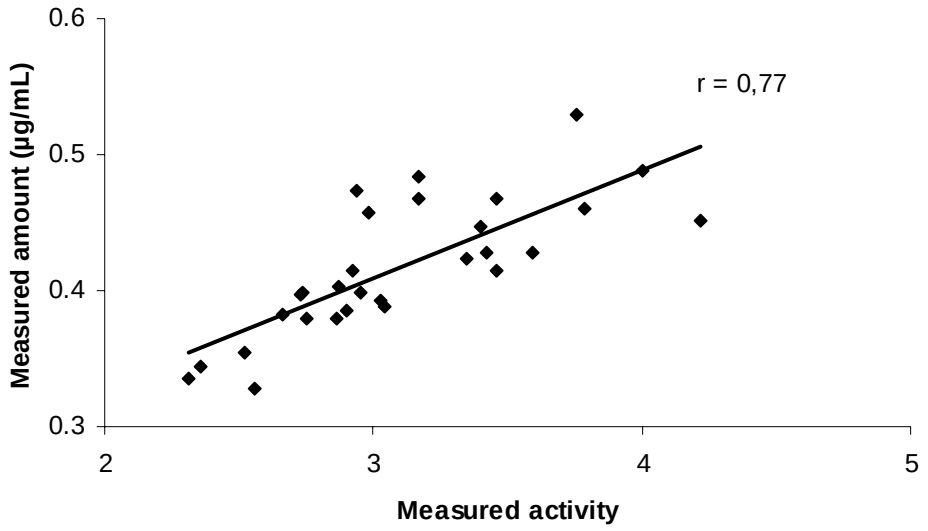

Figure 3. The relationship between the activity and immunologically detectable amount of calpastatin in bovine longissimus sampled at 2 days p.m.

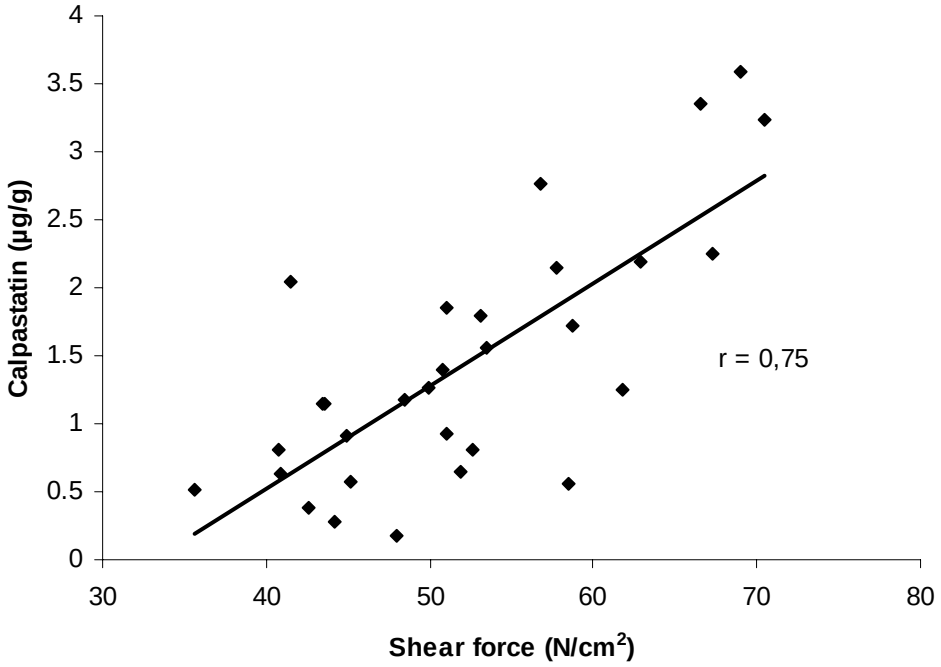

Figure 4. The relationship between the immunologically detectable amount of calpastatin at 1 day p.m. and shear force at 14 days p.m. of bovine longissimus.