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Multiple scanning electrochemical microscopy mapping of tyrosinase in micro-contact printed fruit samples on polyvinylidene fluoride membrane

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ABSTRACT

Herein, we introduce three orthogonal and compatible methods for detecting tyrosinase, a key factor in fruit browning and skin disorders, with high spatial resolution by means of scanning electrochemical microscopy (SECM). All methods are performed subsequently on the same substrate area providing a wide range of relevant information. The first SECM approach that relies on the mapping of a differential pore oxygen concentration induced by the local hydrophobic changes that the adsorption of tyrosinase generates on a porous polyvinylidene fluoride (PVDF) membrane. The second approach is based on the direct monitoring of the enzymatic activity of tyrosinase by detecting amperometrically the reaction products from the enzymatic conversion of L-3,4-dihydroxyphenylalanine (L-DOPA). Finally, tyrosinase was visualized implementing a tyrosinase sandwich immunoassay readout by SECM. The multiple SECM detection strategies were successfully applied to map unequivocally the tyrosinase enzymatic activity of a micro-contact printed banana sample. Furthermore, differential pulse voltammetry and mass spectrometry analyses were employed to elucidate the nature of the electrochemical response obtained during the tyrosinase enzymatic activity experiments.

1. Introduction

Tyrosinase is an enzyme known for catalyzing the hydroxylation of monophenols and their oxidation to the respective quinones in the presence of oxygen. Furthermore, tyrosinase is directly involved in fruit ripening, in the biosynthesis of the skin pigment melanin and in skin disorders such as vitiligo (*i.e.* skin depigmentation due to loss of melanin) [1–6]. Therefore, the accurate and sensitive detection of tyrosinase could offer relevant information for a better understanding of different tyrosinase-related biological processes. The latter represents a demanding task, because tyrosinase, as well as other enzymes, is normally expressed inside cells or tissues in concentration levels that are temporally and spatially dependent [7,8]. In proteomics, a common strategy employed to tackle such situation is based on the extraction, separation and identification of proteins by using protein electrophoresis (*e.g.* isoelectrofocusing (IEF) electrophoresis or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)), protein blotting on a suitable membrane and protein identification by using different labeling protocols [9]. Among the different employed membranes for protein blotting and detection, polyvinylidene fluoride (PVDF) is widely used since it is a highly hydrophobic and porous support with a superior protein binding capacity and a remarkable mechanical and chemical stability. Moreover, PVDF maintains the enzymatic activity of adsorbed proteins and is compatible with different protein labeling protocols including the ones based on Coomassie Blue, silver or gold staining and fluorescent or chemiluminescent dyes [10–16]. Recently, proteins immobilized on PVDF membranes have also been detected and employed for the visualization of human finger prints by scanning electrochemical microscopy (SECM) [17]. For this purpose, silver-staining [11], multi-metal-deposition (MMD) [18] or benzoquinone-tagging [19] strategies have been successfully coupled with SECM for the sensitive and selective spatial detection of immobilized proteins.

SECM is a scanning probe microscopy (SPM) technique, which is based on an ultramicroelectrode (UME) scanned in close proximity to a sample substrate and immersed in a solution containing redox active species or their precursors [20,21]. The recorded amperometric current at the UME can be spatially translated into

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local sample reactivity due to the sample capability to interact (*e.g.* through electron transfer reactions) with the present or generated redox species. In this way, SECM is able to image the chemical reactivity of almost any interface leading to a broad range of applications, such as the screening of electrocatalysts, the study of corrosion processes, the imaging of human fingerprints or the unraveling of different biological processes at cellular level [17,19,22–26].

Additionally, SECM enables a highly resolved spatial detection either in blotted protein separations or in the original samples based on a specific enzymatic activity or a selective tagged protein. The latter is not easily achievable by other protein detection techniques such as colorimetry, fluorescence or chemiluminescence due to additionally present interferences caused by sample color background, oxygen concentration or the presence of suspended particles in the media (*i.e.* scattering).

Herein we present three strategies to further extend the capabilities of SECM as a tool for the detection of adsorbed proteins on PVDF membranes. First, an indirect SECM protein detection method based on local oxygen concentration differences encountered between protein-bound and protein-free PVDF regions was implemented. Furthermore, the enzymatic activity of tyrosinase spots immobilized on PVDF membranes was studied. Finally, a third tyrosinase detection method based on the SECM readout of a tyrosinase specific immune reaction was also developed. The realized strategies were consecutively employed over the same sample region to detect spatially the presence of tyrosinase inside banana peels after microcontact printing (μCP) on PVDF membranes. Thus, the unequivocal characterization and interpretation of the enzymatic activity of tyrosinase and other phenol oxidases on the blotted banana peels were achieved. Finally, differential pulse voltammetry (DPV) and mass spectrometry (MS) analysis were performed in order to unravel the origin of the recorded response when studying the enzymatic activity of tyrosinase by SECM.

2. Experimental

2.1. Chemicals

Tyrosinase (from mushroom, lyophilized powder, >=1000 unit/mg solid), L-3,4-dihydroxyphenylalanine (L-DOPA, 99%), monopotassium dihydrogen phosphate, and dipotassium monohydrogen phosphate were bought from Sigma–Aldrich (Schnelldorf, Switzerland). Sodium phosphate monobasic (99%), sodium chloride (99.5%), and potassium chloride (≥ 99.5%) were obtained from Fluka (St. Gallen, Switzerland). Methanol (≥ 99 %) was purchased from Merck (Dietikon, Switzerland). PVDF membranes for protein blotting were purchased from Bio-Rad (Hercules, CA, USA). Commercial 3,3′,5,5′-tetramethylbenzidine (TMB_{red}) solution was purchased from an enzyme-linked immunochemistry-based assay kit (ABRAXIS, Pennsylvania, USA). All reagents and materials were of analytical grade and used as received. Deionized water was produced by a Milli-Q plus 185 model from Millipore (Zug Switzerland).

2.2. Immobilization of protein spots on PVDF membrane

Squared PVDF membranes (*ca.* 1 cm^2) were first wetted in methanol between 5 to 10 seconds and then transferred to water for about 1 min. Then, the PVDF membrane was taken from the water bath and the excess of water was removed carefully with a filter paper. Afterwards, 0.8 μL of a tyrosinase solution (2 mg/mL) was deposited on the membrane by using a calibrated 2.5 μL micropipette (Eppendorf). To avoid the formation of any significant topographic feature, the tip of the pipette was never in physical contact with the PVDF membrane during the tyrosinase deposition process. After deposition, the immobilized tyrosinase spot on the PVDF membrane was dried under a gentle stream of nitrogen.

2.3. SECM measurements

SECM measurements were carried out using a home-made SECM and a typical three electrodes setup running under SECMx software (G. Wittstock, University of Oldenburg) and comprising an Ivium Compactstat (Ivium Technologies, Netherlands). A silver wire was used as the quasi-reference electrode (QRE), a platinum wire as the counter electrode (CE) and a platinum microelectrode disk (25 μm diameter) as working electrode. All potentials given herein are referred to the QRE. Before each experiment, the platinum microelectrode was mechanically polished with a series of diamond lapping discs starting from 1 μm down to 0.05 μm particle sizes. The quality of the microelectrode and its *RG* (*i.e.* the ratio between the radius of the insulating glass and the radius of the microelectrode (r_T)) were determined with a Laborlux D optical microscope (Leitz, Germany). Data analyses were carried out using MIRA software [27]. The PVDF membrane was fixed on a microscope glass slide and placed on the bottom of an electrochemical setup that was completed by a Teflon body that enclosed the sample into a given volume and where the CE and QRE were placed. Prior to each SECM experiment, a leveling of the sample surface was achieved by comparing the substrate height (determined by SECM approach curves) at three corners of the square area to be scanned. A sample correction angle was applied by using a motorized tilt table (Zaber, Canada) on which the electrochemical cell was placed. All experiments were performed at room temperature (20 ± 2 °C).

2.4. Microcontact printed banana on PVDF membrane

A fresh banana cross section (*ca.* 0.5 mm × 0.5 mm) was cut with a scalpel blade and washed with deionized water. A PVDF membrane was prepared as mentioned above and put in contact with the banana piece for around 1 min (see Fig. S1 in Supporting Information (SI)). After the PVDF membrane was completely dried, a solution of 50 mM phosphate buffer (pH = 6.0) was employed for washing the PVDF membrane, which was then dried again and placed into the electrochemical cell for the performed experiments.

2.5. Indirect SECM detection of adsorbed proteins on PVDF membrane by oxygen reduction method

After the tyrosinase spot was immobilized over the PVDF membrane and placed inside the electrochemical cell, a solution of 50 mM phosphate buffer (pH = 6.0) was added into the system. Then the Pt microelectrode was biased at an electrode potential (E_T) of −0.8 V to monitor the reduction of oxygen while scanning the UME closely over the PVDF sample.

2.6. SECM detection of tyrosinase activity immobilized on PVDF membrane

After the tyrosine spot was deposited on the PVDF membrane, a solution of 2 mM L-DOPA in 50 mM phosphate buffer (pH = 6.0) was added to the system inside the electrochemical cell. Then the Pt microelectrode was scanned close to the sample and biased at 0.7 V to monitor the tyrosinase activity.

2.7. SECM immunoassay of tyrosinase immobilized on PVDF membrane

After the immobilization of a tyrosinase spot on a PVDF membrane, a solution of 1% bovine serum albumin (BSA) in 50 mM

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