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Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals

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Abstract

Laccase from the white rot fungus *Coriolopsis polyzona* was immobilized for the first time through the formation of cross-linked enzyme aggregates (CLEAs). Laccase CLEAs were produced by using 1000 g of polyethylene glycol per liter of enzyme solution as precipitant and 200 μM of glutaraldehyde as a cross-linking agent. These CLEAs had a laccase activity of 148 U g^{-1} and an activity recovery of 60.2% when using 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate. CLEAs formed by co-aggregation with bovine serum albumin (BSA) as a stabilizer showed lower laccase activity and affinity for ABTS than those without BSA. The CLEAs co-aggregated with BSA showed higher residual activity against a protease, NaN_3, EDTA, methanol and acetone. The thermoresistance was higher for CLEAs than for free laccase and also higher for CLEAs co-aggregated with BSA than for simple CLEAs when tested at a pH of 3 and a temperature of 40 °C. Finally, laccase CLEAs were tested for their capacity to eliminate the known or suspected endocrine disrupting chemicals (EDCs) nonylphenol, bisphenol A and triclosan in a fluidized bed reactor. A 100-ml reactor with 0.5 mg of laccase CLEAs operated continuously at a hydraulic retention time of 150 min at room temperature and pH 5 could remove all three EDCs from a 5 mg l^{-1} solution.

Keywords: Laccase; *Coriolopsis polyzona*; Cross-linked enzyme aggregates; Stabilization; Nonylphenol; Bisphenol A; Triclosan

1. Introduction

Laccase (polyphenoloxidase, EC 1.10.3.2) is a class of multicopper lignin-modifying enzymes catalyzing the oxidation of phenol-like compounds, aromatic amines and some inorganics. Over the last decades, the use of laccase has been explored for the biodegradation of xenobiotics (Durán and Esposito, 2000; Torres et al., 2003), for bleaching in the pulp and paper industry (Call and Mucke, 1997; Crestini and Argyropoulos, 1998) and for decolorization in the textile industry (Wesenberg et al., 2003).

Compounds used in personal care products (PCPs) and substances which are either known or suspected endocrine disrupting chemicals (EDCs) can be transformed by laccases. Nonylphenol (4-nonylphenol), bisphenol A (2,2-bis(4-hydroxyphenol)propane), and triclosan (5-chloro-2(2,4-dichlorophenoxy)phenol) are frequently detected in receiving waters downstream of intense urbanization (Boyd et al., 2004; Kolpin et al., 2002). These chemicals can mimic or interfere with the action of animal endogenous hormones by acting as estrogen agonists, binding to the estrogen receptor or eliminating a normal biological response (Ishibashi et al., 2004; Jobling et al., 2003; Soto et al., 1991).

The promise of laccase for the elimination of PCPs and EDCs from both aqueous solutions and polluted soils has been established over the last few years (Cabana et al., 2007; Fukuda et al., 2001; Kim and Nicell, 2006; Tanaka et al., 2000; Tanaka et al., 2001). The resulting chemicals do not have any estrogenic activity (Cabana et al., 2007).

Abbreviations: CLEAs, cross-linked enzyme aggregates; BSA, bovine serum albumin; ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); EDCs, endocrine disrupting chemicals; NP, nonylphenol; p353NP, 4(3′,5′-dimethyl-3′-heptyl)-phenol; BPA, bisphenol A; TCS, triclosan; GLU, glutaraldehyde; CLEA-BSA-0.01, CLEA formed by the co-aggregation of laccase and 0.01 mg of BSA per unit of laccase activity; CLEA-BSA-0.1, CLEA formed by the co-aggregation of laccase and 0.1 mg of BSA per unit of laccase activity; CLEA-BSA-1, CLEA formed by the co-aggregation of laccase and 1 mg of BSA per unit of laccase activity; SEM, scanning electron microscopy

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Efforts to immobilize laccase on solid supports aim at enhancing its industrial utility, including its repeated utilization (Durán et al., 2002). Immobilization generally results in laccase stabilization against thermal and chemical denaturation and in kinetic behavior modifications. A disadvantage of immobilization on a solid support is the low enzyme/support weight ratio. Cross-linked enzyme aggregates (CLEAs) have been recently proposed as an alternative to conventional immobilization on solid supports and to cross-linked enzyme crystals (Sheldon et al., 2007; Schoevaart et al., 2004, 2006; Cao, 2005; Mateo et al., 2004). This immobilization involves the precipitation of the enzyme and the chemical cross-linking of the protein using bifunctional compounds. Cross-linking prevents the solubilization and possible loss of the aggregates after removing the precipitating agent. Some additives have been proposed for the stabilization of CLEAs such as bovine serum albumin (BSA) and polyionic polymers (Shah et al., 2006; Wilson et al., 2004).

The first objective of this study was proving the concept of CLEAs with a crude laccase solution, including the production of laccase CLEAs, the formation of CLEAs stabilized with BSA and their biocatalytic characterization. Since none of the previous reports on applying laccase for the removal of PCPs and EDCs used immobilized enzyme, the second objective of the present study was the utilization of stabilized CLEAs for the elimination of p353NP (a branched isomer of NP), BPA and TCS.

2. Experimental

2.1. Chemical reagents

All chemicals were of analytical grade (or of the highest purity available). 3,5-Dimethyl-3-heptanol and polyethylene glycol were from Alfa Aesar (Karlsruhe, Germany). All other chemicals were from Sigma–Aldrich (Saint-Louis, MO). All solvents were HPLC grade.

2.2. Organism and cultivation conditions

The white rot fungal (WRF) strain *Coriolopsis polyzona* (MUCL 38443) was provided by the Belgian Coordinated Collection of Microorganisms (BCCM^{TM}/MUCL). The inoculum was grown in a rotary shaker at 150 rpm and 27 °C in 250-ml flasks containing 100 ml of standard medium: 10 g l^{-1} glucose, 2 g l^{-1} NH_4NO_3, 0.8 g l^{-1} KH_2PO_4, 0.4 g l^{-1} Na_2HPO_4, 0.5 g l^{-1} $MgSO_4$ $7H_2O$, 2 g l^{-1} yeast extract. The pH was adjusted to 6.0 with 2 M NaOH prior to autoclaving. After 10 days of cultivation the biomass was filtered and the solids were separated by centrifugation at 3000 × *g* for 15 min at 4 °C. Enzymes were precipitated using 600 g l^{-1} ammonium sulfate. This preparation was dialyzed against distilled water using a 13 kDa membrane and then used as the source of laccase.

2.3. Enzyme assay

Laccase activity was determined by monitoring the oxidation of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to its cation radical ($ABTS^{\bullet+}$) at 420 nm ($\varepsilon_{max} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$) (Bourbonnais and Paice, 1990). The assay mixture contained 0.5 mM ABTS. The pH was adjusted to 3 using citric acid/di-sodium hydrogen phosphate buffer and the temperature was set at 30 °C. One unit of activity was defined as the amount of enzyme forming 1 μmol of $ABTS^{\bullet+}$ per min.

2.4. Aggregate activity

Ninety μl of precipitant with proper dilution was added to 10 μl of laccase solution (1 U ml^{-1}). The enzyme was allowed to precipitate for 16 h at 20 °C. Next, 900 μl of phosphate buffer (10 mM, pH 7) was added. A 100 μl aliquot was then transferred into a cuvette to determine the residual laccase activity (Schoevaart et al., 2004). The precipitation step was optimized by testing polyethylene glycol (PEG) (average molecular weight of 1500), ammonium sulfate, ethanol or acetone as precipitants.

2.5. Preparation of CLEAs

CLEAs were prepared using 2 ml of a 1 U ml^{-1} laccase solution, the best precipitant, glutaraldehyde (GLU) as the cross-linking agent, and bovine serum albumin (BSA) as a stabilizer. Different concentrations of GLU and BSA were tested (see Section 3). The solution was stored at 4 °C for 24 h. Subsequently, the solution was centrifuged for 15 min at 1000 × *g*. The pellets were washed repeatedly with distilled water until no laccase activity was detected in the supernatant.

2.6. Determination of kinetic parameters

The Michaelis–Menten kinetic parameters K_m and k_{cat} of laccase CLEAs prepared with and without BSA were determined by measuring the laccase activity using ABTS as substrate. Furthermore, the same kinetic parameters were determined using the selected EDCs as substrate. The parameter values were obtained by curve fitting of the plot of reaction rate versus substrate concentrations using the Sigma Plot 7.0 software (SPSS Inc., Chicago, IL).

2.7. CLEA stability against chemical denaturants

The stability against different denaturants was tested using CLEAs representing 10 μU of laccase activity. The denaturing solutions consisted of NaN_3, $ZnCl_2$, $CoCl_2$, $CaCl_2$, ethylenediamine-tetraacetic acid (EDTA), methanol or acetone in citric acid/di-sodium hydrogen phosphate buffer at pH 3. The tests lasted 30 min at 20 °C. The denaturation with a serine protease from *Streptomyces griseus* was carried out at pH 7 and 30 °C for 6 h.

2.8. Kinetics of thermal inactivation

The time course of thermal inactivation of CLEAs and of soluble laccase was investigated at 40 °C and pH 3. One hundred μU of CLEAs or free laccase were incubated in 500 μl of

buffer solution containing 100 μM of phenylmethylsulfonyl fluoride (PMSF) to inhibit protease activity. Residual activity was determined every 30 min. The inactivation of free laccase and CLEAs was modeled using the 3-parameter model of Aymard and Belarbi (2000):

$$\frac{(A)_t}{(A)_0} = C\,e^{-\alpha t} + (1 - A)\,e^{-\beta t} \quad (1)$$

The ratio $(A)_t/(A)_0$ represents the enzyme activity remaining after time t. The physical meaning of the parameters and their expressions as a function of individual rate constants differs according to the mechanism considered. This expression could be used irrespective of the thermal inactivation mechanism involved (Aymard and Belarbi, 2000). The values of the different parameters of this model were obtained by curve-fitting of the plot of the residual enzyme activity versus time using the Sigma Plot 7.0 software.

2.9. Scanning electron microscopy of CLEAs

Scanning electron micrographs (SEMs) of CLEAs were obtained on Hitachi S-4700 FESEM and S-3000N VPSEM (Tokyo, Japan) electron microscopes. The samples were previously dried at ambient temperature and then coated with platinum using an Emitech K550 (Ashford, UK) sputter coater.

2.10. Synthesis of 4(3′,5′-dimethyl-3′-heptyl)-phenol (p353NP)

The p353NP isomers of NP were synthesized by Friedel-Crafts alkylation from phenol and 3,5-dimethyl-3-heptanol (nonanol). The reaction medium contained 1.88 g phenol, 4.33 g 3,5-dimethyl-3-heptanol, 35 ml BF_3-ether complex and 200 ml anhydrous petroleum ether in a 500 ml two-necked flask equipped with a reflux condenser. The reaction was run for 15 min at 50 °C with stirring, and then stopped by adding 200 ml of water. After intensive stirring for a further 15 min, the aqueous phase was removed and the organic phase was washed five times with water in order to remove non-reacted phenol, and then dried over Na_2SO_4 (Corvini et al., 2004). After removing the petroleum ether under vacuum, the yield of p353NP was 1.72 g with a purity of 98.0% (GC).

2.11. Continuous elimination of endocrine disrupting chemicals by CLEAs

A fluidized bed reactor (FBR) was used for the continuous transformation of each EDC from an aqueous solution of 5 mg l^{-1} whose pH was kept at 5 with citric acid/di-sodium hydrogen phosphate buffer. Catalase (5 U l^{-1}) from *Aspergillus niger* was added to the solution to eliminate hydrogen peroxide. The glass column (1.6 cm diameter, 20 cm height) FBR was fed with the EDC solution (air saturated) at a flow rate of 1.5 ml min^{-1}. The reactor was packed with 0.5 mg of laccase CLEAs and operated at room temperature and at pH 5.

2.12. Extraction of EDCs

The p353NP isomers were extracted with a C_{18} solid phase extraction cartridge conditioned with 4 ml of methanol and 4 ml of distilled water prior to extraction. It was filled with 5 ml of the treated solution and eluted at about 2 ml min^{-1} under vacuum. The p353NP isomers were eluted from the cartridge using 3 ml of ethyl acetate and 6 ml of methylene chloride. BPA and TCS were extracted using methylene chloride in a 1:1 (v/v) ratio with the treated solution. The solutions were acidified to pH 2 with HCl and shaken for 20 min. The organic phase was then separated and evaporated under a gentle stream of nitrogen gas. Each sample was dissolved in 100 μl of methylene chloride prior to quantitative chemical analysis.

2.13. GC–MS analysis

The gas chromatography–mass spectrometry analysis was performed on a G1800A gas chromatograph (Hewlett–Packard, Palo Alto, CA) equipped with an electron ionization detector and an HP-5 MS fused-silica column (30 m × 0.25 mm i.d., 0.25 mm film thickness). Analysis was conducted in full-scan mode over a *m/z* range of 50–450 amu. Helium was used as carrier gas at a flow rate of 0.70 ml min^{-1}. The column was held at 70 °C for 3 min, then temperature increased to 160 °C at a rate of 20 °C min^{-1}, and it finally increased to 300 °C at a rate of 10 °C min^{-1} and held for 7 min (Diaz et al., 2002). The injector was set at 250 °C.

3. Results

3.1. Preparation of laccase CLEAs

The impact of the formation procedures on the activity of laccase CLEAs was studied in two steps: (1) determining the best precipitant and its concentration and (2) determining the impact of cross-linker concentration (Schoevaart et al., 2004). Fig. 1

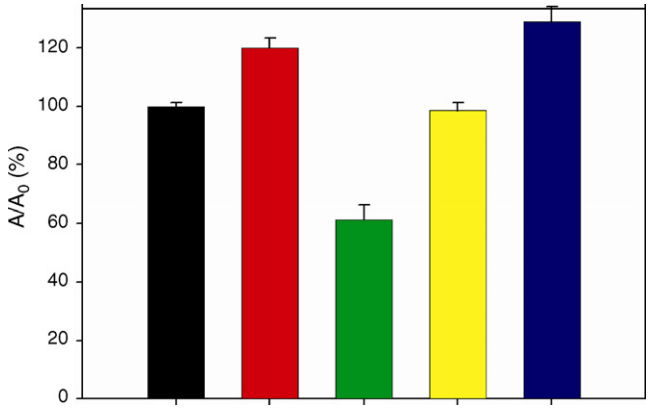

Fig. 1. Laccase activity recovery after a 16-h precipitation at pH 7 and a temperature of 20 °C using (■) buffer, (■) 55% (w/v) ammonium sulfate, (■) 79% (w/v) ethanol, (■) 67% (w/v) acetone or (■) 100% (w/v) PEG as precipitant (mean of triplicates ± standard deviation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Activity of laccase CLEAs and activity recovery using different amounts of BSA[a] x

BSA (mg U^{-1})	Activity (U g^{-1})	Activity recovery (%)
0	148 ± 12	60.2 ± 4.1
0.01	78 ± 10	32.7 ± 2.1
0.1	40 ± 3	28.7 ± 2.1
1	44 ± 4	30.3 ± 3.1

[a] The values represent means of triplicate experiments ± standard deviation.

shows the maximum activity recovery after 16 h of precipitation using PEG, ammonium sulfate, methanol or acetone. The best results (recovery of about 130% of the initial laccase activity) were obtained using 100% (w/v) of PEG (1000 g l^{-1}) as precipitant. Next, in a step combining aggregation and cross-linking, we used a 1000 g l^{-1} solution of PEG and a range of GLU concentrations to produce laccase CLEAs (López-Serrano et al., 2002). GLU at 200 μM led to both higher laccase activity and higher overall recovery (results not shown).

3.2. Formation of laccase CLEAs with the addition of BSA as a stabilizer

Given that the co-aggregation of lipase and penicillin acylase with BSA improved the stability, % recovery and activity of these two enzymes (Shah et al., 2006), CLEAs were made by co-aggregating laccase with different amounts of BSA per unit of activity and using the best conditions found above. The absolute activities initially obtained and the percent activities recovered for the different quantities of BSA added are shown in Table 1. This co-aggregation resulted in a decrease of the laccase activity of the CLEAs formed and a drop in the percentage of activity recovered. At higher BSA supplementations, the addition of 0.01 mg of BSA per unit activity of laccase resulted in the best overall indices of activity and % activity recovery compared to CLEAs without BSA.

3.3. Kinetics of laccase CLEAs

Table 2 presents the different Michaelis–Menten kinetic constants of CLEAs with and without BSA for the oxidation of ABTS. CLEAs had a higher maximum rate of ABTS transformation compared to free laccase. The k_{cat} value of CLEAs not supplemented with BSA was found to be 6 times higher than that of free laccase and 15 times higher than the k_{cat} of CLEAs supplemented with BSA at 0.01 mg U^{-1} (CLEA-BSA-0.01) with ABTS as substrate. When 0.1 and 1 mg U^{-1} of BSA were used, the maximum rates of ABTS transformation by the respective CLEAs decreased significantly. The Michaelis–Menten constants, K_m, of the free and insolubilized forms were about the same. The biocatalytic efficiency of the unsupplemented CLEAs, based on $k_{cat}\,K_m^{-1}$ value, was seven times higher than that of the free laccase for the oxidation of ABTS. CLEA-BSA-0.1 and CLEA-BSA-1 were far less effective catalysts than the other forms of laccase as reflected by their $k_{cat}\,K_m^{-1}$ value which were one and two orders of magnitude lower.

Table 2
Michaelis–Menten kinetic constants of laccase CLEAs for the oxidation of ABTS[a] x

	K_m (μM)	k_{cat} (μmol s^{-1} mg^{-1})	$k_{cat}\,K_m^{-1}$ (l mg^{-1} s^{-1})	R^2
Free	32.0 ± 2.5	10.2 ± 0.5	0.3	0.987
CLEA	28.5 ± 3.8	63.9 ± 2.2	2.2	0.9839
CLEA-BSA-0.01	29.8 ± 6.4	4.3 ± 0.9	0.1	0.9975
CLEA-BSA-0.1	28.5 ± 3.4	0.3 ± 0.01	0.01	0.9831
CLEA-BSA-1	31.9 ± 4.2	0.02 ± 0.02	0.006	0.9806

[a] The values represent means of triplicate experiments ± standard deviation.

Table 3
Michaelis–Menten kinetic constants for the elimination of p353NP, BPA and TCS with laccase CLEAs unsupplemented with BSA at a pH of 5 and a temperature of 20 °C[a] x

Substrate	K_m (mM)	k_{cat} (μmol s^{-1} mg^{-1})	$k_{cat}\,K_m^{-1}$ (l mg^{-1} s^{-1})	R^2
p353NP	0.45 ± 0.002	0.8 ± 0.01	0.018	0.943
BPA	0.23 ± 0.017	0.16 ± 0.01	0.007	0.944
TCS	0.12 ± 0.01	0.24 ± 0.03	0.02	0.961

[a] The values represent means of triplicate experiments ± standard deviation.

Table 3 presents the Michaelis–Menten kinetic parameters for the elimination of the EDCs p353NP, BPA and TCS using laccase CLEAs unsupplemented with BSA. The kinetic constants obtained are of similar value for all three target substrates. Based

Table 4
Residual laccase activity (A/A_0) of laccase CLEAs after incubation with different denaturants[a] x

Denaturant	Free laccase	CLEA	CLEA-BSA-0.01	CLEA-BSA-0.1	CLEA-BSA-1
Protease (2 mg l^{-1})	0.24	0.51	0.56	0.61	0.71
NaN_3 (30 μM)	0.03	0.78	ND	ND	ND
NaN_3 (150 μM)	0.00	0.50	ND	ND	ND
$ZnCl_2$ (10 μM)	0.43	0.47	0.48	0.46	0.44
$CoCl_2$ (10 μM)	0.56	0.53	0.52	0.43	0.53
$CaCl_2$ (10 μM)	0.52	0.54	0.51	0.52	0.57
EDTA (10 μM)	0.41	0.81	0.92	1.07	0.78
Methanol (25%, v/v)	0.50	0.82	0.83	0.79	0.95
Acetone (25%, v/v)	0.57	0.96	0.77	0.62	0.77

ND, not determined.
[a] The values represent means of triplicate experiments.

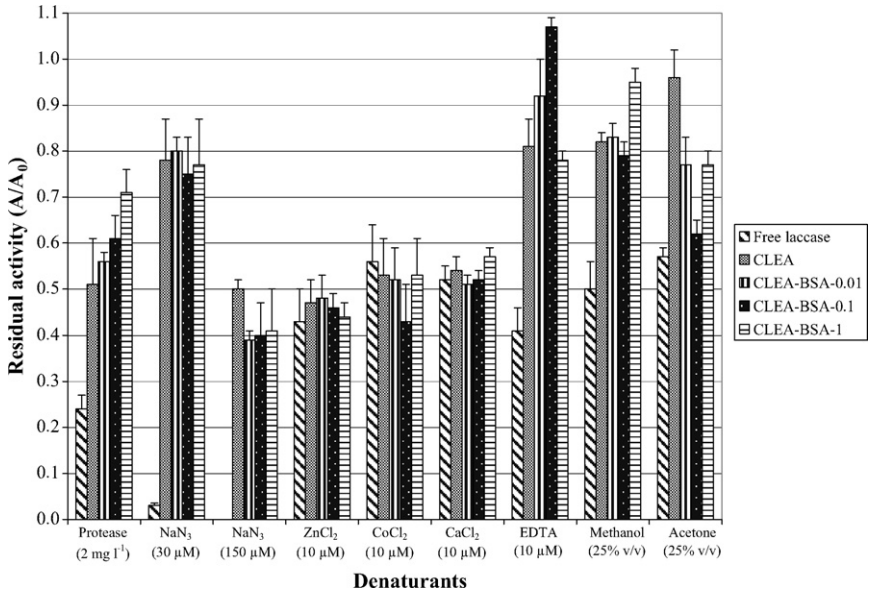

Fig. 2. Residual activity (A/A_0) of laccase CLEAs after incubation with denaturants (mean of triplicates ± standard deviation).

on the affinity and kinetic constants of the CLEAs for the various EDC, these insolubilized biocatalysts have a greater capacity of elimination for TCS, followed by p353NP and by BPA (Table 3).

3.4. Stability against chemical denaturants

Fig. 2 presents the residual activity of the free and immobilized laccases incubated with chemical denaturants and a protease. Unsupplemented CLEAs had higher stability than free laccase in the presence of solutions of 10 μM of EDTA, 30 and 150 μM of NaN_3 and 25% (v/v) methanol and acetone (Table 4). The BSA-supplemented CLEAs had improved stability only against EDTA. Finally, laccase in the form of CLEAs was not more stable against a 10 μM solution of $ZnCl_2$, $CoCl_2$ or $CaCl_2$ than free laccase. The laccase CLEAs displayed a residual activity twice as high as the free laccase against the proteolytic action of a 2 g l^{-1} solution of the serine protease from *S. griseus*. The addition of BSA improved the stability of the immobilized laccase against the proteolytic action. For example, the stability of the CLEA-BSA-1 was 50% higher than that of the CLEAs without BSA (Table 4). In a parallel experiment, BSA-free CLEAs incubated with the different EDCs before their transformation had a stabilizing effect on the enzyme. At the end of the incubation period, the residual activity of the CLEAs in the absence of EDC was 50% of the initial activity versus 85% in their presence for all three substances tested (results not shown).

3.5. Kinetics of thermal inactivation

Fig. 3 presents the temporal variation of activity for the different forms of laccase over a 2-day period. The activity of the immobilized forms of laccase was better conserved than the free form at pH 3 and 40 °C. After a 24-h incubation period, the free laccase had no activity while CLEA, CLEA-BSA-0.01 and

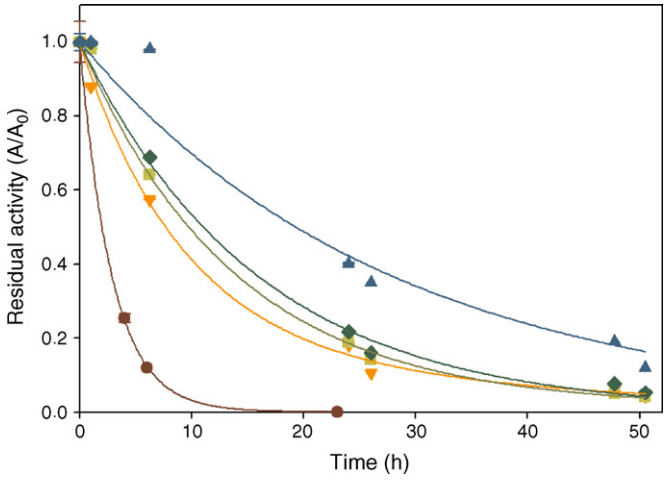

Fig. 3. Thermal inactivation of free laccase (,), CLEA (,), CLEA-BSA-0.01 (,), CLEA-BSA-0.1 (,) and CLEA-BSA-1 (,) subjected to a temperature of 40 °C and a pH of 3. The solid lines represent the inactivation kinetics modeled with the biexponential model proposed by Aymard and Belarbi (2000). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

CLEA-BSA-0.1 still showed 20% of their initial laccase activity versus 40% for the CLEA-BSA-1.

Table 5 presents the kinetic inactivation parameters for free and immobilized laccase. Using these parameters, the half-life

Table 5
Kinetic inactivation parameters for laccase CLEAs and CLEAs formed with addition of BSA, subjected to a temperature of 40 °C and a pH of 3[a] x

	C	α (h^{-1})	β (h^{-1})	R^2	Half-life (h)
Free laccase	0.53	0.35	0.35	0.989	2.0
CLEA	0.80	0.11	0.03	0.977	7.7
CLEA-BSA-0.01	0.99	0.07	0	0.997	9.7
CLEA-BSA-0.1	0.49	0.06	0.06	0.994	11.0
CLEA-BSA-1	0.51	0.04	0.04	0.960	19.3

[a] The values represent means of triplicate experiments.

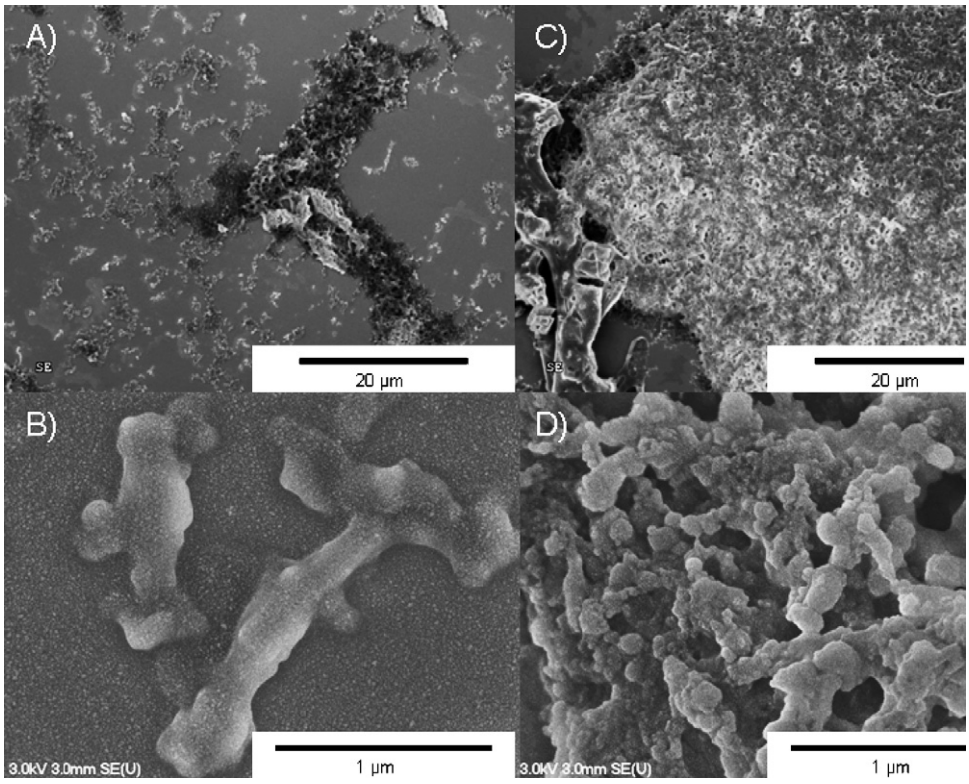

Fig. 4. SEMs of laccase CLEAs and CLEAs formed with 1 mg BSA U^{-1}: (A) CLEA (working distance = 11.4 mm, acceleration voltage = 5 kV); (B) CLEA (working distance = 3.0 mm, acceleration voltage = 3 kV); (C) CLEA-BSA 1 mg U^{-1} (working distance = 11.4 mm, acceleration voltage = 5 kV); (D) CLEA-BSA 1 mg U^{-1} (working distance = 3.0 mm, acceleration voltage = 3 kV).

of the free laccase, CLEA, CLEA-BSA-0.01, CLEA-BSA-0.1 and CLEA-BSA-1 was found to increase in this order, from 2.0 to 19.3 h.

3.6. SEMs of CLEAs

Fig. 4 shows the SEMs of CLEAs of laccase prepared without BSA and with 1 mg of BSA per unit of laccase activity. The BSA-free CLEAs were smaller than those made with the protein feeder, their length being between 1 and 5 μm although some bigger ones up to 50 μm were also observed (Fig. 4(A) and (B)). The CLEAs of laccase made with 1 mg U^{-1} of BSA were bigger (100–200 μm) (Fig. 4(C) and (D)). The SEMs of both types of laccase CLEAs showed a uniform surface structure.

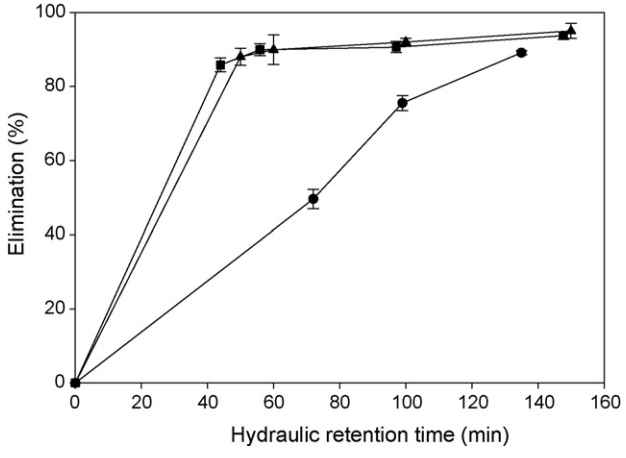

Fig. 5. Continuous treatment of a 5 mg l^{-1} solution of (▲) p353NP, (●) BPA and (■) TCS at pH 5 and at room temperature using 0.5 mg of laccase CLEAs in a fluidized bed reactor as a function of residence time.

3.7. Continuous elimination of EDCs

The continuous elimination of EDCs from a 5 mg l^{-1} solution using 0.5 mg of laccase CLEAs at pH 5 and room temperature in a FBR is shown in Fig. 5. A residence time of 50 min in the FBR allowed a 90% elimination of p353NP and TCS and a 30% removal of the BPA. At a 150-min residence time, more than 95% of the p353NP, BPA and TCS were transformed.

4. Discussion

The results from the precipitation tests indicated that quenching the laccase solution with high precipitant concentrations led to a high recovery of enzyme activity. In the case of other enzymes this fast precipitation procedure has shown higher activity recovery than the slow addition of precipitants (Schoevaart et al., 2004). The higher remaining activity of the laccase precipitated with PEG compared to free laccase could be explained by the hypothesis that this polymer tends to fold on itself thereby allowing to bind more water molecules in its three-dimensional structure (Donato et al., 1996). Denaturation upon precipitation with organic solvents results from the hydrophobic interactions between the solvent and the nonpolar groups of the

laccase which shifts the equilibrium towards the denatured state (Makarov et al., 1995). For thermodynamic reasons ethanol has a higher preferential solvation for enzymes than acetone at the same activity in solution, hence ethanol leads to greater denaturation of laccase compared to acetone (Kovrigin and Potekhin, 1997).

Determining the impact of the cross-linking agent's concentration is necessary due to the potential inactivation of enzymes by this type of chemical. The use of 200 μM GLU as the cross-linking agent gave, among the concentrations tested, the best laccase CLEA activity and recovery compared to higher concentrations. The reduction of the CLEA activity at GLU concentrations higher than 200 μM could be explained by a rigidification of the enzyme's three-dimensional structure that hinders the conformational change needed for the oxidation of substrates at the active site. This trend was also observed recently in the production of laccase crossed-linked enzyme crystals (Roy and Abraham, 2006).

In a study of lipase and alcohol dehydrogenase CLEAs, Schoevaart et al. (2004) showed that concentrations of GLU higher than 8 μM inactivated the alcohol dehydrogenase CLEAs but concentrations between 5 and 100 μM did not significantly change the activity of lipase CLEAs. In our study, the laccase CLEAs formed had an activity of about 150 U g^{-1} and an activity recovery of 60% which corresponds to the levels reported with other enzymes (Cao et al., 2003).

Recently, the formation of CLEAs by co-aggregation of lipase or penicillin acylase with BSA (Shah et al., 2006) and glutaryl acylase or penicillin acylase with polymers (López-Gallego et al., 2005; Wilson et al., 2004) was proposed. These protocols improve the recovery, stability, activity, enantio-selectivity and specificity of the enzyme. In our case, the addition of BSA at levels from 0.01 to 1 mg per unit of laccase activity led to lower CLEA activity, possibly due to inadequate exposure of the laccases catalytic center to ABTS because of shielding by the BSA. In addition, this activity drop could reflect mass transfer limitations from the bulk substrate solution to the laccase active site. This hypothesis is consistent with the visual evidence from the SEMs of laccase CLEAs without BSA and with 1 mg BSA U^{-1}: the surface/volume ratio of CLEAs decreased when increasing the proportion of BSA. The addition of BSA may disturb the internal structure of the aggregates resulting in narrower channels that limit the diffusion of the substrate ABTS in the CLEAs.

The kinetic study showed that the different forms of laccase studied have almost the same affinity, based on K_m values, for the substrate ABTS. CLEA is a better catalyst for the oxidation of ABTS than free laccase when the rate of transformation, k_{cat}, and the efficiency of the process, $k_{cat}\,K_m^{-1}$, are used for comparison. The CLEAs supplemented with BSA had a ten-fold lower efficiency than free laccase. This drop in catalytic activity could also be explained by mass transfer limitations as discussed above.

Testing the stability of CLEAs and free laccase against different denaturants, it seems that CLEA formation diminished the structural alteration of the catalytic site of laccase induced by azide binding (Battistuzzi et al., 2003). The aggregation of laccase as CLEAs improved enzyme stability against the chelator EDTA. This stabilization could be due to hindering mass transfer of EDTA into the CLEA amorphous structure. The stabilities against the chaotropic salts $ZnCl_2$, $CoCl_2$ and $CaCl_2$ were the same for free and insolubilized laccase, likely due to the small dimension of the ions that allowed them to easily migrate into the structure of the CLEAs and inactivate the laccase to an equal extent. Nonetheless, the low concentrations used could explain the salts' low impact. Laccase CLEAs showed higher stability to serine proteases from *S. griseus* than free laccase, possibly because proteolytic enzymes could not efficiently diffuse into the aggregates to hydrolyse the laccase. Furthermore, the addition of BSA as a protective agent significantly enhanced that stability. This could be due to the higher affinity or access of the protease to BSA compared to the laccase present in CLEAs. Finally, the stability against the organic solvents acetone and methanol was higher for the laccase CLEAs than for free laccase. As seen before, the formation of CLEAs stabilizes the enzyme in the presence of organic cosolvents (Tyagi et al., 1999; Wilson et al., 2004) due to the rigidity of the biocatalyst formed.

Laccase CLEAs showed higher stability against thermal denaturation compared to free laccase because the cross-linking (presence of multiple links on the protein surface) prevented the unfolding of laccase under thermal stress conditions (Fernandez-Lafuente et al., 1995). These results differ from those obtained by López-Gallego et al. (2005) who observed the same residual activity for free glutaryl-7-aminocephalosporanic acid acylase compared to the enzyme immobilized as CLEAs. The addition of BSA as a protective agent led to a higher half-life of the CLEAs formed. These findings are in agreement with those of Shah et al. (2006) who obtained more thermally stable CLEAs of penicillin acylase when they added BSA. This higher stability of laccase CLEAs with BSA could be explained by the microenvironment induced by the presence of large amounts of BSA in the immediate surroundings of laccase that enhances the cross-linking and decreases the entropy of the enzyme's denatured state. It is likely that a favourable interaction is established between laccase and BSA and that the cross-linking may be reinforcing it.

The structures of laccase CLEAs formed without BSA and with addition of 1 mg of BSA per unit laccase activity were similar. The structure is of Type 2 as proposed by Schoevaart et al. (2004). The laccase CLEAs formed with the addition of BSA show a structure analogous to CLEAs of lipase from *Pseudomonas cepacia* and from *Candida rugosa* (Schoevaart et al., 2004; Shah et al., 2006). It appears that the aggregates formed with the addition of BSA consisted of the laccase particles without BSA assembled in a macro structure 100–200 μm long compared to BSA-free CLEAs which were only 1–5 μm. This size difference is consistent with a variation in the CLEAs' surface/volume ratio resulting in mass transfer limitations which are greater in CLEAs formed with BSA than for those without.

In this study we investigated the elimination of three known or suspected EDCs: NP, BPA and TCS. Due to the complex mixture of isomers present in technical NP (Wheeler et al., 1997) which gives rise to a complex metabolic pattern, we studied the elimination of p353NP, a branched isomer of NP. In a FBR we could reach more than 90% elimination of p353NP and TCS with

a 40-min hydraulic residence time while 130 min was required for 90% removal of BPA. These findings are in agreement with the kinetic measurements obtained with batch eliminations of EDC using laccase CLEAs. The kinetic constants obtained confirmed that the insolubilized biocatalyst formed had decreasing efficiency towards TCS, p353NP and BPA in that order. We have previously demonstrated that free laccases from *C. polyzona* had a decreasing elimination efficiency for NP, BPA and TCS in that order (Cabana et al., 2007). This difference could be due to a modification of the catalytic properties of the enzyme upon insolubilization (Shah et al., 2006).

5. Conclusion

For the first time, CLEAs of laccase from the WRF *C. polyzona* have been described in detail. The immobilization process was investigated with respect to the precipitation step and the concentration of the cross-linking agent GLU. Furthermore, laccase CLEAs with the addition of BSA as a stabilizer were formed and their performance and stability examined. Finally, the laccase CLEAs were used for the successful elimination of the EDC p353NP, BPA and TCS. Overall, our results show that laccase immobilization via the formation of CLEAs is a promising tool to increase the applicability and reusability of laccase in biotechnology.

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References

Aymard, C., Belarbi, A., 2000. Kinetics of thermal deactivation of enzymes: a simple three parameters phenomenological model can describe the decay of enzyme activity, irrespectively of the mechanism. Enzyme Microbial Technol. 27, 612–618.

Battistuzzi, G., Di, R.G., Leonardi, A., Sola, M., 2003. ^{1}H NMR of native and azide-inhibited laccase from *Rhus vernicifera*. J. Inorg. Biochem. 96, 503–506.

Bourbonnais, R., Paice, M.G., 1990. Oxidation of nonphenolic substrates—an expanded role for laccase in lignin biodegradation. FEBS Lett. 267, 99–102.

Boyd, G.R., Palmeri, J.M., Zhang, S., Grimm, D.A., 2004. Pharmaceuticals and personal care products (PPCPs) and endocrine disrupting chemicals (EDCs) in stormwater canals and Bayou St. John in New Orleans, Louisiana, USA. Sci. Total Environ. 333, 137–148.

Cabana, H., Habib Jiwan, J.L., Rozenberg, R., Elisashvili, V., Penninckx, M.J., Agathos, S.N., Jones, J.P., 2007. Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using enzyme preparation from the white rot fungus *Coriolopsis polyzona*. Chemosphere 67, 770–778.

Call, H.P., Mucke, I., 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). J. Biotechnol. 53, 163–202.

Cao, L., 2005. Immobilised enzymes: science or art? Curr. Opin. Chem. Biol. 9, 217–226.

Cao, L., van Langen, L., Sheldon, R.A., 2003. Immobilised enzymes: carrier-bound or carrier-free? Curr. Opin. Biotechnol. 14, 387–394.

Corvini, P.F.X., Vinken, R., Hommes, G., Schmidt, B., Dohmann, M., 2004. Degradation of the radioactive and non-labelled branched 4(3′,5′-dimethyl-3′-heptyl)-phenol nonylphenol isomer by *Sphingomonas* TTNP3. Biodegradation 15, 9–18.

Crestini, C., Argyropoulos, D.S., 1998. The early oxidative biodegradation steps of residual kraft lignin models with laccase. Bioorg. Med. Chem. 6, 2161–2169.

Diaz, A., Ventura, F., Galceran, M.T., 2002. Development of a solid-phase microextraction method for the determination of short-ethoxy-chain nonylphenols and their brominated analogs in raw and treated water. J. Chromatogr. A. 963, 159–167.

Donato, I.D., Magazu, S., Maisano, G., Majolino, D., Migliardo, P., Pollicino, A., 1996. Hydration phenomena and hydrogen bond connectivity in polymeric aqueous solutions. Mol. Phys. 87, 1463–1469.

Durán, N., Rosa, M.A., D'Annibale, A., Gianfreda, L., 2002. Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. Enzyme Microbial Technol. 31, 907–931.

Durán, N., Esposito, E., 2000. Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. Appl. Catal. B: Environ. 28, 83–99.

Fernandez-Lafuente, R., Rosell, C.M., Rodriguez, V., Guisan, J.M., 1995. Strategies for enzyme stabilization by intramolecular cross-linking with bifunctional reagents. Enzyme Microbial Technol. 17, 517–523.

Fukuda, T., Uchida, H., Takashima, Y., Uwajima, T., Kawabata, T., Suzuki, M., 2001. Degradation of bisphenol A by purified laccase from *Trametes villosa*. Biochem. Biophys. Res. Commun. 284, 704–706.

Ishibashi, H., Matsumura, N., Hirano, M., Matsuoka, M., Shiratsuchi, H., Ishibashi, Y., Takao, Y., Arizono, K., 2004. Effects of triclosan on the early life stages and reproduction of medaka *Oryzias latipes* and induction of hepatic vitellogenin. Aquat. Toxicol. 67, 167–179.

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., Baunbeck, T., Turner, A.P., Tyler, C.R., 2003. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquat. Toxicol. 65, 205–220.

Kim, Y.J., Nicell, J.A., 2006. Laccase-catalyzed oxidation of bisphenol A with the aid of additives. Process Biochem. 41, 1029–1037.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Kovrigin, E.L., Potekhin, S.A., 1997. Preferential solvation changes upon lysozyme heat denaturation in mixed solvents. Biochemistry 36, 9195–9199.

López-Gallego, F., Betancor, L., Hidalgo, A., Alonso, N., Fernandez-Lafuente, R., Guisan, J.M., 2005. Co-aggregation of enzymes and polyethyleneimine: a simple method to prepare stable and immobilized derivatives of glutaryl acylase. Biomacromolecules 6, 1839–1842.

López-Serrano, P., Cao, L., van Rantwijk, F., Sheldon, R.A., 2002. Cross-linked enzyme aggregates with enhanced activity: application to lipases. Biotechnol. Lett. 24, 1379–1383.

Makarov, A.A., Protasevich, I.I., Bazhulina, N.P., Esipova, N.G., 1995. Heat denaturation of pepsinogen in a water–ethanol mixture. FEBS Lett. 357, 58–61.

Mateo, C., Palomo, J.M., van Langen, L.M., van Rantwijk, F., Sheldon, R.A., 2004. A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates. Biotechnol. Bioeng. 86, 273–276.

Roy, J.J., Abraham, T.E., 2006. Preparation and characterization of cross-linked enzyme crystals of laccase. J. Mol. Catal. B: Enzym. 38, 31–36.

Schoevaart, R., Wolbers, M.W., Golubovic, M., Ottens, M., Kieboom, A.P., van Rantwijk, F., van der Wielen, L.A., Sheldon, R.A., 2004. Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs). Biotechnol. Bioeng. 87, 754–762.

Schoevaart, R., Van Langen, L.M., Van Den Dool, R.T.M., Boumans, J.W.L., 2006. Methods for the preparation of cross-linked enzyme aggregates with improved properties. WO Patent 2006046865.

Shah, S., Sharma, A., Gupta, M.N., 2006. Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder. Anal. Biochem. 351, 207–213.

Sheldon, R.A., Sorgedrager, M., Janssen, M.H.A., 2007. Use of cross-linked enzyme aggregates (CLEAs) for performing biotransformations. Chim. Oggi-Chem. Today 25, 62–70.

Soto, A.M., Justicia, H., Wray, J.W., Sonnenschein, C., 1991. *Para*-nonylphenol—an estrogenic xenobiotic released from modified polystyrene. Environ. Health Persp. 92, 167–173.

Tanaka, T., Yamada, K., Tonosaki, T., Konishi, T., Goto, H., Taniguchi, M., 2000. Enzymatic degradation of alkylphenols, bisphenol A, synthetic estrogen and phthalic ester. Water Sci. Technol. 42, 89–95.

Tanaka, T., Tonosaki, T., Nose, M., Tomidokoro, N., Kadomura, N., Taniguchi, M., Fujii, T., 2001. Treatment of model soils contamined with phenolic endocrine-disrupting chemicals with laccase from *Trametes* sp. in a rotating reactor. J. Biosci. Bioeng. 92, 312–316.

Torres, E., Bustos-Jaimes, I., Le Borgne, S., 2003. Potential use of oxidative enzymes for detoxification of organic pollutants. Appl. Catal. B: Environ. 46, 1–15.

Tyagi, R., Batra, R., Gupta, M.N., 1999. Amorphous enzyme aggregates: stability toward heat and aqueous-organic cosolvent mixtures. Enzyme Microbial Technol. 24, 348–354.

Wesenberg, D., Kyriakides, I., Agathos, S.N., 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol. Adv. 22, 161–187.

Wheeler, T.F., Heim, J.R., LaTorre, M.R., Janes, A.B., 1997. Mass spectral characterization of *p*-nonylphenol isomers using high-resolution capillary GC–MS. J. Chromatogr. Sci. 35, 19–30.

Wilson, L., Illanes, A., Abian, O., Pessela, B.C.C., Fernandez-Lafuente, R., Guisan, J.M., 2004. Co-aggregation of penicillin G acylase and polyionic polymers: an easy methodology to prepare enzyme biocatalysts stable in organic media. Biomacromolecules 5, 852–857.