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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Development of a biosensing platform based on a laccase-hydrophobin chimera

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Abstract

A simple and stable immobilization of a laccase from *Pleurotus ostreatus* was obtained through genetic fusion with a self-assembling and adhesive class I hydrophobin. The chimera protein was expressed in *Pichia pastoris* and secreted into the culture medium. The crude culture supernatant was directly used for coatings of polystyrene multi-well plates without additional treatments, a procedure that resulted in a less time-consuming and chemicals reduction. Furthermore, the gene fusion yielded a positive effect with respect to the wild-type recombinant enzyme in terms of both immobilization and stability. The multi-well plate with the immobilized chimera was used to develop an optical biosensor to monitor two phenolic compounds: L-DOPA ((S)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid) and caffeic acid (3-(3,4-dihydroxyphenyl)-2-propenoic acid); the estimation of which is a matter of interest in the pharmaceutics and food field. The method was based on the use of the analytes as competing inhibitors of the laccase-mediated ABTS oxidation. The main advantages of the developed biosensor are the ease of preparation, the use of small sample volumes, and the simultaneous analysis of multiple samples on a single platform.

Keywords Immobilization · Chimera proteins · L-DOPA · Caffeic acid · Self-assembly

Introduction

Laccase (*p*-diphenol-dioxygen oxidoreductases; EC 1.10.3.2) is a well-known multicopper oxidase able to catalyze the oxidation of a wide range of aromatic substrates without additional cofactors, since they use oxygen as co-substrate and produce water as the only by-product (Giardina et al. 2010). This enzyme is a promising biocatalyst with a wide range of possible applications, including bioremediation, chemical synthesis, biobleaching of paper pulp, and biosensing. Laccase immobilization has widened the scope of its application allowing reuse of the biocatalyst with benefits in terms of costs and enzyme performances because the storage and operational stabilities are frequently enhanced. Laccases have been successfully immobilized on various carriers, using methods ranging from covalent binding to adsorption, cross-linking, entrapment, and coating (Rodríguez-Delgado et al. 2015). Several advantages and drawbacks can be described for each immobilization method. The choice of a method of immobilization depends on the target application of laccases. An alternative to conventional methods is the design of a "self-immobilizing" enzyme, a fusion protein built by an enzyme fused to a self-assembling element (Piscitelli et al. 2017c). Hydrophobins (HFBs) are a large family of small self-assembling proteins produced at different growth stages by filamentous fungi. They are very active surface proteins and play a role as coating/protective agents, in adhesion, surface modification, or other types of functions that require surfactant-like properties (Linder 2009). Hydrophobin family can be divided into two classes based on the spacing of the eight conserved cysteine residues and the nature of the amphipathic monolayers that they form. Fibrillar structures formed by class I hydrophobins are extremely robust are disassembled only in strong acids and share structural properties with amyloid fibrils (Kwan et al. 2006; Zampieri et al. 2010). Hydrophobins efficiently adhere to several hydrophobic surfaces, such as Teflon (De Vocht et al. 2002; Askolin et al. 2006; Portaccio et al. 2015), polystyrene (Wang et al. 2010), silicon (De Stefano et al. 2007; De Stefano et al. 2009), steel (Longobardi et al. 2015), and graphene (Gravagnuolo et al. 2015). These appealing properties make them of great interest

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as a versatile and simple platform for the functionalization of different surfaces (Qin et al. 2007; Zhao et al. 2007; De Stefano et al. 2009; Wang et al. 2010; Longobardi et al. 2015; Piscitelli et al. 2017a). The class I hydrophobin Vmh2 from *Pleurotus ostreatus* is able to form spontaneously stable and homogeneous layers on hydrophilic or hydrophobic surfaces, changing their wettability (De Stefano et al. 2007; Gravagnuolo et al. 2016). Chimera proteins are able both to self-assemble and display specific activities, which offer the advantage of additional functions joining the self-assembling Vmh2 hydrophobin via gene fusion (Piscitelli et al. 2017b, c). So far, two Vmh2-protein chimera have been produced to functionalize polystyrene surface. The first one was based on the genetic fusion of Vmh2 to the enzyme glutathione-S-transferase (GST) and was used to quantify toxic compounds in aqueous environmental samples (Piscitelli et al. 2017c). In the other system, Vmh2 was fused to the green fluorescent protein (GFP) and was proven to be an effective tool for the study of Vmh2 self-assembling (Pennacchio et al. 2018) and for the development of a high sensitive biosensor to monitor thrombin in plasma samples (Piscitelli et al. 2017b).

Recently, Fokina and coworkers produced laccase-hydrophobin chimera by fusing the *Aspergillus nidulans* laccase LccC to the class I hydrophobins DewA and DewB and used them to functionalize surfaces (Fokina et al. 2016).

In the present study, a new chimeric protein was designed to combine the hydrophobin Vmh2 to the remarkably stable laccase POXA1b from *P. ostreatus* (Giardina et al. 1999; Garzillo et al. 2001; Pezzella et al. 2017), in order to improve production yield and immobilization efficiency of laccase. The chimera was directly immobilized into multi-well plate without any purification step. Moreover, this procedure was exploited in the development of a rapid and easy-to-use biosensing platform capable of detecting phenolic compounds in different matrices.

Materials and methods

Vector construction

The gene encoding mature POXA1b in frame with the thrombin cleavage site and with mature Vmh2 (1856 bp), optimized according to *Pichia pastoris* codon usage (GenBank accession number MK239757), was synthesized by Thermo Fischer Scientific (Waltham, Massachusetts, USA). The fusion gene was cloned into the pJGG_αkR vector, in frame with the sequence for the α factor (signal peptide) under the control of the constitutive GAP promoter, yielding the recombinant plasmid pJGG_POXA1b-Vmh2. The fusion protein was expressed taking advantage of the recombinant production and secretion system in the yeast *P. pastoris* BG-10. Expression vectors and yeast strains were purchased from BioGrammatics, Ltd. (Carlsbad, CA, USA).

Transformation of *P. pastoris* and screening of the clones

The recombinant plasmid was linearized by *Bsi*WI and transformed into *P. pastoris* competent cells (Piscitelli et al. 2017b). Then, the cell suspension was spread on YPD plates (10 g l^{-1} yeast extract, 20 g l^{-1} Bacto tryptone, 20 g l^{-1} glucose, 15 g l^{-1} agar) containing 900 μg ml^{-1} of geneticin G418 and incubated for 3–7 days at 28 °C until colony formation. For the first screening of cultures in solid phase, the colonies were picked and transferred on solid MD (13 g l^{-1} yeast nitrogen base with ammonium sulfate without amino acids; 4×10^{-4} g l^{-1} biotin; 20 g l^{-1} glucose, 15 g l^{-1} agar, 0.6 mM $CuSO_4$, and 0.2 mM ABTS) to test the laccase expression and YPDS (10 g l^{-1} yeast extract; 20 g l^{-1} Bacto tryptone; 20 g l^{-1} glucose; 182.2 g l^{-1} sorbitol, 15 g l^{-1} agar) supplemented with 900 μg ml^{-1} G418 to test the cell growth.

Expression and characterization of the fused protein

Recombinant clones were inoculated from solid screening cultures in 20 ml YPD medium in a 100-ml baffled shaken flask. This pre-inoculum was grown for 1 day at 28 °C on a rotary shaker (250 rpm), then a volume of suspension sufficient to reach a final OD_{600} value of 0.5 was used to inoculate 250-ml baffled shaken flasks containing 50 ml of BMMY (13 g l-1 yeast nitrogen base with ammonium sulfate without amino acids; 10 g l^{-1} yeast extract; 20 g l^{-1} peptone; 0.5% methanol; 100 mM potassium phosphate pH 6.0; 4×10^{-4} g l^{-1} biotin; 0.6 mM $CuSO_4$). The fusion protein POXA1b-Vmh2 produced by *P. pastoris* was secreted in the culture media. The recombinant protein was recovered after 7–8 days and the cells were removed by centrifugation for 15 min at 6000 rpm at 4 °C. The supernatant was concentrated and dialyzed towards 50 mM Tris-HCl buffer, pH 8.0, using Centricon centrifugal filter units 10 kDa (Merck, Darmstadt, Germany).

The total protein concentration was determined using the Pierce 660 method (Thermo Fischer Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions and using bovine serum albumin (BSA) as the standard.

Laccase activity

Laccase activity was assayed at room temperature, monitoring the oxidation of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) at 420 nm ($\varepsilon_{420nm} = 3.6 \times 10^4$ M^{-1} cm^{-1}): the assay mixture contained 2 mM ABTS, 0.1 M Na-citrate buffer, and pH 3.0.

POXA1b-Vmh2 immobilization into multi-well plates

Several trials were performed immobilizing 100 μl of POXA1b-Vmh2 into polystyrene multi-well plates (96 wells). The enzymatic concentration varied from 0.02 to 2 U ml^{-1} at a protein concentration of 0.1 mg ml^{-1}. The protein was directly deposited into the wells or was mixed to the native Vmh2 at different molar ratios (1:2, 1:5, 1:10). Controls were performed immobilizing 100 μl of POXA1b at the same enzymatic concentration. The fusion protein was immobilized into the polystyrene well by deposition at 28 °C overnight. After incubation, the residual liquid was removed and assayed for laccase activity. Then, the wells were washed fivefold with 50 mM Tris HCl buffer, pH 8.0 to eliminate any unbound proteins, and the activity of the laccase was measured both in the washes and into the wells. The immobilization yield was calculated considering the immobilized enzymatic units with respect to those incubated into the well.

To evaluate the stability of the immobilized laccases, 100 μl of POXA1b-Vmh2 and of POXA1b at 0.5 U ml^{-1} were deposited into the wells and the enzyme activity was measured every day in different wells of a plate stored dry at room temperature.

For microscopic analysis, 20 μl of POXA1b-Vmh2 samples (0.2 mg ml^{-1}) were deposited on polystyrene surface and air-dried. Twenty microliters of 2 mM ABTS were added to one of the dry sample, removed after 1 min, and air-dried again. Fifty millimolars Tris-HCl buffer, pH 8.0, was used as negative control following the same procedure. Morphological analyses of POXA1b-Vmh2 deposited on polystyrene were carried out by means of Zeiss (Oberkochen, Germany) Axio Zoom V16 motorized microscope in transmitted light systems (Microscopy Center at DSC, Università degli Studi di Napoli Federico II). The microscope configuration for this study is with objective Apo Z 1.5×/0.37 FWD 30 mm. The acquisition of images was carried out with AxioCam ICC5 (D) microscopes camera and with Zeiss (Oberkochen, Germany) Axiovision 4 software with Z Stack and extended focus modules.

Western blotting

Protein samples were loaded on SDS-PAGE (12.5%) and transferred to a PVDF membrane using an electroblotting transfer apparatus (Trans-Blot Semi-Dry Transfer Cell, Bio-Rad, Hercules, California, USA). Proteins were detected by using anti-POXA1b antibodies at a 1:2000 ratio, and peroxidase-conjugated anti-rabbit secondary antisera (1:40,000) (Sigma, St. Louis, MO, USA). The membranes were developed by using SuperSignal West Femto Maximum Sensitivity Substrate detection kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA).

L-DOPA assay

Laccase activity against L-DOPA ((S)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid) was assayed at room temperature, monitoring its oxidation at 475 nm ($\varepsilon_{475nm} = 3.7 \times 10^3$ M^{-1} cm^{-1}) in citrate-phosphate buffer at pH 7 containing 5 mM L-DOPA for 15 min.

In the developed indirect assay was to detect L-DOPA, defined volumes (100 μl) of L-DOPA at increasing concentrations (from 5 to 8 mM) in 0.1 M Na-citrate buffer, pH 3 containing 2 mM ABTS, were added to the POXA1b-Vmh2-coated wells and incubated at room temperature for 3 min. Then, the absorbance intensity at 420 nm, relative to the formation of the oxidized ABTS, was measured. Solutions of L-DOPA at varying concentrations were also added to healthy human plasma (supposed to contain undetectable L-DOPA amount) to test the performance of the sensor in complex matrices. Human plasma samples were collected in a citrate anticoagulated tube from healthy subjects. Fibrinogen was removed through precipitation in 3 M ammonium sulfate. The solution was mixed for 3–4 min, then centrifuged, and the supernatant used for L-DOPA assay. The protein amount of the raw plasma and of the eluted solution was evaluated by spectrophotometric measurements at 280 nm and a loss of protein content (40%) was detected after precipitation of fibrinogen.

Caffeic acid assay

Laccase activity against CA (3-(3,4-dihydroxyphenyl)-2-propenoic acid) was assayed at room temperature, monitoring its oxidation at 470 nm ($\varepsilon_{470nm} = 1.8 \times 10^3$ M^{-1} cm^{-1}) in Tris-HCl buffer at pH 8 containing 5 mM CA for 16 h.

In the developed indirect assay to detect CA, one hundred microliters containing different concentrations of CA (from 1 to 4 mM) in 0.1 M Na-citrate buffer, pH 3, supplemented with 2 mM ABTS, were added to POXA1b-Vmh2-coated wells, and incubated at room temperature for 3 min, and then the laccase activity against ABTS was measured.

CA was also measured in real complex matrices, performing the assay in the presence of 10% of ACE juice (Skipper by Zuegg, S.p.A, Verona, Italy) or Tea infusion (Acqua Minerale San Benedetto, Scorzè, Venezia, Italy).

CA content in ACE juice and Tea infusion was also determined using the Folin-Ciocalteu assay as reported by Lettera and coworkers (Lettera et al. 2015). CA at concentrations of 0, 6.25, 12.5, 25, and 50 μg ml^{-1} were used as standard.

Results

Production and characterization of the fusion protein

The chimera POXA1b-Vmh2 was produced in *P. pastoris* and secreted into the culture medium. Its production was compared to that of the laccase POXA1b. Although the growth kinetics (Fig. 1a) of the two recombinant clones were comparable, the level of activity of the fusion protein was systematically lower (about fivefold) than that of POXA1b (Fig. 1b). Western blotting analysis was carried out to evaluate whether such a difference in activity levels were due to a lower production, or to a lower specific activity of POXA1b-Vmh2 with respect to POXA1b (Supplementary Material, Fig. S1). When the same protein amount of the two culture broth samples was loaded, the protein band corresponding to POXA1b was much more intense than that of POXA1b-Vmh2 (see Supplementary Material Fig. S1). On the other hand, when the same enzymatic unities were loaded, the difference in the band intensities was much less evident (see Supplementary Material Fig. S1). These results indicate that lower activity level of POXA1bVmh2 was due to lower concentration rather than specific activity. Taking into account that the pH of *P. pastoris* culture broth decreases during its growth (up to about pH 5), the lower POXA1b-Vmh2 concentration was luckily caused by the Vmh2 propensity to aggregate at pH

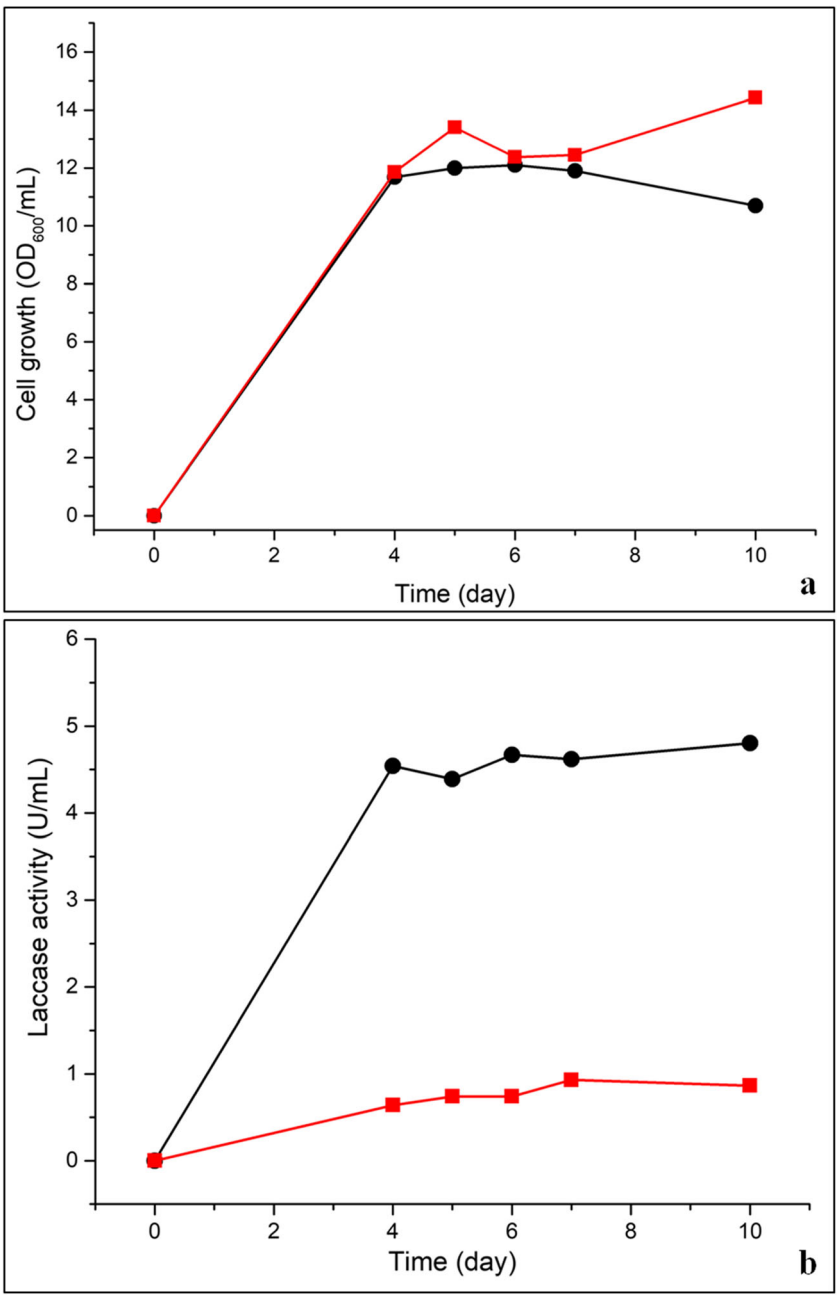


Fig. 1 Recombinant production of POXA1b-Vmh2 (square) and POXA1b (circle) in *P. pastoris* as function of time. **a** Cell growth; **b** laccase activity. Results of two technical replicates of at least three biological replicates were analyzed. Standard deviations among replicates were less than 5%

less than 7 (Gravagnuolo et al. 2016). In such a condition, the recombinant expression system allowed the secretion of about 1000 units of POXA1b-Vmh2 for liter of cultural broth.

The enzymatic affinity of POXA1b-Vmh2 towards ABTS ($K_M = 0.19 \pm 0.02$ mM) was lower than that of POXA1b ($K_M = 0.06 \pm 0.02$ mM) (Lettera et al. 2015) indicating that the presence of Vmh2 affects substrate accessibility.

Immobilization of POXA1b-Vmh2 on polystyrene multi-wells

The fusion protein was directly used for polystyrene coatings without any additional purification steps, exploiting its peculiar adhesive properties. The chimera was easily immobilized by deposition into the wells, and no loss of the total laccase activity was detected during the immobilization procedure. The immobilized enzyme resulted tightly bound to the surface even after several washes with the buffer. When 10 mU were deposited, the highest immobilization yield was obtained, but such a condition resulted into a very low amount of immobilized enzyme (Fig. 2a). Hence, we used 50 mU as a tradeoff between the amount of deposited and immobilized enzyme. Unexpectedly, POXA1b was also able to stick to polystyrene. Nevertheless, the immobilization yield of the fusion enzyme was about 2.5-fold higher (Fig. 2b). The affinity towards ABTS of the immobilized POXA1b-Vmh2 ($K_M = 0.13 \pm 0.02$ mM) did not vary significantly with respect to that of the free counterpart. With the aim to increasing the performances of our system, additional experiments were executed by immobilizing POXA1b-Vmh2 in the presence of different amount of Vmh2, as Vmh2 may act as a spacer that prevents steric hindrance among enzyme molecules (Takatsuji et al. 2013). However, in our case, a comparable or even lower immobilization yield was observed in the presence of Vmh2 (Fig. 2b), as already obtained for other hydrophobin chimera (Fokina et al. 2016; Piscitelli et al. 2017b, c), suggesting a role of competitor rather than of spacer for the free hydrophobin. Therefore, the following experiments were performed immobilizing 100 μl of POXA1b-Vmh2 at 0.5 U ml^{-1} without Vmh2 addition. No significant variation of the activity of the immobilized fusion protein was observed after 40 days at room temperature, while immobilized POXA1b was completely deactivated within the same time interval (Fig. 2c). Thus, a positive effect of the fusion of POXA1b laccase to Vmh2 was registered, both in terms of immobilization yield and stability.

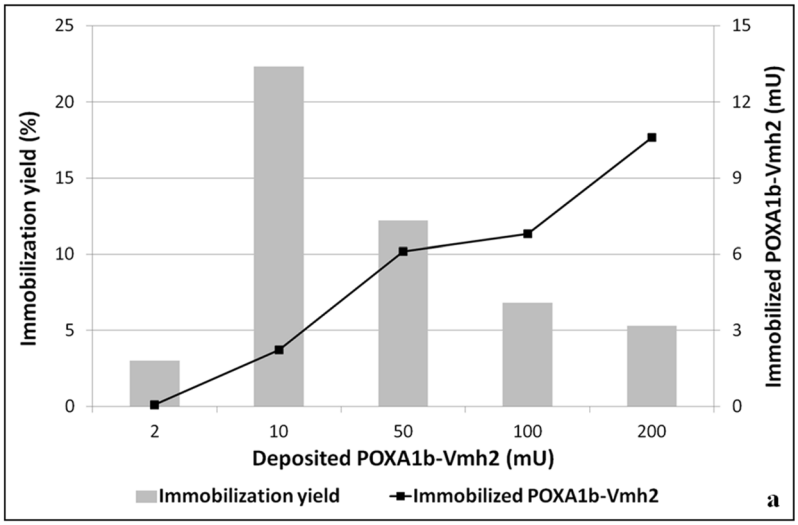
The chimera preserved the ability of Vmh2 to form fibrils when deposited on polystyrene surface. Indeed POXA1b-Vmh2 formed spherical aggregates and long fibrils (Fig. 3b and c), similar to those from native Vmh2 and its recombinant chimera (Gravagnuolo et al. 2016; Pennacchio et al. 2018). Both aggregates and fibrils retained laccase activity, being able to oxidize ABTS (Fig. 3c).

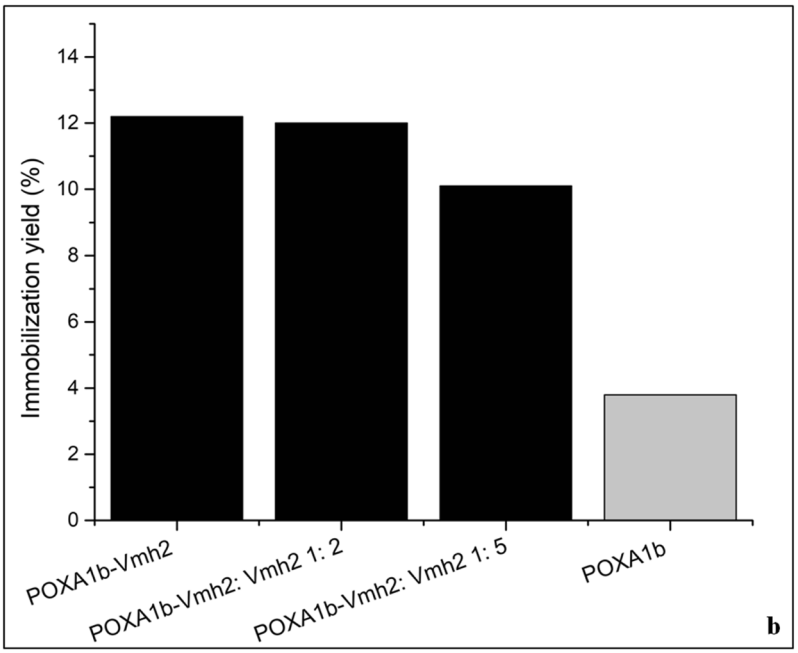
Development of optical biosensors

The multi-well plate with immobilized POXA1b-Vmh2 was used to develop a fast and easy-to-use optical biosensor to monitor two phenolic compounds, selected as examples of analytes the estimation of which is a matter of interest in the pharmaceutics and food field: L-DOPA, an important precursor of the synthesis of dopamine, and CA, a major phenolic compound present in vegetables, fruits, seeds, dark chocolate, and beverages (Eremia et al. 2013) and used as reference compound for phenol measurement.

A spectrophotometric direct detection of the chosen analytes as laccase substrates was hindered by the low sensitivity of the assays and by the long time needed. Taking into consideration the high sensitivity of the activity assay using ABTS as substrate, the analytes were used as competing inhibitors of the laccase-mediated ABTS oxidation in indirect assays.

Calibration curves were constructed reporting the relationship between the oxidized ABTS concentration (ΔA_{420nm}/min) versus analyte concentrations in a logarithmic scale. A linear trend was observed in a wide concentration range of both analytes (from μM to mM), as already observed for the other biosensor developed using the same immobilization method (genetic fusion of a sensing protein to Vmh2 hydrophobin) (Piscitelli et al. 2017b). In particular, a linear trend of concentration–response in the range from 5 μM to 1 mM was observed when L-DOPA was used as analyte (Fig. 4a), and from 50 μM to 4 mM in the case of CA (Fig. 4b). The limit of detection (LOD) was calculated according to the 3sd/m criterion, where m is the slope of the calibration curve and sd is the standard deviation of the blank (Fig. 4). The LOD, calculated in the range of linear proportion of concentration detection of L-DOPA, was of 3 μM, approximately. Since a major challenge for L-DOPA detection is its quantification in complex biological fluids, a comparable calibration curve was developed in plasma samples from healthy humans. The activity assay was slightly affected by the matrix; however, the in plasma response for L-DOPA was very consistent with the in buffer response (Fig. 4a), with a linear proportion of concentration–response in the range from 10 μM to 1 mM, and the LOD was at 2 μM (Table 1). The detection limits calculated for the developed biosensor, both in the buffer and in the real matrix, were comparable to those reported in the literature (Tembe et al. 2006; Saini et al. 2014). Saini and coworkers (Saini et al. 2014) developed an optical tyrosinase-based biosensor, with a derived LOD of 3 μM in comparable concentration of L-DOPA. With regard to the electrochemical biosensor developed by Tembe and coworkers (Tembe et al. 2006), the calculated LOD was of 1 μM, approximately, in a narrower range of linear proportion (from 5 to 30 μM). Regarding the sensitivity of the herein developed CA assay, the calculated LOD was of 1 μM





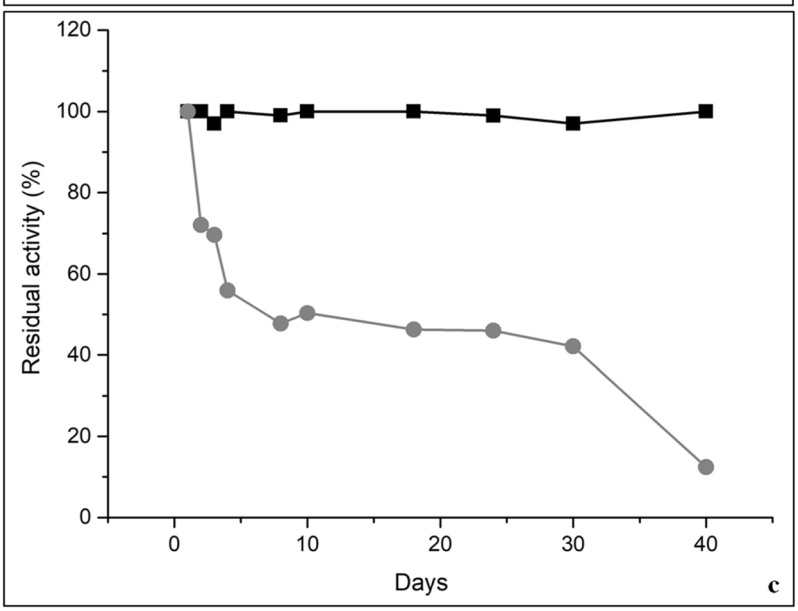


Fig. 2 **a** Immobilization yield (gray bar) and immobilized activity unities (black square) as a function of POXA1b-Vmh2 enzymatic unities deposited in the well; **b** immobilization yield of POXA1b-Vmh2 (black), even after the addition of Vmh2 at different molar ratios, in comparison with that of POXA1b (gray); **c** stability of immobilized POXA1b-Vmh2 (black square) in comparison with that of POXA1b (gray circle). Results of two technical replicates of at least three biological replicates were analyzed. Standard deviations among replicates were less than 5%

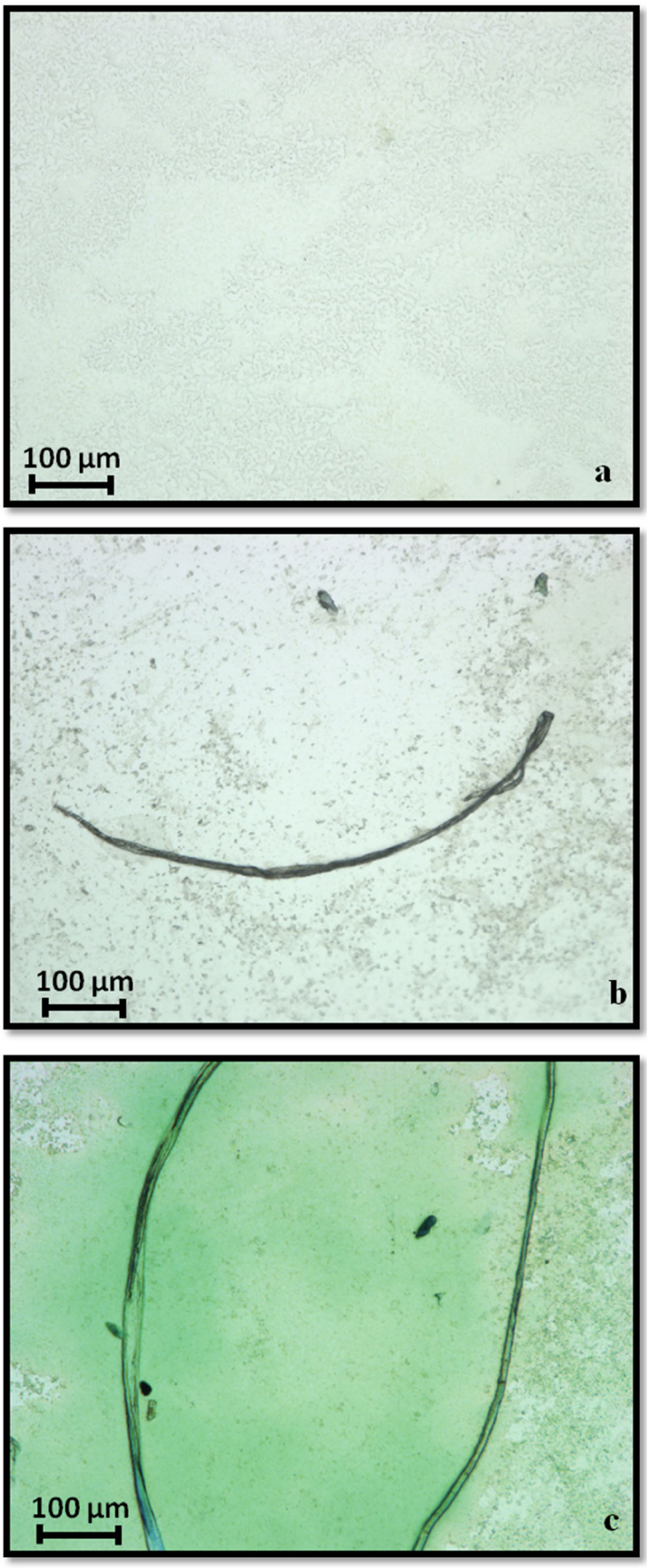


Fig. 3 Stereo microscope images (scale bar 100 µm) of samples deposited on polystyrene surface. **a** Buffer; **b** POXA1b-Vmh2; **c** POXA1b-Vmh2 after addition of ABTS

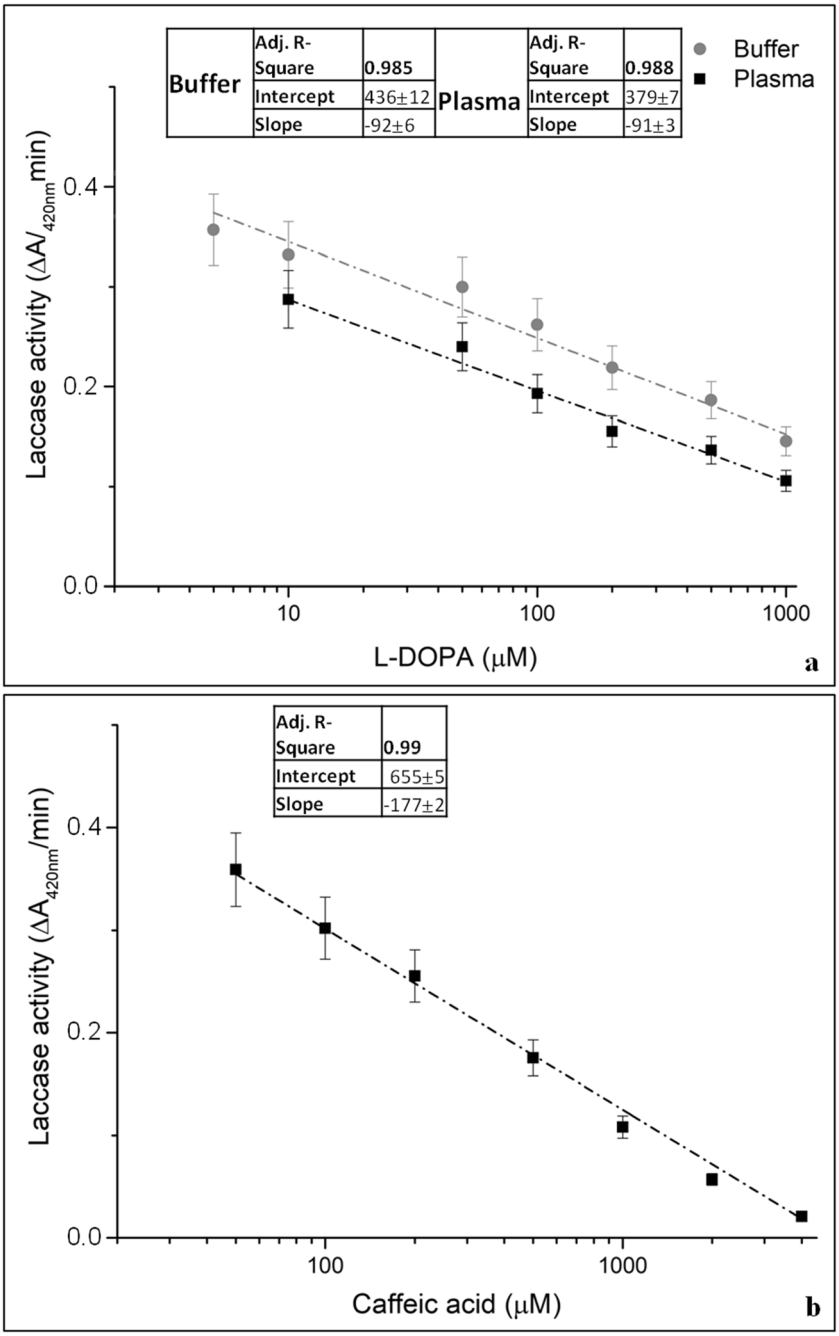


Fig. 4 Calibration curve. **a** L-DOPA; **b** CA

(Table 1), a result that was consistent with that from other reports using laccase–tyrosinase amperometric biosensor (Montereali et al. 2010). A LOD of 0.09 μM and 0.06 μM was reported with an amperometric biosensor described elsewhere (ElKaoutit et al. 2008; Eremia et al. 2013), nevertheless associated with a narrower ranges of linearity.

Table 1 Biosensor performance

Analyte		Linearity range (μM)	LOD (μM)
L-DOPA	Buffer	5–1000	3
	Plasma	10–1000	2
Caffeic acid		50–4000	1

The reliability of the biosensor was tested in the quantification of the phenol content in real matrices. L-DOPA was spiked in the plasma sample and its concentration was evaluated: results confirmed the reliability of this biosensor in quantitative detection with a proximately 100% accuracy at two different concentrations (Table 1; Supplementary Material Table S1).

CA content was evaluated in two different beverages, ACE juice and tea infusion, being well known that their phenol

content affects both their quality and stability (ElKaoutit et al. 2008). Results were compared with those obtained using the standard spectrophotometric method Folin–Ciocalteu, revealing an accuracy of about 90 and 100%, respectively, with ACE juice and tea infusion, (Supplementary Material Table S1).

Discussion

Over the last few years, laccase immobilization has been investigated deeply with the idea of both enlarging and enforcing its applicability. Alongside, the layers of hydrophobins have been employed as an attractive alternative to immobilize several enzymes of industrial interest. Besides direct deposition of proteins on a preformed self-assembled hydrophobin film, a step forward the achievement of homogeneous and more stable layer of immobilized protein has been the exploitation of genetic engineering techniques to combine the hydrophobins features with the specific properties of target proteins (Piscitelli et al. 2017a). Indeed, versatile and simple immobilization methods able to functionalize a wide range of surfaces have been developed selecting suitable target proteins to be fused to hydrophobins. This strategy was herein applied in the design of a new chimera fusing the laccase POXA1b to Vmh2 and heterologously producing it in the yeast *P. pastoris*. Active laccase chimera was secreted in the culture broth with a production yield of about fourfold higher than that obtained by Fokina and coworkers for the fusion of LccC laccase to the hydrophobins DewA (Fokina et al. 2016).

Laccase chimera was directly and tightly immobilized by deposition into the polystyrene wells, avoiding costly and time-consuming additional purification steps. The highest activity of about 0.02 U/cm^2 achieved on polystyrene with POXA1b-Vmh2 was about two orders of magnitude higher than those reported by Fokina and coworkers (Fokina et al. 2016). A further advantage of the immobilized chimera is its enhanced stability with respect to both POXA1b and the same laccase covalently immobilized on epoxy-activated poly(methacrylate) beads (Lettera et al. 2015).

Although not specific, laccases have long-standing applications in the biosensing fields, providing some advantages over other enzymes, such as the absence of additional cofactors, the ability to oxidize substrates in the presence of molecular oxygen, and good stability (Rodríguez-Delgado et al. 2015). The immobilized laccase was used to develop a fast and easy to use optical biosensor to measure L-DOPA and CA for pharmaceutical and food analysis. Laccases have been mainly used for designing electrochemical biosensors; however, optical biosensors can have the advantages of longer stability and higher sensitivity (Rodríguez-Delgado et al. 2015). L-DOPA is the best therapeutic agent for Parkinson's disease which is believed to be related to low levels of dopamine in the brain. Therefore, measuring levels of L-DOPA in biological samples can provide important information relevant to diagnosis and treatment of these disorders (Saini et al. 2014). As for L-DOPA detection, an optical tyrosinase-based biosensor has been developed (Saini et al. 2014), while all the laccase-based developed biosensors have been based on electrochemical transduction methods (Tembe et al. 2006). Caffeic acid was used as standard reference for the determination of the total polyphenolic content from beverages. A variety of electrochemical biosensors have been reported in the literature for CA detection (Odaci et al. 2007; ElKaoutit et al. 2008; Eremia et al. 2013), while, to the best of our knowledge, no report deals with its optical detection.

By the immobilization strategy developed (genetic fusion of the sensing protein to Vmh2 hydrophobin), both biosensors displayed a linear trend of concentration–response in a wide concentration range (from μM to mM), while the other electrochemical developed biosensors displayed narrower ranges of linearity (Tembe et al. 2006; Odaci et al. 2007; ElKaoutit et al. 2008; Eremia et al. 2013). As for the biosensor sensitivity, the limits calculated for L-DOPA detection, both in the buffer and in the real matrix, are comparable to those reported in the literature (Tembe et al. 2006; Saini et al. 2014). On the other hand, more sensitive amperometric biosensors for CA detection have been described (ElKaoutit et al. 2008; Eremia et al. 2013). The accuracy of the biosensor was tested in the quantification of the phenol content in real matrices, such as human plasma, tea, and ACE juice. Accuracies ranging from about 90 to 100% were obtained, confirming the reliability of the developed biosensors.

In conclusion, the genetic fusion of the laccase POXA1b to the self-assembling moiety of Vmh2 is an alternative, effective, and efficient strategy to immobilize the enzyme into multi-well plates. Since Vmh2 self-assembles spontaneously at the interfaces adhering on them, no additional purification steps or other treatments are required.

The biosensors developed herein have the advantages of the ease and effectiveness of the enzyme immobilization procedure and the chance of simultaneous analysis of multiple samples in less than 10 min by non-qualified personnel.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors. Moreover, all procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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