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