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Detection of plasmin based on specific peptide substrate using acoustic transducer

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ABSTRACT

In this work we report the detection of plasmin protease by means of the thickness shear mode (TSM) acoustic method. The biorecognition element consists of a peptide substrate (PS) specific to plasmin immobilized on a piezoelectric quartz crystal electrode. After enzymatic reaction with plasmin, it cleaves a short fragment of the peptide causing increase in the resonance frequency of the piezo crystal. Plasmin was detected in the range of concentrations 1–20 nM, a target interval in which its presence presumably affects the quality of milk. The PS exhibited negligible response against to similar protease trypsin. This has been confirmed also by electrochemical detection method. Limit of detection of this acoustic transducer was found to be 0.65 nM. Formation of the sensing surface and kinetic effect of plasmin on the peptide substrate was studied by atomic force microscopy (AFM). The PS response was also validated in pretreated milk samples spiked by known concentrations of plasmin achieving an average recovery of 63 ± 0.6%.

1. Introduction

Proteolytic activities in milk comprise both, positive and negative impacts on industrial applications. For instance, it has been demonstrated that degradation of milk proteins highly influences cheese ripening through development of desirable changes in flavor and consistency. In contrast, proteolysis is undesirable when it results in gelation and bitterness due casein breakdown as observed in ultra high temperature (UHT) treated milk [1]. Proteolysis of milk has been connected to the natural serine protein plasmin, the most abundant endogenous protease transferred from blood into bovine milk [2]. Despite of the quantification of plasmin activity in dairy products could have important industrial impacts, not enough information has been yet provided about the amounts of plasmin present and its effect on the properties of the product. Additionally, since plasmin is a part of an intricate enzymatic-inhibitory system its detection is not a simple task. Factors such as cow characteristics, processing conditions, other milk components, storage conditions, bacterial proteases and mutual interaction of other components of the plasmin system could compromise the accurate and effective detection of this protease [3].

Up to date, plasmin has been mainly detected by immunological and spectroscopic methods [4]. Separation techniques such as reversed-phase HPLC [5], gel electrophoresis [6], tri-nitro-benzene-sulfonic acid (TNBS) [7] and fluorometric methods [8] have also been reported. Fluorescence technique based on fluorescamine has the advantage of the lowest detection limit compared with the other methods, whereas gel electrophoresis has shown to be the best qualitative method; however none of these is as sensitive as HPLC. The TNBS method is recommended for use in routine laboratory analysis on the basis of its accuracy, reliability and simplicity, but lacks high sensitivity and exhibits low limit of detection [7]. Recently, a peptide substrate (PS) consisting of four amino acids specifically synthesized for cleavage of plasmin has been implemented as biorecognition element in electrochemical and photometric biosensors [9,10]. Although highly effective and sensitive, these methods are not completely adaptable to in situ and real time conditions usually required when analyzing food samples. Furthermore, confrontation with other techniques

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less depended on interferences and surrounding cross-reactivity is highly demanded for validating the response of a biosensor on plain buffer and turbid samples, as it is the case of milk.

The thickness shear mode (TSM) acoustic transducer is a well established tool for the detection of mass and interfacial viscosity of affinity interactions at surface. TSM works in a closed fluidic framework and it is based on the application of a high frequency AC voltage across an AT-cut quartz crystal on which due to the piezoelectric effect, an acoustic shear wave is generated and propagated through the sensing layer. The viscous forces result in friction between the surrounding liquid and the layer immobilized on the surface of the piezo crystal electrode. This effect is reflected as changes in the motional resistance (R_m) and in the resonant frequency (f_s) respectively [11]. In contrast with traditional QCM technique, TSM allows the analysis of complex electrical impedance to receive information about the layer properties even when relatively small molecules adsorb to the surface, which do not contribute to the mass but only to viscoelastic properties of the layer [12]. Until nowadays, TSM transducers have been extensively used for sensitive monitoring of several biological processes including: adhesion [13,14], affinity interactions [15] (reversible sensing of insulin in this work has been achieved by method based on imprinted polymers and antibody replicae), adsorption-desorption [16,17] or cleavage [18,19] since they offer exceptional response in real time surface monitoring. Particularly, TSM has been applied to real-time direct detection of probiotic bacteria in fermented dairy products [20]. Another acoustic biosensor application was recently published demonstrating direct label-free immunosensor using a quartz crystal microbalance (QCM) transducer for detection of staphylococcal enterotoxins in food matrices of milk and cheese [21].

On the other hand, a complementary technique for controlling biosensing surface events is atomic force microscopy (AFM). AFM is a useful tool for collecting surface information about topography, ultrastructure and changes in morphology after biochemistry interaction with surfaces [22,23], therefore it is ideal method for imaging of very fragile and sensitive biological samples such as live cells, oligonucleotides or proteins.

We report an acoustic method of detection based on a specific peptide substrate for in situ monitoring of enzymatic activity of plasmin. Additionally, the activity of plasmin on the peptide substrate is visualized and examined in real time conditions by AFM. These imaging experiments served for optimizing the preparation of sensing layer as well as helped to elucidate the mechanism into which the peptide undergoes by the action of plasmin at different intervals of time. According to our knowledge, there is no acoustic detector reported so far for detection of plasmin. Moreover, in contrast with other detection methods previously developed, acoustic and imaging techniques provide a deeper insight occurring at the sensing surface. Therefore, we believe that the proposed approach constitutes a useful tool for protease analysis and it could eventually contribute to practical and specific detection of low concentrations of plasmin in milk samples, a concern with industrial implications that is still under subject of study.

2. Materials and methods

2.1. Reagents

Spectrozyme substrate was obtained from American Diagnostica (Germany), urokinase from human urine was purchased from Merck (Germany). Plasminogen bovine, 6-mercapto-1-hexanol (MCH) and trypsin was supplied by Sigma–Aldrich (Germany). Ammonia (26% NH_4OH), hydrogen peroxide (30% H_2O_2) and ethanol were obtained from Slavus (Slovakia). All reagents and solvents were of analytical grade and used without further purification.

Peptide substrate (PS) with primary structure: Lys-Thr-Phe-Lys-Gly-Gly-Gly-Gly-Gly-Gly-Cys and the same sequence with a ferrocene conjugate at the Lys end (PS-Fc) was synthesized according to the work reported by [9]. Underlined sequence is the specific part for plasmin cleavage. Plasmin was freshly prepared before each experiment according to the procedure reported by [10]. The concentration of plasmin was determined according to the protocol published in [24]. In all experiments 10 mM PBS (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl and 137 mM NaCl) pH 7.4 was used as working and running buffer and it was filtrated by a 0.22 μm membrane filter (Merck-Millipore, Germany) prior to use. Highly purified deionised water was prepared by PureLab Classic UV (Elga Water Systems, UK) and used in all experiments.

2.2. Preparation of biosensing layer

The gold electrodes of QCM crystals were cleaned by exposure to basic piranha mixture (H_2O_2:NH_3:H_2O in a 1:1:5 ratio) for 25 min, followed by carefully rinsing with MilliQ water. This process was repeated 3 times. Subsequently, the electrodes were rinsed thoroughly by MilliQ water and ethanol and then dried under nitrogen. Thus cleaned QCM crystals were immersed in 1 mM aqueous solution of peptide substrate (PS) terminated by thiol group of Cys and let incubated overnight. After chemisorption of thiols, surface was rinsed by MilliQ water and ethanol and dried under nitrogen stream. Naked gold was blocked by co-thiol layer of 1 mM 6-mercapto-1-hexanol (MCH) at $T = 45\ ^{\circ}C$ during 1 h. After this step crystal was rinsed several times by PBS buffer and dried under nitrogen. Modified PS-MCH QCM electrode was mounted between two silicon o-rings in the flow-through cell in such a way that the analyte solution was added only at one side of the electrode. The effective volume of the cell was 100 μl. Running PBS buffer and analyte were introduced into the cell by means of a Genie Plus syringe pump (Kent Scientific, USA). PBS buffer was allowed to flow over the PS-MCH modified piezo crystal at flow rate 50 $\mu L\ min^{-1}$ until a stable base line was obtained. This step was necessary to remove any weakly adsorbed peptide at the QCM electrode surface. After obtaining the steady state condition, certain concentration of plasmin prepared in PBS buffer was allowed to flow through the cell and the resonant frequency (f_s) and motional resistance (R_m) changes were continuously and simultaneously measured. One peptide-modified surface by one concentration of plasmin was performed for every experiment. Plasmin concentrations were as follows: 1, 2, 5, 10 and 20 nM. After each addition of plasmin and establishment of steady-state conditions the sensing surface was again rinsed by flowing PBS buffer for eliminating any possible residues. In order to examine the non-specific interactions, protease trypsin was allowed to react with the PS-MCH layer surface in the same range of concentrations used for plasmin and it followed the same incubation conditions. A schematic representation of sensing surface is given in Fig. 1.

2.3. Thickness shear mode method (TSM)

In our experiments, the measurement of f_s and R_m were performed using a network analyzer 8712ES (Agilent Technology, USA) with a measuring procedure published elsewhere [25]. The accuracy of measurement taking in account the background noise was 3 Hz and 0.1 Ω for frequency and motional resistance, respectively. AT-cut quartz crystals (ICM, Oklahoma City, OK, USA) with fundamental frequency of 8 MHz covered with polished gold electrodes (working area 0.2 cm^2) at both sides (QCM electrodes) were used for peptide immobilization. PS layer was formed only at one crystal side. All experiments were performed in flow mode. The flow cell