Multitechnique study on a recombinantly produced *Bacillus halodurans* laccase and an S-layer/laccase fusion protein

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(Received 18 February 2011; accepted 19 April 2011; published 2 June 2011)

Methods for organizing functional materials at the nanometer scale are essential for the development of novel fabrication techniques. One of the most relevant areas of research in nanobiotechnology concerns technological utilization of self-assembly systems, wherein molecules spontaneously associate into reproducible supramolecular structures. For this purpose, the laccase of *Bacillus halodurans* C-125 was immobilized on the S-layer lattice formed by SbpA of *Lysinibacillus sphaericus* CCM 2177 either by (i) covalent linkage of the enzyme to the natural protein self-assembly system or (ii) by construction of a fusion protein comprising the S-layer protein and the laccase. The laccase and the S-layer fusion protein were produced heterologously in *Escherichia coli*. After isolation and purification, the properties of the proteins, as well as the specific activity of the enzyme moiety, were investigated. Interestingly, the S-layer part confers a much higher solubility on the laccase as observed for the sole enzyme. Comparative spectrophotometric measurements of the enzyme activity revealed similar but significantly higher values for rLac and rSbpA/Lac in solution compared to the immobilized state. However, rLac covalently linked to the SbpA monolayer yielded a four to five time higher enzymatic activity than rSbpA/Lac immobilized on a solid support. Combined quartz crystal microbalance with dissipation monitoring (QCM-D) and electrochemical measurements (performed in an electrochemical QCM-D cell) revealed that rLac immobilized on the SbpA lattice had an approximately twofold higher enzymatic activity compared to that obtained with the fusion protein.
[DOI: 10.1116/1.3589284]

I. INTRODUCTION

Being composed of a single protein or glycoprotein species, bacterial cell surface layers (S-layers) represent the simplest biological membrane developed during evolution.[1–3] Based on the remarkable intrinsic feature of S-layer proteins to self-assemble and the possibility for genetic modifications, S-layer proteins were exploited as a component for the development of novel immobilized biocatalysts based on fusion proteins comprising S-layer proteins of *Bacillaceae* and various enzymes.[4,5] The well-defined arrangement of fused functions on S-layer lattices and the repetitive physicochemical properties down to the nanometer scale allow the binding of functional molecules (e.g., enzymes, antibodies, antigens, and ligands) and nanoparticles with unsurpassed spatial control.[6–8] Moreover, S-layers can be used as structural basis for a biomolecular construction kit involving all major species of biological molecules (proteins, lipids, glycans, nucleic acids, and combinations of them).[1,8–11]

Laccases belong to a large family of multicopper oxidases capable of oxidizing a wide range of inorganic and aromatic compounds, while reducing molecular oxygen to water.[12] This enzyme has a widespread application. In industrial fields, laccases have a great potential in pulp, paper, textile, and food industries as well as for the removal of phenolic pollutants and polycyclic aromatic hydrocarbons in wastewater and soil.[13,14] In nanobiotechnology, their most important contribution can be seen in the development of tiny and highly efficient biosensors due to controlled and specific adsorption of biomolecules on different types of solid supports.[15] Owing to the above characteristics, the laccase is a functional element for biosensors and bioelectrochemical detections. Such biosensors consist of a solid support on which an enzyme is attached or bound and a specific readout system. Further interest in laccase biosensors is place in its application as biofuel cell in combination with cellobiose dehydrogenase on the anode and laccase on the cathode.[16] According to this need, immobilization methods allow the partition of the enzyme catalyst without difficulty from the reaction mixture and can reduce the costs of enzymes significantly. Therefore, moderate immobilization yields and high operational stability are preferred.[17] Although for adsorption of macromolecules, a specific linker or attachment system is needed, laboratory handling is elegant and easy to perform. However, another advantage is the repeated application that decreases toxicological risks caused by daily handling and preparation.[18] In literature, a huge variety of immobilization methods for laccases on solid surfaces is available. One possibility is the direct attachment of laccases on various modified substrates such as Zr^{4+} on polycrystalline gold, indium-doped tin oxide, and silver as reported by Mazur *et al.*[19]

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Another technique is the chemical binding of laccases via specific linkers [silanes or thiol-functionalized self-assembled monolayers (SAMs)].[20–22] Furthermore, a preferred method is the incorporation of laccases inside matrixes such as functionalized or conducting polymers,[23] magnetic core shells,[24] and composite metal complexes.[25] According to all these techniques, no ordered structure or distinct immobilization is provided. Owing to this fact, one major interest in our group is the enzyme-sensor technology based on crystalline S-layer proteins that provide a regular structure for enzyme-surface attachment. Different covalent immobilization studies on S-layer lattices showed that regarding the binding density, retained activity, and biospecificity, the optimal activation method is strongly dependent on the respective enzyme, antibody, or ligand.[26–28] Current research activities are focused on the production of fusion proteins between S-layer proteins of *Bacillaceae* and enzymes from extremophiles for the development of novel immobilized biocatalysts. The aim is the controllable display of biocatalytic epitopes, storage stability, and reuse[4,5] as required for a great variety of applications (e.g., biocatalytic processes and diagnostics) in chemical, pharmaceutical, and food industry.

The S-layer protein SbpA of mesophilic *Lysinibacillus sphaericus* CCM 2177 consists of 1268 amino acids, including a 30 amino acids long signal peptide.[8] By producing various C-terminally truncated forms and performing surface accessibility screens, it became apparent that amino acid position 1068 is located on the outer surface of the square lattice. This C-terminally truncated form fully retained the ability to self-assemble into a square S-layer lattice with a center-to-center spacing of the tetrameric morphological units of 13.1 nm.[8] Therefore, the C-terminally truncated form $rSbpA_{31-1068}$ was used as a base form for the construction of various S-layer fusion proteins. An advantage of the SbpA system for nanobiotechnological applications is that the recrystallization is dependent on the presence of calcium ions, thus allowing control over lattice formation.[29]

Due to the much higher thermostability of bacterial laccases, biocatalysts based on these enzymes may have advantageous properties compared to classical laccases.[30] The electrocatalytic properties of the laccase Lbh1 of alkalophilic *Bacillus halodurans* C-125 opened up the potential of using this enzyme for the development of cathodes for enzyme-catalyzed biofuel cells.[15,31] The bacterial laccase Lbh1 shows laccaselike activity, oxidizing 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), syringaldazine (SGZ), and hydroquinone (HQ). The enzyme shows an alkaline *p*H optimum with SGZ as the substrate and is stimulated rather than inhibited by chloride. Since Lbh1 is optimally active at alkaline *p*H, it may be expected that this enzyme is also less susceptible to inhibition by other anions.[15] Therefore, the fusion protein rSbpA/Lac, comprising the truncated S-layer protein SbpA of *Lysinibacillus sphaericus* CCM 2177, a glycine linker, and the C-terminally fused laccase Lbh1, was constructed [see supplementary material, Fig. S1 (at http:dx.doi.org.10.1116/1.3589284 for a schematic represetation of the rSbpA\Lac fusion protein). The aim of this study was to obtain a completely covered electrode with recrystallized rSpbA/Lac and, consequently, an oriented exposed enzyme array on the electrode surface with maximum accessibility for the substrate. As an architectural reference, chemical linkage of the sole laccase to the S-layer lattice was pursued. In this case, the S-layer lattice with accessible groups on its surface (e.g., 1.6 carboxylic acid groups/nm^2)[32] constituted an anchoring layer similar to SAMs without providing the advantage of a defined orientation of the laccase.

II. EXPERIMENT

A. Cloning and expression

Detailed descriptions of the cloning procedure (culture conditions,[33,34] plasmide construction, and gene expression, isolation, and purification)[35,36] are given in the supplementary material, Secs. A1–A3, at http:dx.doi.org.10.1116/1.3589284.

B. Investigation of the self-assembly and recrystallization properties of rSbpA/Lac

1. Transmission electron microscopy

For investigating the self-assembly properties of the chimaeric protein, sample preparation was carried out as described elsewhere.[8] In brief, purified and lyophilized rSbpA/Lac was dissolved at a concentration of 300 μg/ml in 0.7 ml 5*M* guanidine hydrochloride (GHCl) in 50 mM Tris/HCl buffer (*p*H 7.2), which was subsequently removed by dialysis against 10 mM $CaCl_2$ in aqua purificata (A. purif.; Milli-Q grade; resistance: >18.2 MΩ cm) at 4 °C for 18 h in order to form self-assembly products. Sample preparation for the investigation of recrystallization properties was also carried out on peptidoglycan-containing sacculi (PGS). The preparation of PGS and the extraction of secondary cell wall polymer (SCWP) were performed as previously described.[37,38] For recrystallization of rSbpA/Lac on PGS, the same procedure was carried out as described above for self-assembly products, except that 300 μg PGS from *Ly. sphaericus* CCM 2177 were added. For transmission electron microscopy (TEM) analysis, the samples were transferred onto Formvar-filmed carbon-coated copper grids and after negative staining with 1% uranyl acetate,[39] the specimen were inspected with a Philips CM12 transmission electron microscope (Philips, Eindhoven, The Netherlands) operated at 80 kV in a low-dose mode.

2. Atomic force microscopy

1 mg lyophilized rSbpA/Lac was dissolved in 0.7 ml 5*M* GHCl in 50 mM Tris/HCl buffer (*p*H 7.2). Dialysis against A. purif. for 2 h at 4 °C was followed by centrifugation (36 000*g*, 20 min, 4 °C). The concentration of the soluble protein in the clear supernatant was determined at 280 nm and adjusted to 0.05 mg ml^{-1} with crystallization buffer (0.5 mM Tris-HCl, 10 mM $CaCl_2$ at *p*H 9.0).

The recrystallization of the fusion protein was performed on silicon wafers (IMEC, Belgium), gold wafers, and gold

wafers coated with SCWP of *Ly. sphaericus* CCM 2177. Wafers were cleaned with ethanol (70%) and A. purif. The different supports were incubated with rSbpA/Lac solution (0.05 mg ml^{-1} protein in crystallization buffer) overnight at room temperature and subsequently stored in A. purif.

Atomic force microscopy (AFM) images were recorded at room temperature using a Nanoscope IIIa multimode (Vecco Instruments Inc., Santa Barbara, CA) equipped with a J-scanner (nominal scan size: 130 μm). To avoid electrostatic repulsion between tip and sample, the scanning was carried out in contact mode in aqueous solution containing 100 nM $CaCl_2$. Standard 200-μm-long oxide-sharpened silicon nitrile cantilevers (NP-S, NanoProbes, Digital Instruments) were used. AFM was also used for quality control of recrystallized rSbpA/Lac and SbpA (for further covalent binding of rLac) on sensor surfaces.

C. Enzymatic assays

1. Determination of the enzymatic activity of rLac and rSbpA/Lac

The specific enzyme activity of the laccase and that of the fusion protein were determined for different substrates: DMP (ε_{468}=14 800 l mol^{-1} cm^{-1}), SGZ (ε_{530}=64 000 l mol^{-1} cm^{-1}), ABTS (ε_{420}=38 000 l mol^{-1} cm^{-1}), and HQ.[40] Control measurements without the respective protein were performed to calculate the possible chemical oxidation of the substrates. Quantification of enzymatic activity was done under different conditions (*p*H, temperature, and buffer). The *p*H of the buffer used in the assays was adjusted at 30, 40, and 45 °C, respectively. The specific enzyme activity was defined as the amount of laccase that oxidizes 1 μmol substrate/min (U mg^{-1}) under standard assay conditions. The difference in molecular masses of rLac and rSbpA/Lac was taken into account.

2. Preparation of the proteins for the enzymatic assays

The lyophilized proteins (rLac and rSbpA/Lac) were dissolved in 7*M* GHCl in 50 mM Tris/HCl buffer (*p*H 7.2) and dialyzed against A. purif. containing 1 mM copper sulfate. In order to get rid of the copper in the protein solution, an additional dialysis step against A. purif. was done. Precipitated protein was removed by centrifugation and the protein concentration in the clear supernatant was determined by UV absorbance at 280 nm using the sequence-derived extinction coefficients of 54 320 and 132 600 for rLac and rSbpA/Lac, respectively. As reference experiment for all enzymatic assays, the native S-layer protein SbpA from *Ly. sphaericus* CCM 2177 (Czech Collection of Microorganisms) was used. The isolation and growth of SbpA is described elsewhere.[1,41] For recrystallization of SbpA or rSbpA/Lac on solid supports, the protein solution was mixed with crystallization buffer and a protein concentration of 0.1–0.2 mg ml^{-1} was used.

D. Sensor surfaces and experimental setups

Screen-printed electrodes (223AT, Drop Sens, Oviedo, Spain) were used for the electrochemical screening of laccase activity in solution. The working electrode, the reference electrode, and the counterelectrode were made up of gold, silver, and platinum, respectively. The connection was provided by a specific connector (ref. DSC, Drop Sens, Oviedo, Spain) acting as an interface between the electrodes and the potentiostat (CH Instruments, CHI660c, Austin, TX). Before each experiments, the sensors were cleaned by repeated application of cyclic voltammetry in 0.05*M* sulfuric acid and extensively washed with A. purif. before performing the experiments. Each electrode was used for one experimental series only. For quartz crystal microbalance with dissipation monitoring (QCM-D) measurements, 5 MHz gold coated quartz crystal sensors (QSX 301; from Q-Sense AB, Gothenburg, Sweden) were used. Before use, the QCM-D sensors were cleaned with a cleaning solution according to Kern and Puotinen,[42] stored in a 2% Hellmanex II solution (Hellma) over night, rinsed with A. purif., dried under a nitrogen stream, and finally cleaned by ozone plasma using a plasma cleaner (Plasma Prep2, Gala, Gabler Labor Instruments, Germany).

E. Immobilization of rSbpA/Lac as well as SbpA for further covalent linkage of rLac

Recrystallization of SbpA and rSbpA/Lac onto the QCM-D gold sensor surface was performed with a protein concentration of 0.1–0.2 mg ml^{-1} at room temperature either for 3 h or overnight. Two sensors were coated with SbpA; the first one was used as the reference surface and the second sensor was used for the immobilization of rLac. In order to bind rLac to the SbpA lattice, the carboxyl groups of the S-layer lattice were activated using a solution containing 200 mM *N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide (EDC) (Sigma) and 50 mM *N*-hydroxysuccinimide (NHS) (Sigma). Subsequently, rLac was subjected to the activated surface in an acetate buffer *p*H 4.0 with a concentration of 0.02 mg ml^{-1}. The third sensor was functionalized with rSbpA/Lac. A schematic illustration is given in Fig. 1.

F. QCM-D and electrochemical QCM-D

Standard QCM-D flow cells and an electrochemical QCM-D cell (EQCM-D) (QEM-401, QSense AB) were utilized. QCM-D measurements were carried out using an E4 instrument (Q-Sense AB). The shifts in frequency (Δf) and dissipation (ΔD) were monitored by the Q-SOFT 401 software (version 2.5.7.505). All frequency data were normalized to the corresponding overtone. For analysis of the QCM-D data, the frequency and dissipation shifts of the seventh overtone were used. The EQCM-D cell consisted of three electrodes: the working electrode was the QCM-D gold sensor itself, a platinum plate auxiliary electrode, and an Ag/AgCl, KCl saturated reference electrode which was placed in the outlet of the flow cell. The utilized potentiostat was the same as for the screen-printed electrodes. Both cells were con-

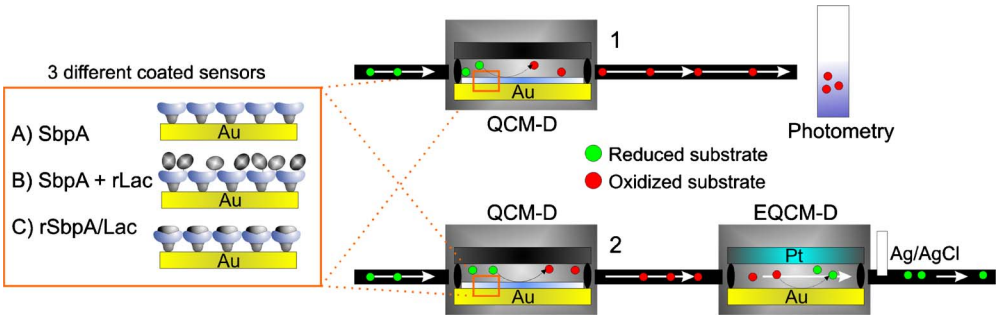

FIG. 1. (Color online) Experimental setup for the determination of the enzymatic activity on QCM-D crystals either by photometry (1) or amperometry (2) on SbpA (a), SbpA and chemically bound rLac (b), and rSbpA/Lac (c).

nected to a peristaltic pump. For analysis of the adsorbed mass of the S-layer protein SbpA as well as rSbpA/Lac, the Sauerbrey equation was used.[43]

G. Spectrophotometric measurements of rSbpA/Lac and rLac

For measurements in solution, the reaction mixture (100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, supplemented with 100 mM NaCl and the respective protein solution) was preincubated at 30 or 45 °C. The reaction was started by adding the substrate to a final concentration of 1 mM for DMP and ABTS or 22 μM for SGZ. After incubation for 5 min, the concentration of the oxidized substrates was calculated using a photometer adjusted to the respective wavelength. Citrate buffer (50 mM) was used in the reaction mixture for the determination of the activity with ABTS as the substrate.[15] For the detection of the enzymatic activity on solid supports, the proteins were first immobilized on QCM-D crystals (see Sec. II F) and subsequently subjected to the same procedure as described above (see also Fig. 1).

H. Electrochemical measurements

1. Voltammetry on screen-printed electrodes

The electrochemical determination of the enzyme activity for HQ was performed by using linear sweep voltammetry on screen-printed electrodes. The temperature (room temperature up to 40 °C) as well as the *p*H (*p*H 6–7.8) were varied. All experiments were performed in potassium phosphate buffer (20 mM) with 100 mM sodium chloride. For all measurements, the concentration of HQ was 1 mM and the protein concentrations of rSbpA/Lac and rLac were 7.6 and 1.8 μg ml^{-1}, respectively. For measurements at higher temperatures, the whole cell holder was transferred into an incubator. The applied potential between the reference and the working electrodes had a scan rate of 10 mV s^{-1}. The occurring current peak (from −0.2 to 0.25 V) was evaluated in the absence and presence of laccase in solution. For comparison, the current peaks, 4 min before and 4 min after adding the laccase, were also evaluated.

2. Electrochemical impedance spectroscopy

Using an EQCM-D cell, the protein coverage of QCM-D gold sensors was determined as described by Diniz *et al.*[44] Briefly, the frequency range was 10 mHz–100 kHz. An ac potential of 15 mV was applied at a dc bias voltage of 500 mV. The electrolyte consisted of 10 mM potassium phosphate buffer, *p*H 7.4 (Sigma) containing 100 mM NaCl. In all experiments, the redox system $K_3Fe(CN)_6/K_4Fe(CN)_6$ (Sigma) was present in the solution at a concentration of 1 mM. The obtained impedance spectra were fitted to the classical equivalent circuit described by Randles[45] and Barsoukov and Macdonald.[46] For the detection of the defect area of rSbpA/Lac, the sensor was also used as a working electrode.

3. Amperometric detection

For the determination of the enzymatic activity on QCM-D sensor surfaces, the experimental procedure was performed as described by Vianello *et al.*[47] The sensors were coated with SbpA functionalized with covalent bound rLac, rSbpA/Lac, as well as SbpA as a reference. Afterward, the temperature was increased to 40 °C and the surfaces were flushed with potassium phosphate buffer, *p*H 7.6. Each QCM-D cell was connected to an electrochemical cell via a 100 μl tube. Hence, the effective volume was 200 μl. To indicate again, the counterelectrode was a platinum plate and the reference electrode an Ag/AgCl electrode. Before the amperometric detection, the reduction potential of −200 mV of HQ was determined by cyclic voltammetry (data not shown). The cells were rinsed with a 10 mM HQ solution in the same buffer and subsequently incubated for 5 min. Subsequently, the cells were rinsed at a flow rate of 100 μl min^{-1} and the current response was recorded in the electrochemical cell at the applied reduction potential against the time. In all steps, special care was taken to prevent air bubble formation. A scheme of the performed experimental procedure is given in Fig. 1. The amount of converted substrate was determined by calibrating the electrochemical cell with respect to the oxidized substrate as described by Vianello *et al.*[47] Owing to this fact, the concentrations of Q could be detected in the range of 1 μM–10 mM.

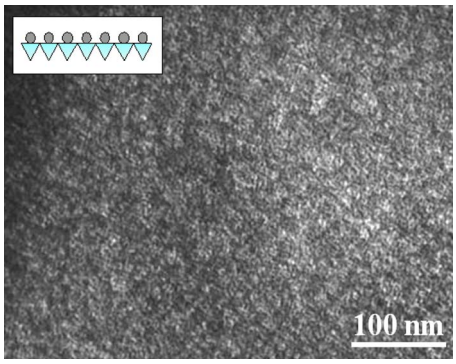

FIG. 2. (Color online) TEM image of a negatively stained preparation of rSbpA/Lac fusion proteins recrystallized as a crystalline monolayer on peptidoglycan-containing sacculi of *Lysinibacillus sphaericus* CCM 2177.

III. RESULTS AND DISCUSSION

A. Cloning and expression

For production of the laccase and the rSbpA/Lac fusion protein, the constructs pET28a(+)/rLac and pET28a(+)/rSbpA$_{93-3204}$/Lac were cloned in *E. coli* TG1 and expressed in *E. coli* One Shot® BL21 Star™ (DE3). rSbpA/Lac encodes a protein of 1545 aa with a theoretical molecular weight of 166 315 Da and a theoretical pI of 4.52. After induction of expression, biomass samples were harvested at distinct points in time and subjected to sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. In comparison to *E. coli* cells harvested before induction of protein expression [see supplementary material, Fig. S2, left, lane b at http:dx.doi.org.10.1116/1.3589284], an additional protein band was observed in the *E. coli* host cells induced to express rSbpA/Lac [see supplementary material, Fig. S2, left, lanes c–e at http:dx.doi.org.10.1116/1.3589284] and rLac (data not shown). In such samples, the apparent molecular masses of the additional protein bands corresponded well to the calculated molecular masses of the respective proteins. As shown by the SDS-PAGE analysis, the fusion protein [see supplementary material, Fig. S2, right, lanes d and e at http:dx.doi.org.10.1116/1.3589284] and the enzyme [see supplementary info Fig. S2, right, lanes b and c at http:dx.doi.org.10.1116/1.3589284] could be isolated from the insoluble cytoplasmic fraction of *E. coli* One Shot® BL21 Star™(DE3) and purified by gel permeation chromatography according to the procedure described in literature.[36]

B. Investigation of the self-assembly and recrystallization properties of rSbpA/Lac for coating electrodes

As shown by negative staining and subsequent electron microscopic investigations, rSbpA/Lac reassembled into flat sheets. The square lattice structure was clearly visible on self-assembly products (not shown). The fusion protein was capable of recrystallizing into the square lattice structure on PGS of the A4α-chemotype from *Ly. sphaericus* CCM 2177 (Fig. 2), although the lattice was not as distinctly and visibly compared to the wild-type SbpA. Concerning the coating of different solid supports (silicon wafers, gold wafers, and gold wafers coated with SCWP), rSbpA/Lac mainly showed structures composed of small patches forming a closed proteinaceous layer (data not shown). However, the square lattice structure of the S-layer protein could no longer be distinguished on the AFM images. This observation might be explained by an organization of the rSbpA/Lac with the S-layer part sitting on the solid support and the fused laccase facing the aqueous environment. Hence, the fused laccase, with a molecular mass of 56 kDa, constitutes the outermost surface topology, which is imaged by AFM. The laccase molecules, fused with a linker to the S-layer protein, do not obviously reproduce the underlying S-layer protein lattice structure and it is not known where the laccase is exactly located on the SbpA lattice. To conclude, although the previously described arrangement of the rSbpA/Lac on the solid support is highly desired, the lattice structure of the underlying SbpA cannot, unless with TEM, be visualized by AFM. Nevertheless, the solid supports could entirely be covered with a closed proteinaceous layer. Furthermore, when rSbpA/Lac was recrystallized on a gold surface for determining the defect area and compared with recrystallized SbpA, a complete coverage of the electrode surface was observed.

By using QCM-D and electrochemical impedance spectroscopy (EIS), this defect area can be verified. For this reason, the adsorption of rSbpA/Lac on gold as substrate could be compared with the wild-type SbpA, which exhibits excellent recrystallization properties. Information on the blocking effect or defect area of recrystallized S-layer proteins can be obtained by determining the charge transfer resistance R_{ct} compared to a bare gold electrode in the presence of an electroactive species such as potassium ferrocyanide. EIS was utilized for the measurement of R_{ct}. The ratio of the detected R_{ct} of gold and S-layer protein is an indication of the amount of area-wide defects within the spread layer [see Eq. (1)], as described in literature.[44,48,49] Recrystallized S-layer proteins reduced the accessibility of the electrode area for electroactive species and, hence, R_{ct} was increasing,

$$\Delta R_{ct} = \frac{R_{ct(gold)}}{R_{ct(gold+S\text{-}layer)}} \times 100. \quad (1)$$

The determined change in the charge transfer of SbpA was 0.36% and that of rSbpA/Lac was 0.51%. These results indicate that even though no continuous lattice structure was visible, rSbpA/Lac entirely covered the electrode surface. Moreover, the calculated mass for a crystalline monolayer for SbpA and rSbpA can be compared to the determined masses by QCM-D, respectively. This comparison evidenced the formation of a closed monolayer on the sensor surface [see also Sec. III C 2 and the supporting material, Sec. B at http:dx.doi.org.10.1116/1.3589284 for calculation of the theoretical mass[50] of Sbp4/Lac, respectively on QCM-D sensors].

TABLE I. Enzymatic activity as analyzed by spectrophotometry. Maximum laccase activity obtained with different substrates in solution (A). Maximum laccase activity obtained for immobilized enzymes on the gold sensor (QCM-D) crystal (B).

				Specific activity (U mg^{-1})	
	T (°C)	pH	Substrate	rSbpA/Lac	rLac
(A) Solution	30	7.5	DMP	9.0 ± 0.33	10.21 ± 0.05
	45	7.5	DMP	25.42 ± 0.08	30.91 ± 0.78
	45	7.9	SGZ	0.41 ± 0.11	0.18 ± 0.01
	T (°C)	pH	Substrate	rSbpA/Lac[a]	rLac on S-layer lattice[b]
(B) Gold sensor	45	7.5	DMP	0.62 ± 0.07	2.78 ± 1.09

[a]Recrystallized rSbpA/Lac.
[b]rLac covalently linked to recrystallized SbpA.

C. Enzymatic assays

1. Electrochemical and spectrophotometric measurements with proteins in solution

Laccase activity of the enzyme moiety in the rSbpA/Lac fusion protein was measured spectrophotometrically using SGZ and DMP as substrates; the latter turned out to be the most efficient substrate (Table I). Consequently, the main studies were carried out using DMP. Starting with a pH [Fig. 3(a)] and temperature screening (data not shown), the best conditions for the activity tests were determined, revealing, in line with previous findings,[15] that rLac and rSbpA/Lac show the best activity at 45 °C and pH 7.5. Hence, the S-layer protein fused to the enzyme does not influence the nature of the enzyme, but it is important to emphasize that the rSbpA/Lac fusion protein revealed a ten times higher solubility at room temperature than sole rLac. Water-soluble rSbpA/Lac protein as present in the absence of calcium ions revealed a specific activity of 25 U mg^{-1}, while the value for sole rLac was 31 U mg^{-1}. The differences in activity might be due to different diffusion rates between substrate and rLac and the fused enzyme in rSbpA/Lac, respectively, but also a conformational change of the laccase in rSbpA/Lac might lower the laccase activity to some extent.

Besides the previously mentioned substrates, we observed a limitation of the activity for ABTS (data not shown), which can be explained according to Ozgen *et al.*, who reported that the stability of ABTS at pH 7.4 is problematic.[51] However, DMP is suitable for photometrical detection but utilized in electrochemical methods, an polymerization effect occurs at the oxidation potential.[52] Owing to this crucial property, HQ was used for the electrochemical screening experiments in solution. In water, the reduction of Q as well as the oxidation of HQ can be represented by a single two-electron wave.[53] HQ is relatively good soluble in water and the electrochemical behavior is well known. The pH dependence of chemically attached HQ to SAMs in aqueous solutions was investigated by cyclic voltammetry.[54] For this reason, the redox reaction at the electrode interface can be performed

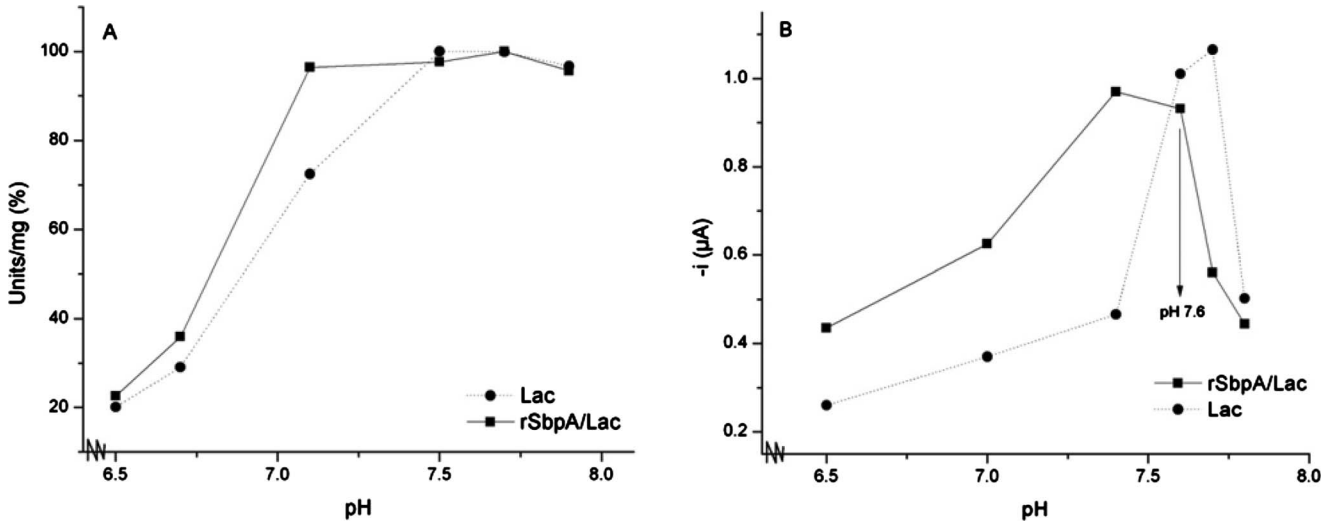

FIG. 3. pH-dependent activity profile of recombinantly produced rLac and of the S-layer fusion protein rSbpA/Lac for two different substrates determined (a) photometrically (substrate DMP) and (b) electrochemically (substrate HQ).

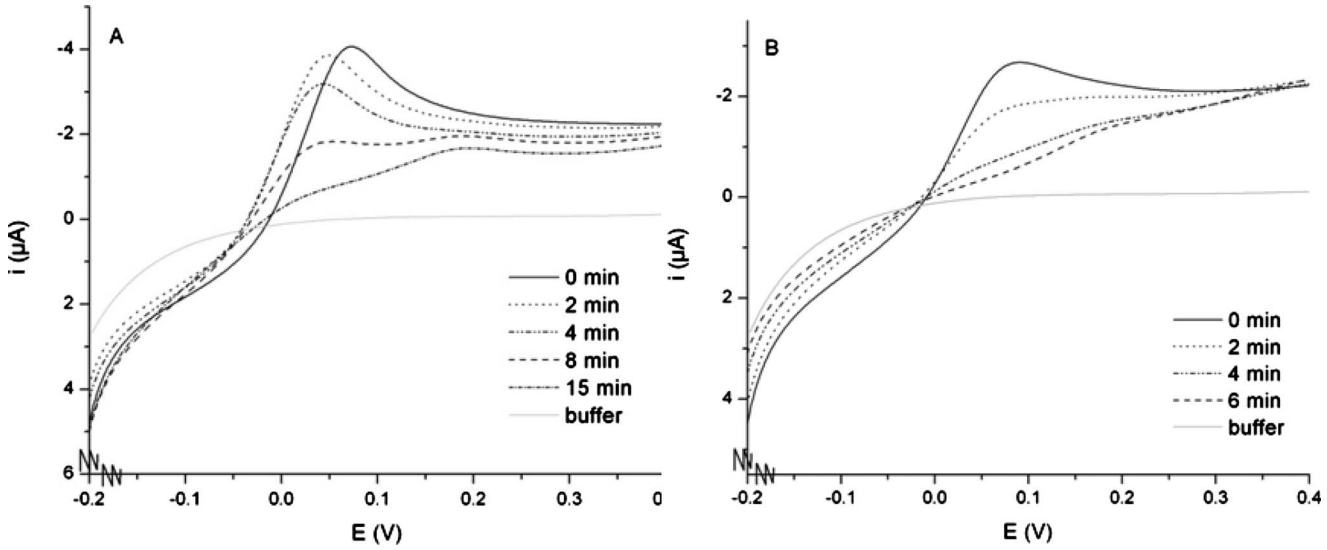

FIG. 4. Voltammograms of HQ at 40 °C and *p*H 7.7 after the addition of rSbpA/Lac (a) and rLac (b). The applied sweep rate was 10 mV s^{-1} in the potential range of −0.2–0.4 V.

within a wide *p*H range (*p*H 2–12) and HQ can be used as substrate for laccase Lbh1 even at higher *p*H values. By using cyclic voltammetry, the competitive reaction between the laccase and the working electrode is visible according to the recorded decrease of the substrate and the increase of the product, respectively. Owing to this fact, the decrease of the anodic current peak can be evaluated after the addition of the enzyme. If no enzyme is added, no change in the current peak occurs (data not shown). In this study, only the anodic peak was investigated because these experiments were done qualitatively in order to screen the best conditions [Figs. 3(b) and 4] for the subsequent activity measurements on solid supports. In Figs. 4(a) and 4(b), voltammograms indicating the time dependent decrease of the anodic current peak of HQ at 40 °C and *p*H 7.7 caused by the addition of rSbpA/Lac and rLac, respectively, are shown. Klis *et al.*[55] worked on a different type of laccase but showed a similar tendency of the anodic current peak after the addition of the enzyme. The results obtained in this study showed a response in the nanoampere range due to the utilization of microelectrodes.

The *p*H screening revealed the highest enzymatic activity for the fusion protein and the sole enzyme in the range from *p*H 7.4 to 7.7. Owing to the fact that both proteins exhibited nearly the same activity at *p*H 7.6, this condition was chosen for further characterization. Measurements performed at temperatures below 40 °C showed less enzymatic activity (data not shown). The present results, which indicate that the highest activity of *B. halodurans* Lbh1 occurs at higher *p*H values, correlate with the findings of Martins *et al.*[30]

2. Determination of the enzymatic activity on gold crystals (QCM-D)

Based on the determination of the defect areas on QCM-D gold sensors covered either by rSbpA/Lac or native SbpA, complete coverage could also be observed for the fusion protein, although no satisfying square lattice structure was detected by AFM. Therefore, gold sensors were coated with SbpA, rSbpA/Lac, and rLac (covalently linked to the SbpA-layer via amine coupling) in order to calculate the activity of immobilized enzyme compared to the enzyme in solution.

QCM-D was utilized to monitor the adsorbed mass of the proteins on the sensor surface (Figs. 1 and 5). Two sensors were coated with SbpA; the first one was used as reference surface and the second sensor was used for the immobilization of rLac via amine coupling by EDC/NHS to the SbpA-layer. The third QCM-D sensor was functionalized by recrystallization of rSbpA/Lac (see Sec. II E). A decrease in frequency adverse to an increase in dissipation upon protein adsorption and recrystallization on the gold sensor was observed (Fig. 5).

SbpA shows dissimilar recrystallization properties compared to the fusion protein according to the difference in the frequency and the dissipation as a function of time (Fig. 5). Immediately after subjecting the protein solutions to the sensors, the frequency decreases due to the adsorbed mass associated with protein deposition and at the same time, the dissipation slightly increases, indicating the formation of a more or less rigid layer. While the frequency change of SbpA is 88.19 ± 0.03 ($n=3$) Hz with a dissipation of 2.52 ± 0.08 ($n=3$), the frequency change of rSbpA/Lac corresponded to 110.71 ± 15.48 ($n=3$) Hz and 6.35 ± 1.12 ($n=3$). The latter value leads to the conclusion that rSbpA/Lac shows higher viscoelastic properties compared to SbpA. In Fig. 5, the frequency and dissipation of the immobilizations are shown for each sensor surface.

With respect to the Sauerbrey equation, the mass per unit area was calculated from overall frequency changes of the seventh overtone, revealing 1561 ng cm^{-2} for SbpA, 1960 ng cm^{-2} for rSbpA/Lac, and 132 ng cm^{-2} for the sole enzyme, which was chemically linked to SbpA. In terms of the generation of a protein monolayer, special emphasis has to be placed on the comparison between the ratios of the

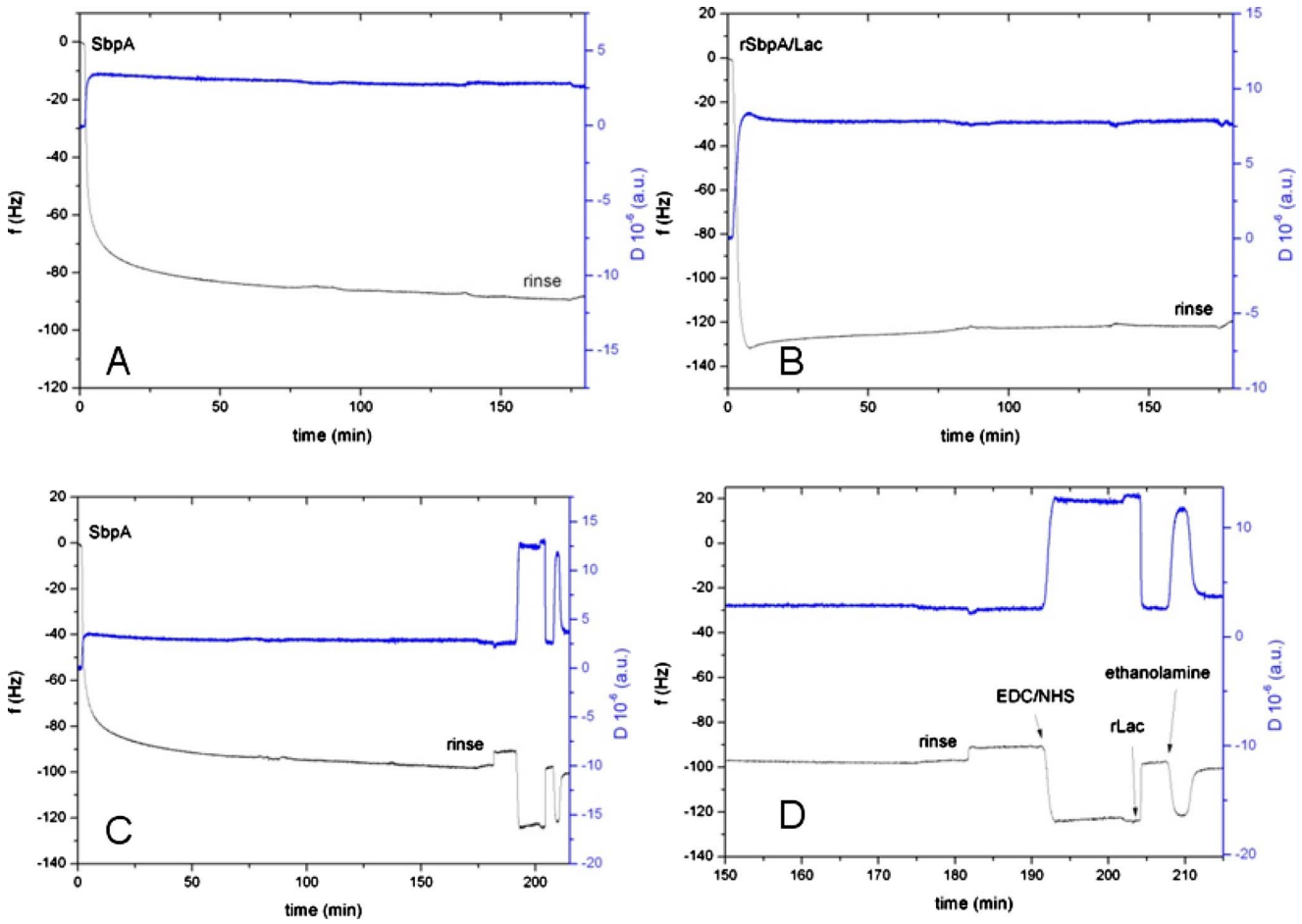

FIG. 5. (Color online) Frequency and dissipation shifts of recrystallized SbpA (a), rSbpA/Lac (b), and rLac covalently linked to SbpA [(c) and (d)]. In (d), the frequency and dissipation shifts during the linking procedure are shown at a more detailed time scale.

general molar mass between SbpA and rSbpA/Lac, which is 0.8. This value is in keeping with the obtained mass ratio determined by QCM-D (1561 ng cm^{-2} versus 1960 ng cm^{-2}). In addition to these findings, the mass of a crystalline monolayer can be calculated by the lattice parameters of SbpA.[56] The measured mass of both SbpA and rSbpA/Lac are in good accordance with the calculated mass of a crystalline monolayer [see supplementary material, Sec. B, at http:dx.doi.org.10.1116/1.3589284 for calculation of the theoretical mass].

a. Spectrophotometric measurements Subsequent to the preparation of the QCM-D gold sensors, the activity of the immobilized enzymes was measured photometrically (Fig. 1). Therefore, the temperature was set to 45 °C and the sensors were rinsed with MOPS buffer. Followed by collecting the oxidized substrate in tubes, the proteins immobilized on the QCM-D crystals were incubated with the reaction mixture (100 mM MOPS buffer, supplemented with 100 mM NaCl and 1 mM DMP). The amount of converted DMP was determined spectrophotometrically (Table I).

rLac chemically linked to the S-layer lattice revealed the four- to fivefold activity compared to the fusion protein immobilized on the QCM-D gold sensor surface. The high standard deviation for rLac on an S-layer lattice compared to rSbpA/Lac might reflect that the latter presented the laccase in an oriented, water-exposed fashion, whereas the chemically immobilized rLac is bound in a totally random orientation on the S-layer lattice.

b. Electrochemical measurements The enzymatic activity was determined by amperometric detection with HQ as substrate. The detection was performed at the potential at which the reduction of the oxidized species occurs.

By using QCM-D, the adsorbed mass of the fusion protein and the chemically bound enzyme can be determined for specific activity calculations. Combined with an electrochemical cell, amperometric detection of the product can be performed. In the experiment, two sensor surfaces were coated with SbpA, one acted as blank and the other one was functionalized with laccase via reactive ester coupling. On the third sensor, the fusion protein rSbpA/Lac was recrystallized. Figure 1 shows the experimental setup in detail. The current response of quinone was detected at −200 mV.

The calibration curve resulted in the following linear relationship [see also supplementary material, Fig. S3 at http:dx.doi.org.10.1116/3589284 for a plot of this relationship]:

TABLE II. Calculation of the specific activity derived from QCM-D data and amperometric detection.

Surface	i (μA)	c (mM)	Substrate (μ mol)	U mg^{-1}
rSbpA/Lac[a]	17.80 ± 0.47	0.198 ± 0.005	0.040 ± 0.001	10.78 ± 0.29
rLac on S-layer lattice[b]	5.77 ± 0.99	0.064 ± 0.011	0.013 ± 0.002	17.66 ± 3.05

[a]Recrystallized rSbpA/Lac.
[b]rLac covalently linked to recrystallized SbpA.

$$i_{\text{peak}} = 9^{-5} c_{\text{quinone}} \quad (r = 0.9999),$$

where i is the current response in amperes and c_{quinone} is the concentration of the converted substrate in mM concentration.

The limit of detection within this setup is 1 μM. This experiment revealed that the enzyme moiety in the fusion protein, as well as the sole enzyme, is active on solid supports (Table II). The specific activity could be determined due to the insertion of the current responses in the calibration formula. The determined specific activity of the sole enzyme is approximately two times higher than for rSbpA/Lac. However, the standard deviation of rLac bound to SbpA is more than 17% compared to less than 3% for rSbpA/Lac. These results are similar to the photometric measurements with DMP.

To summarize, the specific activity of rLac and rSbpA/Lac on solid supports revealed that HQ is a preferred substrate compared to DMP. rLac immobilized on SbpA has an approximately twofold higher activity compared to the fusion protein using HQ (see Table II) and a fourfold higher activity using DMP (see Table I). This discrepancy can be explained by the detection limit of the spectrophotometer according to the low coverage of the surface by the enzyme.

IV. SUMMARY AND CONCLUSIONS

The aim of this study was to obtain an electrode completely covered with recrystallized rSbpA/Lac fusion protein. Spectrophotometric measurements revealed the highest enzymatic activity of the fusion protein with DMP as substrate at 45 °C and *p*H 7.5. Electrochemical studies revealed the highest activity with HQ as the substrate at 40 °C and *p*H 7.6. By determining the defect areas of QCM-D gold sensors covered either by rSbpA/Lac or native SbpA, complete coverage could also be evidenced for the fusion protein. Consequently, gold sensors were coated with native SbpA, rSbpA/Lac, and/or rLac (covalently linked to the SbpA-layer via amine coupling) in order to calculate the specific activity of immobilized enzyme compared to the enzyme in solution. Spectrophotometric measurements of the specific enzyme activity revealed similar but significantly higher values for rLac and rSbpA/Lac in solution compared to the immobilized state. However, rLac covalently linked to the SbpA monolayer yielded a four to five times higher enzymatic activity than rSbpA/Lac immobilized on a solid support. The enzyme activity was for all substrates except SGZ higher for rLac compared to rSbpA/Lac which might be explained by an altered conformation of the laccase when fused to the rSbpA and by different diffusion rates between the substrate and either the rLac or the rSbpA/Lac. HQ turned out to be the preferred substrate compared to DMP. Results from electrochemical measurements showed that rLac immobilized on the SbpA lattice had an approximately twofold higher activity compared to that obtained with the fusion protein. To sum up, the laccase from *B. halodurans* C-125 is an interesting biocatalyst in applications for which classical laccases are unsuited. Concerning the present application, the preferred use of S-layers can be seen in providing a regular matrix for chemical linkage of the enzyme laccase.

ACKNOWLEDGMENTS

The authors thank Dietmar Haltrich for providing the plasmid pEBhL1, Jacqueline Friedmann for her skillful assistance in AFM imaging, and José L. Toca-Herrera for fruitful discussions. This work was supported by the Austrian Science Fund (FWF) Project No. P20256-B11, the U.S. Air Force Office of Scientific Research (AFOSR) Project Nos. FA9550-07-0313 and FA9550-10-1-0223, and the FP7 Collaborative Project MEM-S (Grant No. 244967) funded by the European Commission.

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