Short communication

Stabilization of enzymes in micro-emulsions for ultrasound processes

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A B S T R A C T

Oil-in-water proteinaceous micro-emulsions are described as novel methodology for the stabilization of enzymes. Proteins are tightly packed at the oil–water interface of micro-emulsions and it was found that micro-emulsions of laccases enzymes have enhanced stability under high temperatures and ultrasound fields (see graphical abstract scheme and data). This stabilization technique seems to be a promising methodology to apply on other enzyme-based processes where the operational conditions required high levels of mass transport.

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1. Introduction

Laccase is a polyphenol oxidase, which belongs to the family of multicopper oxidases. It catalyzes the oxidation of a range of inorganic and aromatic compounds with the concomitant reduction of molecular oxygen to water [1–3]. The combination of ultrasonic energy with enzymatic treatments has become a promising approach to improve enzyme efficiency, accelerating mass transfer during some textile processing steps such as desizing, scouring, bleaching and dyeing, preserving however the integrity of the fabrics [4–6]. In literature, studies put forward a dependence of the enzymatic enhancement with the intensity or long sonication time [7–9]. Enzymatic activity of laccases is strongly affected upon ultrasound treatment, the formation of aggregates leads to the inactivation of the enzyme caused by the radicals resulting from the cavitation phenomenon [4]. It has been reported that laccase in the presence of ultrasound could be stabilized using specific stabilizers such as polyvinyl alcohol (PVA) or polyethylene glycol (PEG) [10]. However, they can hinder the oxidation of the textile substrate and in the presence of ultrasound, might contribute to non-enzymatic bleaching effects.

The stabilization of proteins has been an important driving force for development of protein formulations [11]. Often the increased stability, selectivity and activity of the proteins are obtained by combining techniques from immobilization to formulation as well as emulsifying properties [12,13]. The structural organization of proteins adsorbed at fluid interfaces has been recognized as main influence to control the stability of emulsion-based products [13].

The main focus of this work is to find new stabilization methods to improve the stability of laccase enzymes under ultrasonic fields. Previously we found that oil micro-droplets (50–500 nm) can be stabilized with proteins under high shear forces of ultrasound [14]. Globular proteins like Serum Albumin didn’t show any change of the relative amounts of secondary structure and several layers of protein were present at the water oil interface [15]. At the interface of micro-emulsions, it can be assumed that external layers are made of fully folded active proteins. By a suitable selection of the system components and preparation method, thermodynamically and kinetically stable systems can be obtained [16,17]. The micro-emulsions were produced by high pressure homogenization method [16] and their physical and morphologic characterization was carried out. Proteins are tightly packed at the oil–water interface of micro-emulsions [17] and it can be expected a considerable stabilization in operational conditions that require high shear forces. An application example is given for ultrasound-assisted pre-treatment of cotton fabrics with laccase stabilized on these micro-emulsions.

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2. Materials and methods

2.1. Materials

Laccase (EC.1.10.3.2) from ascomycete Myceliophthora thermophila was provided by Novozymes, (Denmark). The 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and BSA were obtained from Sigma, Spain. The bleaching reagents and desized cotton were kindly supplied by ACATEL, Portugal.

2.2. Micro-emulsions preparation

The laccase micro-emulsions were prepared by contact of an organic (vegetable oil: Oleo Fula—food grade from Sovena, SA) and an aqueous phase (protein, bovine serum albumin, in acetate buffer pH5). Micro-emulsions in a ratio of 0.5 oil/99.5 H2O were prepared using different concentrations of BSA (2.5; 5 and 10 g/L) and of laccase (1.5; 2.5; 5 and 10 g/L) to form an oil-in-water (O/W) emulsion. The two phases once mixed were homogenized (39 cycles—13 min) using a homogenizer (EmulsiFlex-C3—Avestin, Canada) under high pressure (1000 bar).

2.3. Physical and morphologic characterization of BSA/laccase micro-emulsions

The zeta-potential and size distribution of micro-emulsions were determined at 25 ± 0.1 °C using a Malvern zeta-sizer NS (Malvern Instruments) by electrophoretic laser Doppler anemometry and DLS, respectively. The morphologic characterization was evaluated using the transmission electron microscopy (TEM) technique.

2.4. Measurement of laccase activity

The laccase activity was determined using ABTS as substrate. The oxidation of the substrate was monitored spectrophotometrically by the increase in the absorbance at 420 nm for 2 min [18] at water bath at 50 °C. Enzymatic activity (U) was expressed as U = μmol of ABTS oxidized per min.

2.5. Thermal stability and pH profile of laccase formulated into micro-emulsion and for free form

Thermal stability was estimated from 30 min of incubation (40, 50 and 65 °C) as the residual activity. The residual laccase activity was determined under standard assay (see above Section 2.4) conditions and the final result was displayed by half-life time values. pH profile was measured in the range of 2.0–7.0 using acetate buffer 0.1 M using the fresh enzyme or the formulation. Half-life times of enzyme activity were estimated assuming a first order kinetic decay with data from 30 min incubation.

2.6. Enzyme stability during cotton bleaching under ultrasound

In order to evaluate the stability and efficiency of laccase micro-emulsions, desized fabrics were pre-treated in the ultrasound bath sonicator (VWR Ultrasonic Cleaner) with fixed frequency 45 kHz and power intensity at 120W. One sample was incubated with 2 U/mL of free laccase and other with 2 U/mL of laccase micro-emulsion, in 0.1 M acetate buffer pH 5, at 50 °C during 30 min. The activity was determined as mentioned in Section 2.4. Cotton samples were further washed with distilled water at 80 °C and dry at room temperature. The pre-treated cotton fabrics were bleached afterwards following the recipe: 1 g/L anti-wrinkle, 0.5% wetting agent, 1.5 g/L sequestrant, 4 g/L NaOH, 8 g/L H2O2, 3 g/L equalizer, 1% optical brightener (o.w.f). The bleaching was carried out at 80 °C for 1 h, assisted by ultrasounds equipment (45 kHz; 120 W).

2.7. Measurement of whiteness index

The whiteness index Berger (W*) of the samples was determined by using a reflectance Data-Colour apparatus at standard illuminant daylight D65.

3. Results and discussion

Taking advantage of BSA emulsifying properties, micro-emulsions made of laccase and BSA were prepared, using different concentrations of both proteins, forming O/W (0.5/99.5) emulsions. Supplementary Fig. S1 represents the physical appearance of native laccase and the correspondent micro-emulsion where proteins, BSA and laccase, are highly dispersed in the oil as micro-order dispersions. In this process, several factors in the process have been taken into account, namely pressure, temperature, number of passages and flow rate [19,20].

Angel et al. studied the assembly of BSA with other proteins on microspheres production [21]. They suggest that two proteins can form together the microsphere’s walls from fluorescence data. TEM showed an average particle size of around 250 nm (Fig. 1), corresponding to the values obtained by DLS measurements (see Supplementary Fig. S2). It illustrates that the proteinaceous micro-emulsions are spherical with a regular surface. This morphology would allow a higher protein stabilization, since the protein is packed sphere with contained movements and a minimal contact with water [22].

The influence of proteins concentration was analysed in terms of particle size and polydispersity index (PDI). The results presented in Fig. 2 show that the z-average diameter and PDI of micro-emulsions is dependent of the BSA and laccase concentration used for each sample. Thus, best results with lower z-average diameter and PDI were obtained for the formulation 5/10 of BSA/laccase g/L (250 nm; PDI: 0.132). BSA presents a high content of hydrophobic residues and it is known that only high concentrations yield a narrower size distribution and lower particle’s size [15,17,19].

Since emulsions can suffer aggregation over time, the stability of produced micro-emulsions was measured in terms of particle size and zeta-potential. These results displayed that laccase micro-emulsions were maintained stable during 10 weeks presenting...
good particle stability at room temperature (Table 1). Laccase lost almost 50% of its activity while the formulated enzyme had 40% decay when left at room temperature. Both enzymes showed great stability when kept at 4 °C with half-lives over 2 years.

The formulation of laccase presented in this study leads to recover 35% of the initial enzyme activity used in process for the considered best formulation. This value might seems to be lower but actually it is quite high considering that there are several layers of protein [15] at the interface oil–water and it is reasonable to expect that only the most external layer would contribute for the enzyme activity of the emulsion. The extra minutes obtained as half-life time of the enzyme under ultrasound (Table 2) makes the all difference in effectiveness for textile bleaching (see data below in Table 3 after bleaching). The thermal and pH stability were expressed as the half-life time for free and laccase micro-emulsion using 30 min of exposure (Table 2). Half-life time was calculated from exponential decay (graphical abstract) and the time at which half of the initial activity was lost was mentioned as half-life time. The presence of laccase in the micro-emulsions enhanced considerably the activity of enzyme under extreme conditions (pH and temperature) when compared with the free enzyme. Under pH 3, pH 6, 50 °C and 60 °C, the formulated laccase showed higher stability (high half-life time) when compared to the free form. Stability enhancement through formulated laccase was evident when exposed at extreme conditions in 30 min of incubation (graphical abstract). However, pH profile (see Supplementary Fig. S3) was evaluated at time points in the range pH 2–7 and was similar for free and micro-emulsion of BSA/laccase. BSA and laccase have similar isoelectric points (around pH 4.7–4, respectively) [23,24], which might indicate that both proteins assume very similar charges with pH [25]. Those assumptions were made, because it is well known that the charge of molecules solvated around enzyme-proteins induce significant changes at pH profile of enzymes [26].

Table 1
Stability of the free laccase and laccase micro-emulsion left at room temperature as activity, z-average diameter, polydispersity and zeta-potential, during 10 weeks.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Formulated laccase</th>
<th>Free laccase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccase activity (U/mL)</td>
<td>Time 0 (weeks)</td>
<td>Time 10 (weeks)</td>
</tr>
<tr>
<td>pH 7</td>
<td>6250 ± 353.6</td>
<td>3500 ± 707.1</td>
</tr>
<tr>
<td>pH 6</td>
<td>50.9 ± 353.6</td>
<td>3500 ± 707.1</td>
</tr>
<tr>
<td>pH 5</td>
<td>3500 ± 707.1</td>
<td>3500 ± 707.1</td>
</tr>
<tr>
<td>pH 4</td>
<td>3500 ± 707.1</td>
<td>3500 ± 707.1</td>
</tr>
<tr>
<td>pH 3</td>
<td>3500 ± 707.1</td>
<td>3500 ± 707.1</td>
</tr>
<tr>
<td>pH 2</td>
<td>3500 ± 707.1</td>
<td>3500 ± 707.1</td>
</tr>
<tr>
<td>Z-average (d nm)</td>
<td>233.5 ± 2.19</td>
<td>236.7 ± 3.32</td>
</tr>
<tr>
<td>PDI</td>
<td>0.132 ± 0.001</td>
<td>0.183 ± 0.002</td>
</tr>
<tr>
<td>Zeta-potential (mV)</td>
<td>−19.8 ± 0.35</td>
<td>−20.2 ± 0.57</td>
</tr>
</tbody>
</table>

Table 2
Effect of temperature, pH and ultrasound (45 kHz, 120 W) in free and laccase micro-emulsion diluted in buffer. Values of half-life time were estimated from 30 min of exposure.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Half-life time (min)</th>
<th>Free laccase</th>
<th>Laccase micro-emulsion</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH values at room temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7</td>
<td>9.2</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td>pH 6</td>
<td>95.4</td>
<td>294.5</td>
<td></td>
</tr>
<tr>
<td>pH 5</td>
<td>214.0</td>
<td>259.8</td>
<td></td>
</tr>
<tr>
<td>pH 4</td>
<td>111.4</td>
<td>176.5</td>
<td></td>
</tr>
<tr>
<td>pH 3</td>
<td>72.3</td>
<td>169.6</td>
<td></td>
</tr>
<tr>
<td>pH 2</td>
<td>23.4</td>
<td>21.2</td>
<td></td>
</tr>
<tr>
<td>Temperature values at pH 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 °C</td>
<td>108.8</td>
<td>176.5</td>
<td></td>
</tr>
<tr>
<td>50 °C</td>
<td>46.3</td>
<td>82.7</td>
<td></td>
</tr>
<tr>
<td>65 °C</td>
<td>23.3</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td>pH 5–50 °C under ultrasound</td>
<td>25.3</td>
<td>51.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 3
Values of whiteness Berger (W*) level for bleaching of samples pre-treated with free and laccase micro-emulsion under ultrasound.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Whiteness (W*) after enzymatic pre-treatment</th>
<th>Whiteness (W*) after enzymatic pre-treatment and bleaching</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw cotton fabric</td>
<td>27.2 ± 0.071</td>
<td>160.8 ± 0.058</td>
</tr>
<tr>
<td>Control (acetate buffer)</td>
<td>30.9 ± 0.071</td>
<td>163.0 ± 0.071</td>
</tr>
<tr>
<td>Free laccase</td>
<td>35.1 ± 0.141</td>
<td>151.3 ± 0.071</td>
</tr>
<tr>
<td>Laccase in micro-emulsion (5/10 g/L of BSA/laccase) (after preparation)</td>
<td>35.2 ± 0.141</td>
<td>170.1 ± 0.071</td>
</tr>
<tr>
<td>Laccase in micro-emulsion (5/10 g/L of BSA/laccase) (after being left in lab for 4 weeks)</td>
<td>35.2 ± 0.071</td>
<td>172.7 ± 0.058</td>
</tr>
</tbody>
</table>

Fig. 2. Particle size and polydispersity index of BSA/laccase micro-emulsions using different ratio concentrations.
Once the final target of produced micro-emulsions is their application on textile pre-treatment bleaching processes, to preserve their catalytic activity against ultrasound constitutes a crucial parameter. As mentioned in literature, the loss of catalytic activity at ultrasound application implies to stabilize the enzyme [10]. Therefore, to evaluate the stability of the laccase micro-emulsions in the presence of ultrasounds, the laccase activity was measured during 30 min, for the incubation in the ultrasound bath. The comparison between the native and the formulated protein activity was made using equal activity amounts of enzyme. Comparing with free laccase, formulated laccase into micro-emulsion presents a double half-life under the ultrasound field (Table 2 and graphical abstract).

This fact is just sufficient to yield an enough turnover to make the bleaching process at ultrasound field a viable methodology. The stabilization of a protein is effectively reached when specific formulations are applied [27,28].

Laccase was described as a pre-treatment to enhance the whiteness after a bleaching step with hydrogen peroxide [10]. It is believed that yellowish phenolic compounds can be polymerized and those can be better removed by a further bleaching step [10]. The efficiency of the enzymatic pre-treatment was evaluated by measuring the whiteness of samples after bleaching (Table 3). Laccases in the micro-emulsions showed a considerable stabilization towards temperature and operational conditions that involve high shear forces. Better results were obtained after bleaching for those micro-emulsions. The formulation tested in cotton after 4 weeks of storing yield similar results as the fresh formulation. From this, it can be stated that laccase is efficiently stabilized by BSA/laccase micro-emulsions for textile bleaching and the formulation yield good storage stability levels under room temperature. The technical benefit of the formulated enzyme is clear for textile bleaching and the enzyme based bleaching process is discussed in previous publications [10,29].

4. Conclusions

In this work, we have prepared mixed proteinaceous O/W micro-emulsions of BSA and laccase using high pressure homogenization technique. The combination of these two proteins with vegetable oil made possible the achievement of stable micro-emulsions. The catalytic activity of the enzyme emulsions was far greater than free laccase, when exposed at different temperatures and pH values. Their great stability was also observed during application in ultrasound bath promoting an enhancement of enzyme inactivation at 50 °C, which double the enzyme half-life time. The laccase micro-emulsion allowed a significant increase of enzyme stability making it able to better polymerize cotton natural phenolics, thus increasing the subsequent step of bleaching. This new methodology seems promising for the stabilization of enzymes where the operational conditions require high levels of molecular agitation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2014.09.011.

References