Bioelectrochemistry 76 (2009) 93–99

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Label free capacitive immunosensor for detecting calpastatin — A meat tenderness biomarker

Kinga Zór [a,*], Roberto Ortiz [a], Ebru Saatci [b], Ronald Bardsley [c], Tim Parr [c], Elisabeth Csöregi [a], Mihaela Nistor [a]

[a] Department of Analytical Chemistry, University of Lund, 221 00 Lund, Sweden
[b] Department of Biology, University of Erciyes, 38039 Kayseri, Turkey
[c] Division of Nutritional Sciences, University of Nottingham, LE12 5RD, UK

ARTICLE INFO

Article history:
Received 19 December 2008
Received in revised form 27 May 2009
Accepted 4 June 2009
Available online 10 June 2009

Keywords:
Meat tenderness
Calpastatin
Capacitive method

ABSTRACT

An immunological capacitive biosensor for calpastatin was developed, optimized and applied for the analysis of meat extract samples. Anti-calpastatin antibody was immobilized on a gold electrode modified with a self-assembled monolayer of mercaptoundecanoic acid and Protein A from *Staphylococcus aureus*, and the obtained immunosensor was inserted as the working electrode in an electrochemical cell of a flow injection system. The dynamic range of the sensor was 20 to 160 ng/mL calpastatin. The electrode could be regenerated and re-used for more than 7 days with minimal reduction in sensitivity. For the analysis of real samples, the target analyte was extracted from the Longissimus dorsi muscle from beef carcasses directly after slaughtering. The extract was analyzed both with the developed immunosensor and microtiter plate ELISA, and a good correlation was obtained. However the immunosensor offers advantages of speed, simplicity, sensitivity and possibility for miniaturization over conventional assays for calpastatin quantification.

1. Introduction

Meat tenderness is one of the most important palatability characteristics for consumers. Besides genetic factors, meat tenderization is influenced by the nature of feeding, age of the animal, degree of stress prior to slaughter, carcass chilling [1], ageing time and cooking method [2]. Final tenderness is determined by the rate and extent of post-mortem proteolysis of key myofibrillar proteins in the muscle.

The calpain system (calpain-I, calpain-II and calpastatin) is the principal contributor to post-mortem proteolysis which is closely related to meat tenderness. Among the factors affecting the tenderness, post rigor calpastatin activity has the largest effect (~40%) on aged beef Longissimus muscle [3,4].

Currently, there is no available method on the market which can measure meat tenderness in a fast, accurate and objective way. The measurement is usually made either by using taste panels which are expensive, time consuming and subjective, or by Warner–Bratzler Shear Force method, which is rather simple but destructive and is mostly evaluates the resistance of the meat during cutting without providing direct meat tenderness determination [5]. Since this method requires removing a piece of steak from the carcass for performing the test, a non-destructive method for predicting the tenderness would be more desirable [6].

Hydrophobic and ion-exchange chromatography have conventionally been used for quantification of the activities of the calpain proteolytic system [7]. Shackelford et al. estimated the heritability of calpastatin activity and its genetic relationship to tenderness by developing a procedure for the quantification of calpastatin activity by ion-exchange chromatography [3]. It has also been found that calpastatin activity is linearly related to the amount of calpastatin in heated skeletal muscle extracts [8]. Quantification of calpastatin as a meat tenderness biomarker has been performed using different methods such as ELISA [8], surface plasmon resonance [9] or fluorescence resonance energy transfer-based immunosensors [10]. Recently, the development of an optical fiber [11] and a capillary-based biosensor for calpastatin detection in heated meat samples [12] have been reported.

This work investigates the use of a capacitance-based immunosensor as an easy-to-use and sensitive alternative for the determination of calpastatin. Immunosensors are based on the interaction between an immobilized recognition element and the analyte of interest, using an appropriate method for detecting the specific binding. Immunological sensors are in general based on labels attached either to the antibody or to the antigen, but there are also several methods based on label free detection, making the assay simpler, more user-friendly, and in most of cases less expensive.

* Corresponding author. Tel.: +46 46 222 80 98; fax: +46 46 222 4713.
E-mail addresses: Kinga.Zor@analykem.lu.se (K. Zór), Roberto.Ortiz@analykem.lu.se (R. Ortiz), Saatci@erciyes.edu.tr (E. Saatci), Ronald.Bardsley@nottingham.ac.uk (R. Bardsley), Tim.Parr@nottingham.ac.uk (T. Parr), Elisabeth.Csoregi@analykem.lu.se (E. Csöregi), Mihaela.Nistor@analykem.lu.se (M. Nistor).

1567-5394/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2009.06.001

Capacitive label-free immunosensors have been successfully applied in different fields [13–15], which makes this technique a promising tool for direct calpastatin quantification.

The aim of the present work was to develop a fast analytical method for the determination of calpastatin as a key marker of tenderness. The calpastatin protein which is present in the meat extract is recognized by the antibodies immobilized on the electrode surface, this interaction generating changes of the interfacial capacitance. By applying pulse sequences, the calpastatin can be quantified. The quantity of calpastatin measured with the developed immunosensor was in good correlation with the results obtained by ELISA using a spectrophotometric detection.

The biosensor design, the simplicity of the assay, and the possibility for further miniaturization, make this method potentially suitable for a rapid grading of bovine carcasses in terms of tenderness, an important parameter for the meat industry.

2. Materials and methods

2.1. Chemicals

Ethylenediamine tetra-acetic acid (EDTA), dithiothreitol (DTT), 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), leupeptine, 1-3-(dimethylamino) propyl-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) 98%, glycine hydrochloride, sulfuric acid (H_2SO_4) 95–98%, hydrogen peroxide (H_2O_2) 30% and potassium chloride (KCl) 99% were purchased from Sigma-Aldrich (Steinheim, Germany). Bovine serum albumin 96% and protein A from *Staphylococcus aureus* soluble (PrA) were from Sigma Chemical Co. (St. Louis, MO, USA). 11-mercaptoundecanoic acid (MUA) (95%), and the gold rods (Ø 3 mm) 99.99% used as electrode material were purchased from Aldrich (Steinheim, Germany). 0.1 μm alumina slurry AP-D suspension was obtained from Struers (Ballerup, Denmark). Absolute ethanol (99.7%) was purchased from Solveco AB (Rosersberg, Sweden) and hydrochloric acid (HCl) 37% from Merck (NJ, USA).

The bovine recombinant calpastatin (46 kDa) was produced at the University of Nottingham under the European Project contract COOP-CT-2006-032696 and recombinant human calpastatin (70 kDa) was purchased from Fitzgerald Industries International, Inc. (USA). Two types of antibodies were used; the first one, monoclonal anti-calpastatin produced in mouse (clone 1F7E3D10) was from Sigma, and the second type, also a monoclonal anti-calpastatin (clone 2G11D6) was from Fitzgerald.

ELISA coating buffer powder, 5× ELISA diluent solution, wash buffer powder, Super AquaBlue ELISA substrate and Nunc MaxiSorp T flat-bottom 96-well plates were purchased from eBioscience (San Diego, CA, USA). Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H + L) was from Jackson ImmunoResearch Laboratories (Suffolk, UK). All other used chemicals were of analytical grade. All buffers were prepared with distilled water obtained from a Milli-Q system, preceded by a reverse osmosis (Millipore, Bedford, MA) and degassed.

2.2. Immunosensor preparation

The biosensor development includes several steps: cleaning of the active surface of the plain sensor; formation of the self-assembled monolayer (SAM) on the sensor surface with thiols containing active groups (able to be covalently coupled to the protein); attachment of PrA; antibody immobilization; and capacitive measurements of the specific signal on the immunosensor (see Fig. 1).

One of the main requirements to achieve a good quality biosensor for capacitive measurements (i.e., to obtain an ideal SAM and an optimal immobilization of the antibody, and to reduce as much as possible the background noise during capacitance measurements) is the cleanliness of the electrode surface. Therefore, chemical, mechanical and electrochemical cleaning procedures were used, since the combination of these techniques gives the best reproducibility of gold surfaces [16]. The gold electrodes were dipped for 3 min in freshly prepared piranha solution (1:3 H_2O_2:H_2SO_4) in order to remove all the organic materials from the surface prior to polishing. Using 0.1 μm alumina slurries in suspension (1 mg in 100 and 200 mL, respectively), the electrode surface was polished using an in-house made polishing machine and then, rinsed with water and dried under N_2. The electrodes were placed in a Teflon holder and cleaned electrochemically in 0.5 M H_2SO_4 solution by cycling the applied potential from −0.2 to 1.7 V versus Ag/AgCl reference electrode with a scan rate of 100 mV/s (14 cycles). After the electrochemical treatment, the electrodes were placed in plasma cleaner (PDC-3XG, Harrich, NY, USA) for 15 min to obtain a sterile surface. Immediately after, the electrodes were immersed in MUA solution (1 mM in ethanol) and left to react overnight [17–19].

For a better orientation of the antibody on the electrode surface, several authors reported the pre-immobilization of PrA [20,21]. PrA binds specifically to the Fc region of antibody molecules, leading to a proper orientation of the bound antibody on surfaces. A favorable orientation of the immobilized protein makes easier for the antigen to reach active sites of the immobilized antibody. This theoretically increases the degree of the antibody–antigen interaction and, in this way, the specific signal.

Prior to covalent coupling with PrA (2 mg/mL in buffer for 2 h), the carboxylic groups from the SAM were activated using 0.2 M EDC/0.5 M NHS solution [22] for 1 h. After each step the electrodes were rinsed and dried under N_2.

The immobilization of the biorecognition element was carried out by placing a 25 μL drop of the anti-calpastatin antibody solution on the top of the electrode surface after PrA immobilization. The reaction took place overnight; the electrodes were stored in a glass beaker covered with a plastic foil for protecting the immobilized surface and to avoid evaporation. All the immobilization steps have been performed at room temperature, except the antibody immobilization which took place at 4 °C. Before inserting the electrodes in the electrochemical cell, they were rinsed with 10 mM phosphate buffer pH 7.4, which was also used as running buffer in the capacitance system. For long term storage, the sensors were kept in the same buffer solution at 4 °C. Additional blocking of the unspecific sites on the immobilized surface was reported [14,23]; however, the procedure was tested but not used since it proved not to be necessary, probably due to the long chain thiols used for SAM formation on the electrode surface.

2.3. Capacitive measurements

The antibody-modified gold electrode was inserted as the working electrode in an electrochemical detection cell with an internal volume of 10 μL, as shown in Fig. 2.

The capacitive system was built on a 4 electrode setup, containing a working, an Ag/AgCl reference, a Pt counter (Ø 0.5 mm), and an auxiliary Pt reference electrode (Ø 15 mm). The fourth electrode, the auxiliary Pt reference electrode had no defined potential, and the Ag/AgCl electrode, placed in the outlet flow stream, was only used to correct the potential of the auxiliary Pt reference electrode. The computer matched the potential of the auxiliary Pt wire and the Ag/AgCl reference electrode, before application of the potential pulse. A 50 mV potential step was applied for 20 ms in order to perturb the interface layer at the working electrode. During the 50 mV potential pulse, the resulting current response was collected and processed by a Keithley 575 measurement and control system equipped with an AMM2 master analogue to digital converter module. An acquisition frequency of 50 kHz allowed sampling of 1000 values during the 20 ms potential pulse applied. The potential was stepped back to rest (0 V versus Ag/AgCl) between two consecutive pulses. This procedure was repeated, and an average of 10 pulses was sent to a computer with compatible software and visualized as a plot of

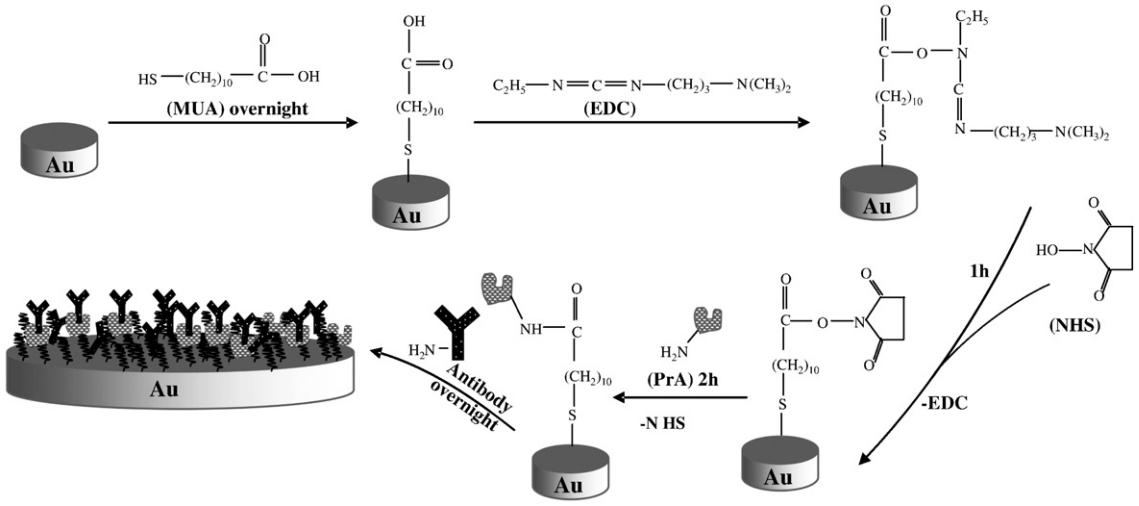

Fig. 1. Immobilization of the antibody on the SAM formed on gold electrodes.

capacitance versus time. Simultaneously, data were stored in the computer for manual processing. The resulting current (Eq. (1)) was measured:

$$i(t) = u / R_s \exp(-t / R_s C_{total}) \quad (1)$$

where $i(t)$ is the current in the circuit as a function of time, u is the applied pulse potential, R_s is the dynamic resistance of the self-assembled layer, C_{total} is the total capacitance measured at the working electrode/solution interface, and t is the time elapsed after the potentiostatic step was applied [24]. The total capacitance, the capacitance at an electrode/solution interface, C_{total}, can be described by Eq. (2):

$$1 / C_{total} = 1 / C_{ins} + 1 / C_{rec} + 1 / C_{dl}. \quad (2)$$

The first capacitor (C_{ins}) represents the insulating layer created by the thiol (MUA) immobilization. C_{rec} includes the capacitance contribution from the recognition element (antibody) with or without antigen binding. The capacitance of the double layer (C_{dl}) extending out in the bulk solution is created by the formation of a space-charge by the ions in buffer solution. When the antigen binds to the antibody, a hydrophobic layer is created, which moves the double layer further out in the solution. The lowest capacitance will dominate the total capacitance, thus the capacitance of the insulating layer should be as large as possible to make the change caused by the antigen binding to be dominant [23,25].

The total capacitance was measured every minute and plotted as a function of time. The binding of the antigen to the immobilized antibody causes a decrease in capacitance. The change due to the binding (ΔC) was calculated by subtracting C_{total} after binding ($C_{total\ Ab–Ag}$) from the C_{total} before binding ($C_{total\ Ab}$). The surface specific capacitance (ΔC_s) was calculated according to Eq. (3), by dividing ΔC to the active surface of the electrode ($S = 0.071\ cm^2$).

$$\Delta C_s = \left(C_{total\ Ab} - C_{total\ Ab–Ag}\right) / S = \Delta C / S \quad (3)$$

2.4. Cyclic voltammetry measurements

Cyclic voltammograms were recorded in 10 mM $[Ru(NH_3)_6]Cl_3$, 0.1 M KCl (applied potential from −0.5 to 0.2 V) or in 10 mM $K_3[Fe(CN)_6]$, 0.1 M KCl, (applied potential from −0.2 to 0.6 V versus Ag/AgCl). The measurements were carried out in a batch cell, with the unmodified/modified gold electrode as the working electrode, an Ag/AgCl as the reference electrode, and a platinum wire as the auxiliary electrode. The scan rate used for all scans was 100 mV/s. The electrodes were connected to an AutoLab potentiostat–galvanostat PGSTAT 12 (Utrecht, Netherlands).

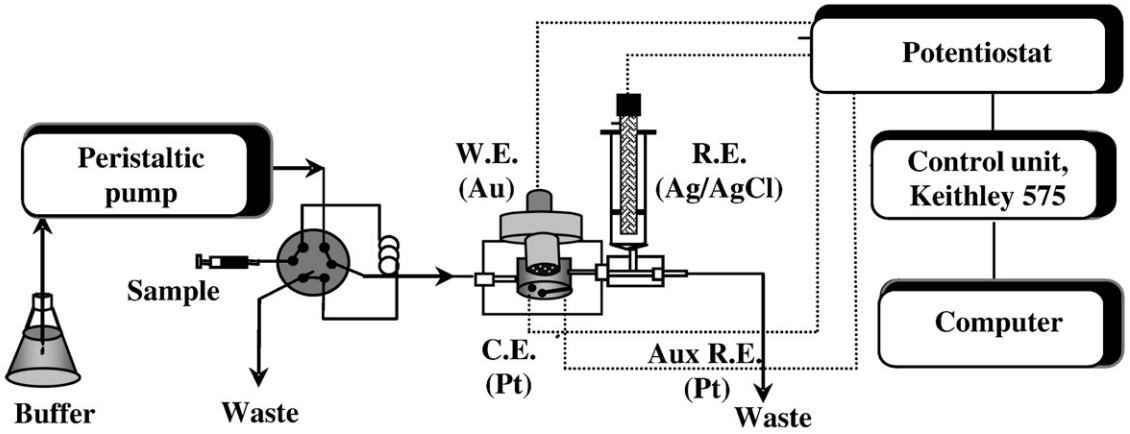

Fig. 2. Schematic drawing of the capacitive system Symbols: W.E. — working electrode, R.E. — reference electrode, Aux R.E. — auxiliary reference electrode, and C.E. — counter electrode.

2.5. ELISA measurements

A previously described method based on ELISA [8,26] for calpastatin detection was modified and used for validating the capacitive measurements.

Nunc MaxiSorpT flat-bottom 96-well plates were coated with calpastatin standard or heated meat extract diluted in coating buffer and incubated overnight at 4 °C. The plates were blocked with 5× ELISA diluent solution diluted in Milli-Q-grade, and incubated at room temperature for 1 h. Calpastatin mouse monoclonal primary antibody was incubated for 2 h at room temperature followed by 2 h incubation with HRP-conjugated goat anti-mouse IgG both diluted in 5× ELISA diluent solutions. Between each modification step a washing step (250 µL of washing buffer/well; repeated 5 times/washing step) was introduced. Finally, Super AquaBlue ELISA substrate was added to the plate and the product of the reaction was recorded with a microplate reader (BioTek ELx800) at 405 nm.

2.6. Preparation of meat extracts

1 g bovine Longissimus dorsi muscle was sampled as soon as possible after slaughter and dropped into liquid nitrogen for storage at −80 °C. Frozen tissues were ground in a small amount of liquid nitrogen in a pestle and mortar and homogenized (Polytron, 2×20 s bursts), cooling the homogenate on ice with five volumes of ice-cold 100 mM Tris–HCl, 10 mM EDTA pH 8.3, containing 2 mM DTT, and protease inhibitors such as AEBSF (1 mM) and leupeptin (1 mg/mL) [27]. The homogenates were centrifuged at 14000 ×g at 4 °C for 20 min and the supernatant was filtered through glass wool. This supernatant was further heated in a water bath until it reached 95 °C and was held at this temperature for 10 min. The precipitated protein was removed by centrifugation (14000 ×g at 4 °C for 20 min), and calpastatin (being heat stable) remained in the supernatant. This boiled supernatant was used as an analytical sample for determination of calpastatin.

3. Results and discussion

3.1. Characterization of insulating properties of immobilized layers

For obtaining capacitive immunosensors with high sensitivity and selectivity, it is important to have a good electrically insulated SAM and a proper orientation of the biorecognition element on the electrode surface. SAM formation of thiols on gold surfaces have been widely studied and it has been found that increasing the chain length of the used thiols increases the SAM organization, the surface coverage and packing density, achieving a well organized, good insulating layer on the electrode surface [17,19,28]. Characterization with electrochemical impedance spectroscopy showed that the SAM formed using long chain thiols (e.g., MUA) versus short chain thiols is more effectively blocking the electron transfer to the surface [29]. In the present work, after self-assembling MUA on the gold surface and its activation of the free carboxyl groups by using EDC/NHS, PrA was used for specific binding to the Fc region of antibody molecules, for a proper orientation of the immobilized antibody [20,22,30] and for an easy regeneration of the immunosensor [31]. Through the PrA, the active sites of the antibodies are more easily reachable for the antigen.

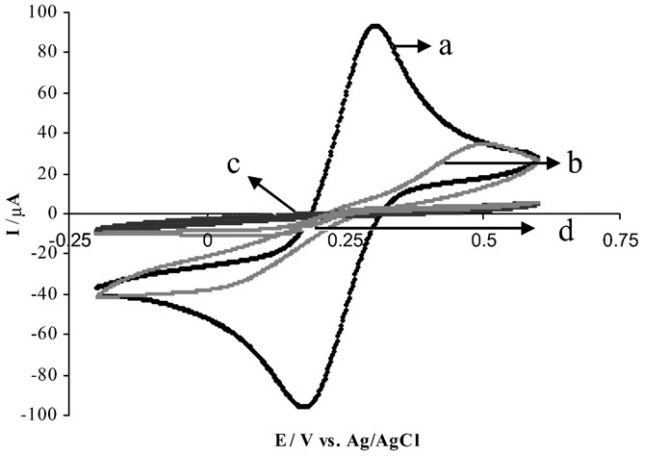

Fig. 3. Cyclic voltammograms recorded for a clean gold electrode (a), a gold modified with SAM of MUA without (b) or with an additional layer of PrA (c) and with the final immobilized antibodies (d). Measurements were performed in 10 mM $K_3[Fe(CN)_6]$, 0.1 M KCl, with a scan rate of 100 mV/s.

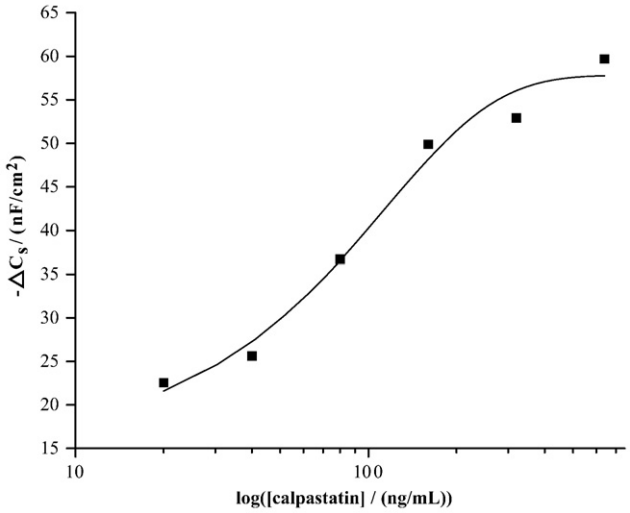

Fig. 4. Calibration curve for bovine recombinant calpastatin using an electrode modified with anti-calpastatin antibody.

The blocking degree of the electrode surface was evaluated using cyclic voltammetry, employing two redox couples with different charges ($Ru(NH_3)_6^{+2/+3}$ and $Fe(CN)_6^{-3/-4}$) in solution. On a clean gold surface, both redox couples are reversibly oxidized and reduced at the metal surface during cycling. When the electrode was modified with the negatively charged MUA-SAM, different behaviour to the electron transfer was observed for the tested couples: the blocking feature obtained in $Fe(CN)_6^{-3/-4}$ was more drastic as compared with the one obtained in $Ru(NH_3)_6^{+2/+3}$ solution. This phenomenon could be explained knowing that ferrocyanide anions were repelled by the negatively charged COO^- groups and the ruthenium complex cations were attracted and could thus further penetrate through the MUA monolayer to the gold surface [32]. Since the experiments in the presence of $Ru(NH_3)_6^{+2/+3}$ did not give a clear indication on the presence/ absence of the SAM, blocking of the electrode transfer between the redox couple and the electrode was further evaluated only in the presence of $Fe(CN)_6^{-3/-4}$. Fig. 3 shows how the blocking increases with each additional layer immobilized on the electrode surface.

3.2. Optimisation steps

Antibody–antigen interactions are usually performed in buffers with a relatively high salt concentration (about 150 mM equivalents NaCl) which mimics the physiological conditions and allows the best possible binding. Moreover, the high salt concentration considerably reduces the possibility of non-specific interactions of other proteins than the target with the modified electrode surface, which is an important aspect when developing immunoassay based on direct interaction, such as capacitance-based immunosensors.

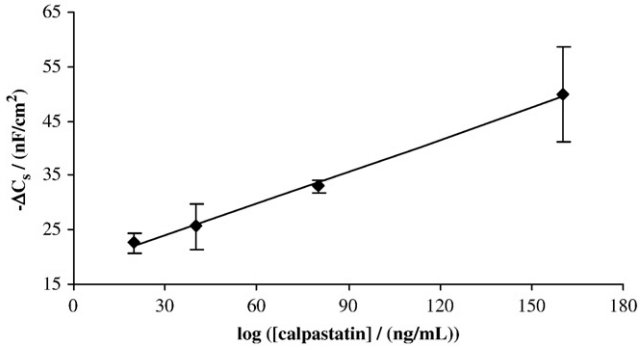

Fig. 5. The linear range of the calibration curve using electrodes modified with anti-calpastatin antibody. The error bars show the reproducibility between three different immunosensors; the linear regression factor being 0.9975.

The initial experiments were carried out with 10 mM phosphate buffer pH 7.4 containing 100 mM NaCl, both as a sample solvent and carrier buffer. Unfortunately, the high salt concentration resulted in an unstable baseline and high noise. The signal noise and drifting, the background capacitance and the time for signal stabilization were considerably reduced in the absence of NaCl, which made the detection more sensitive and accurate. An optimum concentration (10 mM) of the buffer solution was previously reported in the literature [13,14,33]. The effect of high salt concentration on the capacitive measurements was studied by Berggren et al. [23] and proved that relatively low concentrations of electrolyte are required for capacitance measurements.

The immunosensors were further optimised with respect to the flow rate of the carrier buffer, the injection volume of the standard/sample solution and the amount of the immobilized antibody on the electrode surface (data not shown). The optimum conditions found were: buffer flow rate 100 μL/min, injection volume 250 μL and 1:4 antibody dilution.

3.3. Characterisation of the calpastatin immunosensor

Considering that the signal intensity of the capacitive measurement based on antibody–antigen interaction depends on the antigen structure, charge, molecular weight and the affinity constant of antibody–antigen complex [13], two antigens (recombinant human calpastatin and recombinant bovine calpastatin) were tested against two monoclonal calpastatin antibodies (i.e., Fitzgerald and Sigma) in order to find the best antibody–antigen couple for the development of the capacitive immunosensor.

Fig. 4 shows the calibration curve of the recombinant bovine antigen having anti-calpastatin antibody (Fitzgerald) immobilized on the electrode surface. As shown in Fig. 5, the linear range for the sensor was from 20 up to 160 ng/mL, the values shown being the mean of three equally prepared immunosensors. The error bars show that there is a high variability (almost 20%) between electrodes, which means that a calibration is necessary for each immunosensor before its use for analysis of real samples. The dynamic range of the sensor based on the commercially available antigen (recombinant human calpastatin) was found to be from 100 to 600 ng/mL. Decreasing the detection limit allows the real samples (heated meat extracts) to be diluted in order to diminish the effect of interfering compounds.

The introduction of a regeneration step between injections was necessary in order to be able to perform several analyses with the same sensor, thus, reducing the cost of the analyses, as well as generating more accurate results, because the variability of the measurements performed with different sensors is higher than the one obtained with the same electrode. According to Berggren et al. [13], the regeneration reagent (100 mM glycine/HCl pH 2.8) used to break the antibody–antigen interaction destroys the electric insulation layer of the sensors. Other publications report that the electrodes can be re-used after several regeneration steps [14,15]. In our experiments, when regenerating the calpastatin immunosensor after each analyte injection with 25 mM glycine–HCl, pH 2.4 [34], the electrodes could be used for several consecutive measurements; no damage on the insulating layers was observed, thus avoiding any decrease in sensitivity (see Fig. 6).

The non-specific response of the developed immunosensor was tested and no signal was obtained for either the extraction buffer or for a similar concentration of bovine serum albumin (up to 100 ng/mL) — a model protein used as reference.

To establish the optimal dilution factor to be used in order to eliminate the possible effects of interfering compounds from the meat extract, the samples were diluted to different levels with carrier buffer and analyzed with sensors containing or lacking the specific antibody immobilized on the surface of the electrode. As shown in Fig. 7 at a dilution lower than 5 times, no non-specific response was observed. The samples were thus diluted at least 5-fold with the carrier buffer prior to further analysis.

The storage and operational stability of the developed capacitive immunosensor was also tested. A decrease in sensitivity of only about 8.5% could be observed after 7 days of storage at 4 °C between measurements. Below 10% decrease in capacitance was displayed after 6 h of consecutive injections of calpastatin solutions with different concentrations from the linear range of the sensor.

3.4. Determination of calpastatin in real samples

The amount of calpastatin can vary not only between species [35], but also between breeds of the same species [3] as well as within various muscles of the same animal [36,37]. It has also been observed that the immunologically detectable calpastatin decreased faster during post-mortem storage that the detectable amount in the enzyme assay [9,38], which could be due to the fact that calpastatin

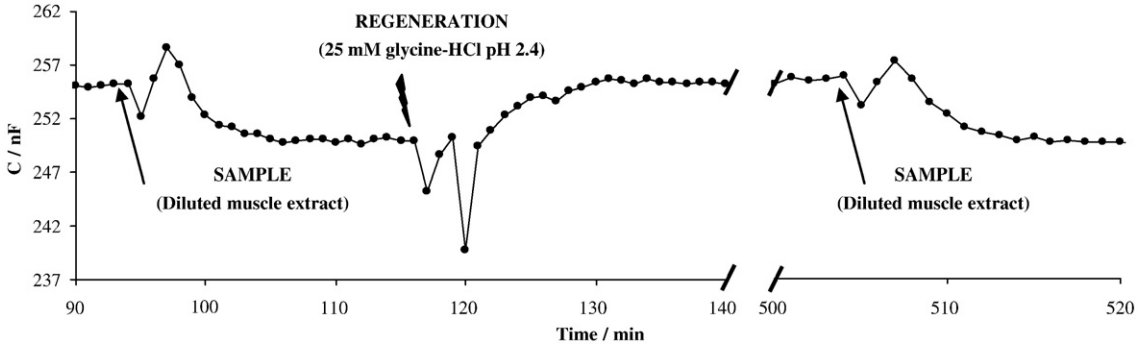

Fig. 6. Baseline stability and signal repeatability before and after 10 injection/regeneration cycles during 8.5 h of continuous measurements. The injected sample was a 2 time diluted heated muscle extract.

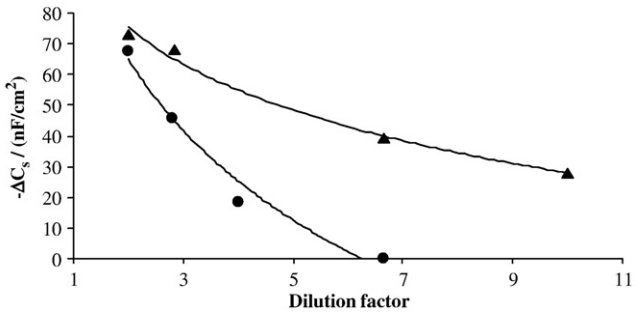

Fig. 7. Specific (▲) and non-specific (●) interaction of the heated meat sample on an electrode modified with (▲)/or without (●) anti-calpastatin antibody, for different sample dilutions. In the last case the gold electrode was modified with SAM of MUA and PrA.

degradation fragments still posses some inhibitory activity [38]. Taking into consideration this fact, muscle samples were taken directly after slaughtering, when the best correlation between calpastatin quantity and activity is obtained [9]. The muscle sample was taken from bovine Longissimus dorsi, the most frequently analyzed muscle type for this purpose. Calpastatin concentration in heated muscle samples was found to be 482 ng/mL ($N=3$, CV = 9.5%). This value is in good correlation with the calpastatin amount found in heated bovine muscle (Longissimus dorsi) samples by Geesink et al. [9] where the calpastatin quantity was shown to vary between 350 and 550 ng/mL in non-dialysed heated samples.

The results obtained with the capacitive system (482 ng/mL) have been compared with the data obtained from ELISA, where the amount of immunoreactive calpastatin was found to be 428 ng/mL ($N=3$, CV = 6.3%).

4. Conclusions

The aim of this work was to develop a label free capacitive sensor for direct detection of calpastatin from meat sample extracts. The results yielded by the optimized sensor were in good agreement with the ELISA methods used as a reference. In comparison with other described techniques for calpastatin quantification, the capacitive sensor described here is simpler to prepare, easy to use and offers the possibility of further miniaturization. The immunoassay developed by Doumit et al. [8] allows the analysis of many samples at the same time; however, it is very difficult to miniaturize it for its possible use in a portable detector prototype. The calpastatin sensors developed by Grant et al. [10] based on fluorescence resonance energy transfer, and by Bratcher et al. [11,12] require protein labeling which makes them more expensive. As reported for the SPR biosensor developed by Geesink et al. [9], the capacitive immunosensor could be adapted to a platform giving the possibility of several measurement at the same time, increasing more chances for a future usage of these techniques in the meat industry.

Acknowledgement

The authors are grateful to the European Commission (FP6) for financial support within the framework of the Tendercheck project (COOP-CT-2006-032696; web page: http:// Tendercheck.cric-projects.com).

References

[1] S.G. May, H.G. Dolezal, D.R. Gill, F.K. Ray, D.S. Buchanan, Effect of days fed, carcass grade traits, and subcutaneous fat removal on postmortem muscle characteristics and beef palatability, J. Anim. Sci. 70 (1992) 444–453.
[2] D.M. Wulf, J.B. Morgan, J.D. Tatum, G.C. Smith, Effects of animal age, marbling score, calpastatin activity, subprimal cut, calcium injection, and degree of doneness on the palatability of steaks from limousin steers, J. Anim. Sci. 74 (1996) 569–576.
[3] S.D. Shackelford, M. Koohmaraie, L.V. Cundiff, K.E. Gregory, G.A. Rohrer, J.W. Savell, Heritabilities and phenotypic and genetic correlations for bovine postrigor calpastatin activity, intramuscular fat content, Warner–Bratzler shear force, retail product yield, and growth rate, J. Anim. Sci. 72 (1994) 857–863.
[4] G. Whipple, M. Koohmaraie, M.E. Dikeman, J.D. Crouse, Predicting beef-longissimus tenderness from various biochemical and histological muscle traits, J. Anim. Sci. 68 (1990) 4193–4199.
[5] W.J. Platter, J.D. Tatum, K.E. Belk, P.L. Chapman, J.A. Scanga, G.C. Smith, Relationships of consumer sensory ratings, marbling score, and shear force value to consumer acceptance of beef strip loin steaks, J. Anim. Sci. 81 (2003) 2741–2750.
[6] M. Koohmaraie, G.H. Geesink, Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system, Meat. Sci. 74 (2006) 34–43.
[7] M. Koohmaraie, Quantification of Ca2(+)-dependent protease activities by hydrophobic and ion-exchange chromatography, J. Anim. Sci. 68 (1990) 659–665.
[8] M.E. Doumit, S.M. Lonergan, J.R. Arbona, J. Killefer, M. Koohmaraie, Development of an enzyme-linked immunosorbent assay (ELISA) for quantification of skeletal muscle calpastatin, J. Anim. Sci. 74 (1996) 2679–2686.
[9] G.H. Geesink, J.G.P. Van der Palen, M.P. Kent, E. Veiseth, G. Hemke, M. Koohmaraie, Quantification of calpastatin using an optical surface plasmon resonance biosensor, Meat Sci. 71 (2005) 537–541.
[10] S.A. Grant, R.C. Stringer, S. Studer, D. Lichlyter, C.L. Lorenzen, Viability of a FRET dual binding technique to detect calpastatin, Biosens. Bioelectron. 21 (2005) 438–444.
[11] C.L. Bratcher, S.A. Grant, R.C. Stringer, C.L. Lorenzen, Correlation between a novel calpastatin biosensor and traditional calpastatin assay techniques, Biosens. Bioelectron. 23 (2008) 1429–1434.
[12] C.L. Bratcher, S.A. Grant, J.T. Vassalli, C.L. Lorenzen, Enhanced efficiency of a capillary-based biosensor over an optical fiber biosensor for detecting calpastatin, Biosens. Bioelectron. 23 (2008) 1674–1679.
[13] C. Bergreen, G. Johansson, Capacitance measurements of antibody–antigen interaction in a flow system, Analytical Chemistry 69 (1997) 3651–3657.
[14] M. Hedstrom, I.Y. Galaev, B. Mattiasson, Continuous measurements of a binding reaction using a capacitive biosensor, Biosens. Bioelectron. 21 (2005) 41–48.
[15] W. Limbut, P. Kanatharana, B. Mattiasson, P. Asawatreratanakul, P. Thavarungkul, A comparative study of capacitive immunosensors based on self-assembled monolayers formed from thiourea, thioctic acid, and 3-mercaptopropionic acid, Biosens. Bioelectron. 22 (2006) 233–240.
[16] R.F. Carvalhal, R.S. Freire, L.T. Kubota, Polycrystalline gold electrodes: a comparative study of pretreatment procedures used for cleaning and thiol self-assembly monolayer formation, Electroanalysis 17 (2005) 1251–1259.
[17] D.L. Allara, R.G. Nuzzo, The application of reflection infrared and surface enhanced Raman spectroscopy to the characterization of chemisorbed organic disulfides on Au, J. Electron Spectrosc. Relat. Phenom. 30 (1983) 11.
[18] R.G. Nuzzo, D.L. Allara, Adsorption of bifunctional organic disulfides on gold surfaces, J. Am. Chem. Soc. 105 (1983) 4481–4483.
[19] M.D. Porter, T.B. Bright, D.L. Allara, C.E.D. Chidsey, Spontaneously organized molecular assemblies. 4. Structural characterization of n-alkyl thiol monolayers on gold by optical ellipsometry, infrared spectroscopy, and electrochemistry, J. Am. Chem. Soc. 109 (1987) 3559–3568.
[20] E. Briand, M. Salmain, J.M. Herry, H. Perrot, C. Compere, C.M. Pradier, Building of an immunosensor: how can the composition and structure of the thiol attachment layer affect the immunosensor efficiency? Biosens. Bioelectron. 22 (2006) 440–448.
[21] T. Moks, L. Abrahmsen, B. Nilsson, U. Hellman, J. Sjöquist, M. Uhlen, Staphylococcal protein A consists of five IgG-binding domains, Eur. J. Biochem 156 (1986) 637–643.
[22] A. Tlili, A. Abdelghani, S. Hleli, M.A. Maaref, Electrical characterization of a thiol SAM on gold as a first step for the fabrication of an immunosensors based on a quartz crystal microbalance, Sensors 4 (2004) 105–114.
[23] C. Berggren, B. Bjarnason, G. Johansson, An immunological interleukine-6 capacitive biosensor using perturbation with a potentiostatic step, Biosens. Bioelectron. 10 (1998) 1061–1068.
[24] C. Berggren, B. Bjarnason, G. Johansson, Instrumentation for direct capacitive biosensors, Instrumentation Science & Technology 27 (1999) 131–139.
[25] C. Berggren, B. Bjarnason, G. Johansson, Capacitive biosensors, Electroanalysis 13 (2001) 173–180.
[26] H. Yokota, M. Katayama, F. Hino, I. Kato, E. Takano, M. Maki, M. Hatanaka, T. Murachi, Direct measurement of calpastatin subtypes by sandwich enzyme immunoassay using monoclonal antibodies, Mol. Cell. Probes 5 (1991) 261–269.
[27] T. Parr, P.L. Sensky, R.G. Bardsley, P.J. Buttery, Calpastatin expression in porcine cardiac and skeletal muscle and partial gene structure, Arch. Biochem. Biophys. 395 (2001) 1–13.
[28] J.J. Gooding, F. Mearns, W. Yang, J. Liu, Self-assembled monolayers into the 21st century: recent advances and applications, Electroanalysis 15 (2003) 81–96.
[29] R.K. Mendes, R.S. Freire, C.P. Fonseca, S. Neves, L.T. Kubota, Characterization of self-assembled thiols monolayers on gold surface by electrochemical impedance spectroscopy, J. Braz. Chem. Soc. 15 (2004) 849–855.
[30] B. Lu, M.R. Smyth, R. O Kennedy, Oriented immobilization of antibodies and its applications in immunoassays and immunosensors, Analyst 3 (1996) 29R–32R.
[31] J. Quinn, P. Patel, B. Fitzpatrick, B. Manning, P. Dillon, S. Daly, R. O Kennedy, M. Alcocer, H. Lee, M. Morgan, K. Lang, The use of regenerable, affinity ligand-based surfaces for immunosensor applications, Biosens. Bioelectron. 14 (1999) 587–595.
[32] K. Takehara, H. Takemura, Y. Ide, Electrochemical studies of the terminally substituted alkanethiol monolayers formed on a gold electrode: effects of the terminal group on the redox responses of $Fe(CN)_6^{3-}$, $Ru(NH_3)_6^{3+}$ and ferrocenedimethanol, Electrochim. Acta 39 (1994) 817–822.

[33] I. Bontidean, C. Berggren, G. Johansson, E. Csoregi, B. Mattiasson, J.R. Lloyd, K.J. Jakeman, N.L. Brown, Detection of heavy metal ions at femtomolar levels using protein-based biosensors, Anal. Chem. 70 (1998) 4162–4169.
[34] W. Limbut, M. Hedström, P. Thavarungkul, P. Kanatharana, B. Mattiasson, Capacitive biosensor for detection of endotoxin, Anal. Bioanal. Chem. 389 (2007) 517–525.
[35] A. Ouali, A. Talmant, Calpains and calpastatin distribution in bovine, porcine and ovine skeletal muscles, Meat Sci. 28 (1990) 331–348.
[36] R.G. Taylor, M. Koohmaraie, Effects of postmortem storage on the ultrastructure of the endomysium and myofibrils in normal and callipyge longissimus, J. Anim. Sci. 76 (1998) 2811–2817.
[37] C.L. Lorenzen, M. Koohmaraie, S.D. Shackelford, F. Jahoor, H.C. Freetly, T.L. Wheeler, J.W. Savell, M.L. Fiorotto, Protein kinetics in callipyge lambs, J. Anim. Sci. 78 (2000) 78–87.
[38] M.E. Doumit, M. Koohmaraie, Immunoblot analysis of calpastatin degradation: evidence for cleavage by calpain in postmortem muscle, J. Anim. Sci. 77 (1999) 1467–1473.