Special Issue Article

Journal of Molecular Recognition

Received: 6 November 2013, Revised: 6 May 2014, Accepted: 7 June 2014, Published online in Wiley Online Library: 3 February 2013

(wileyonlinelibrary.com) DOI: 10.1002/jmr.2405

Aldehyde PEGylation of laccase from *Trametes versicolor* in route to increase its stability: effect on enzymatic activity†

Karla Mayolo-Deloisa[a], Mirna González-González[a], Jesús Simental-Martínez[a] and Marco Rito-Palomares[a]*

Laccase is a multicopper oxidase that catalyzes the oxidation of phenolic compounds. Laccase can be used in bioremediation, beverage (wine, fruit juice, and beer) processing, ascorbic acid determination, sugar beet pectin gelation baking, and as a biosensor. Recently, the antiproliferative activity of laccase toward tumor cells has been reported. Because of the potential applications of this enzyme, the efforts for enhancing and stabilizing its activity have increased. Thus, the PEGylation of laccase can be an alternative. PEGylation is the covalent attachment of one or more molecules of methoxy poly(ethylene glycol) (mPEG) to a protein. Normally, during the PEGylation reaction, the activity is reduced but the stability increases; thus, it is important to minimize the loss of activity. In this work, the effects of molar ratio (1:4, 1:8, and 1:12), concentration of laccase (6 and 12 mg/ml), reaction time (4 and 17 h), molecular weight, and type of mPEG (20, 30, 40 kDa and 40 kDa-branched) were analyzed. The activity was measured using three substrates: ABTS, 2,6-dimethoxyphenol, and syringaldazine. The best conditions for laccase PEGylation were 12 mg/ml of laccase, molar ratio 1:4, and 4 h reaction time. Under these conditions, the enzyme was able to maintain nearly 100% of its enzymatic activity with ABTS. The PEGylation of laccase has not been extensively explored, so it is important to analyze the effects of this bioconjugation in route to produce a robust modified enzyme. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: laccase; *Trametes versicolor*; PEGylation; N-terminal PEGylation reaction

INTRODUCTION

Laccase (EC 1.10.3.2, benzenediol : oxygen oxidoreductase) is one of the few enzymes that have been studied since the 19th century (Baldrian, 2006). These enzymes catalyze the one-electron oxidation of a wide variety of organic and inorganic substrates, including mono-, di-, and polyphenols, amino-phenols, methoxyphenols, aromatic amines, and ascorbate, with the concomitant four-electron reduction of oxygen to water (Madhavi and Lele, 2009). Laccases are common in fungi. Actually, they have been found in higher fungi. They occur in fungal causative agents of soft rots, in most bracket fungi causing white rot, and in many agarics, including cultivated edible fungi: champignon, *pleurotus*, and medicinal shiitake *Lentinula edodes*. Other laccase producers are wood-degrading fungi as *Trametes versicolor* (Morozova *et al*., 2007). Typical fungal laccase is a protein of approximately 60–70 kDa with acid isoelectric point around 4.0 (Baldrian, 2006). Potential uses of laccases include textile-dye bleaching, pulp bleaching, food improvement, bioremediation of soils and water, polymer synthesis, the development of biosensors and biofuel cells, chemical synthesis, and as a tool for medical diagnostics (Rodríguez-Couto and Toca-Herrera, 2006; Zou *et al*., 2012). Recently, the antiproliferative activity against tumor cells of laccase from a white root fungus *Abortiporus biennis* has been reported (Zhang *et al*., 2011). Because of the potential applications of this enzyme, the efforts for enhancing and stabilizing its activity have increased.

PEGylation defines the modification of a protein or peptide by the linking of one or more methoxy poly(ethylene glycol) (mPEG) chains, a nontoxic and nonimmunogenic polymer (Veronese and Pasut, 2005; Veronese and Mero, 2008). This strategy has been mainly exploited in the pharmaceutical area. PEGylation changes the physical and chemical properties of the biomolecule, such as its conformation, electrostatic binding, size, and hydrophobicity; resulting in an improvement in the pharmacokinetic behavior of the protein (Fee and Van Alstine, 2006; Veronese and Mero, 2008). Most of these phenomena can be explained by the greatly expanded hydrodynamic volume of the PEG-protein conjugate as a result of the ability of mPEG to coordinate numerous water molecules and of the high flexibility of the mPEG chain (Gaberc-Porekar *et al*., 2008). Because of the versatility of this technique, its application has been extended to areas other than therapeutics.

Although laccase is an enzyme widely studied, few reports about its conjugation with mPEG are known. Up to now, the PEGylation of laccase from *Coriolopsis gallica*, *Trametes hirsuta*, *Myceliophthora thermophila* and *T. versicolor* have been reported. In all cases, chemical conjugation of the lysine free amino groups was carried out using mPEG of 5 kDa. Under these conditions, PEGylation slightly improved the thermostability of laccase from

* *Correspondence to: M. Rito-Palomares, Centro de Biotecnología-FEMSA, Tecnológico de Monterrey. Campus Monterrey, Ave. Eugenio Garza Sada 2501 Sur, Monterrey, NL 64849, México.*
E-mail: mrito@itesm.mx

† *This article is part of a special issue dedicated to Affinity 2013.*

a *K. Mayolo-Deloisa, M. González-González, J. Simental-Martínez, M. Rito-Palomares Centro de Biotecnología-FEMSA, Tecnológico de Monterrey, Campus Monterrey, Ave. Eugenio Garza Sada 2501 Sur, Monterrey, NL, 64849, México*

M. thermophila and decreases its inactivation rate in organic solvents. While laccase from *C. gallica* conjugated with mPEG had a higher catalytic activity on syringaldazine than the native enzyme. In the case of laccase of *T. hirsuta*, the PEGylation resulted in an enhanced enzyme stability while with increasing molecular weight of attached mPEG (5, 2 and 1.10 kDa) the substrate affinity for the laccase conjugated decreased (Vandertol-Vanier *et al*., 2002; Lopez-Cruz *et al*., 2006; Schroeder *et al*., 2006; Kim *et al*., 2013). The PEGylation reaction used in the above mentioned reports is usually designated as random and results in complex mixtures of conjugates with different numbers and distributions of bound mPEG chains (Gaberc-Porekar *et al*., 2008).

During the PEGylation reaction, the activity is reduced but the stability increases; thus, it is important to minimize the loss of activity. In this work, the influence of time of the aldehyde PEGylation reaction on the total enzyme activity of laccase was analyzed after 4 and 17 h. The activity was measured using three substrates: ABTS, syringaldazine, and 2,6-dimethoxyphenol. Additionally, different molecular weights of mPEG were used: 20, 30, 40 kDa and 40 kDa-branched. The PEGylation of laccase has not been extensively explored so it is important to analyze the effects of this bioconjugation on the enzymatic activity using laccase from *T. versicolor* as a model.

EXPERIMENTAL

Materials

Laccase from *T. versicolor* (catalogue number 51639), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (DMP) and 4-hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine, SYR) were purchased from Sigma–Aldrich (MO, USA). Methoxy poly(ethylene glycol) propionaldehyde (mPEG) with nominal molecular weight of 20, 30, 40 lineal, and 40 kDa Y-shaped (branched) came from JenKem Technology (Beijing, China). PEGylation reaction buffer was prepared from sodium phosphate monobasic and dibasic salts (Mallinckrodt Baker, Mexico) and sodium cyanoborohydride (Fluka, Switzerland).

Laccase PEGylation reaction

Laccase was PEGylated in triplicates according to the procedure reported by Daly *et al.* and previous works of our group (Daly *et al*., 2005; Cisneros-Ruiz *et al*., 2009; González-Valdez *et al*., 2011; Mayolo-Deloisa *et al*., 2012). Briefly, 7.0 ml of 12 mg/ml (unless otherwise specified) laccase from *T. versicolor* reacted with 20, 30, 40 lineal, or 40 kDa-branched mPEG-propionaldehyde in a pH 5.10, 100 mM sodium phosphate buffer with 20 mM sodium cyanoborohydride. A 2 ml control reaction without mPEG was also conducted in every experiment. The reactions were stirred rapidly for 4 or 17 h at 4 °C. After 10 min of mixing, the reagents were completely dissolved, and an aliquot (namely time 0 h) was taken, as well as at the endpoint of the reactions (namely time 4 or 17 h, respectively). These samples were centrifuged at 15 000 × g and 4 °C for 5 min, and then they were syringe-filtered (0.2 μm) for future analyses.

SDS-PAGE analysis

The samples for the SDS-PAGE analysis were assembled as follows: 0.012 ml of a 4X loading buffer were added to 0.036 ml

Table 1. Molar ratio effect (protein : 20 kDa mPEG) on the total activity recovery using ABTS

Molar ratio (protein-mPEG)	Protein (mg/ml)	mPEG (mg/ml)	Recovery of enzymatic activity (%)	SD
1:4	6	7.5	43.39	3.68
			39.43[a]	2.73[a]
1:8	6	15.0	58.65	2.61
			36.71[a]	1.47[a]
1:12	6	22.5	52.91	4.86
			35.01[a]	1.18[a]

mPEG = methoxy poly(ethylene glycol); SD = standard deviation; ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).
[a]Control reaction without mPEG.

Table 2. Effect of protein concentration and 20 kDa mPEG on the total enzyme activity recovery using three substrates. 1:4 molar ratio

		ABTS		DMP		SYR	
Protein (mg/ml)	mPEG (mg/ml)	Recovery of enzyme activity (%)	SD	Recovery of enzyme activity (%)	SD	Recovery of enzyme activity (%)	SD
6	7.5	43.39	3.68	33.32	0.64	17.67	1.41
		39.43[a]	2.74[a]	20.18[a]	0.58[a]	5.66[a]	0.73[a]
12	15.0	**65.92**	3.89	40.89	1.27	36.11	3.62
		57.87[a]	1.03[a]	37.16[a]	2.16[a]	20.03[a]	0.74[a]

mPEG = methoxy poly(ethylene glycol); SD = standard deviation; ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DMP = 2,6-dimethoxyphenol; SYR = syringaldazine.
[a]Control reaction without mPEG.

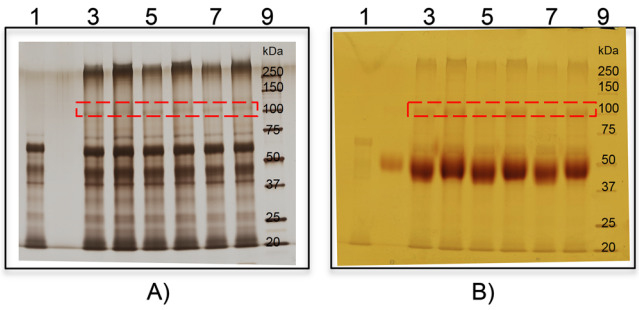


Figure 1. SDS-PAGE analysis of PEGylated laccase reaction at time 0 and 17 h with silver staining for protein detection (A) and I_2-$BaCl_2$ staining for mPEG detection (B). The samples are: laccase standard 1.5 μg (lane 1), standard mPEG 20 kDa 1.5 μg (lane 2), laccase reaction at 0 h (lanes 3, 5 and 7), laccase reaction at 17 h (lanes 4, 6, and 8), and molecular weight marker precision plus protein all blue standards 1.5 μg (lane 9).

of the sample, mixed, and heated for 10 min at 99 °C in a digital heatblock. The gels, Mini-Protean TGX precast 10% gels (Bio-Rad Laboratories, Inc., CA, USA), were run at 80 V for 150 min and were first silver-stained and then dyed with barium iodine to visualize the mPEG as previously reported (González-González *et al*., 2012).

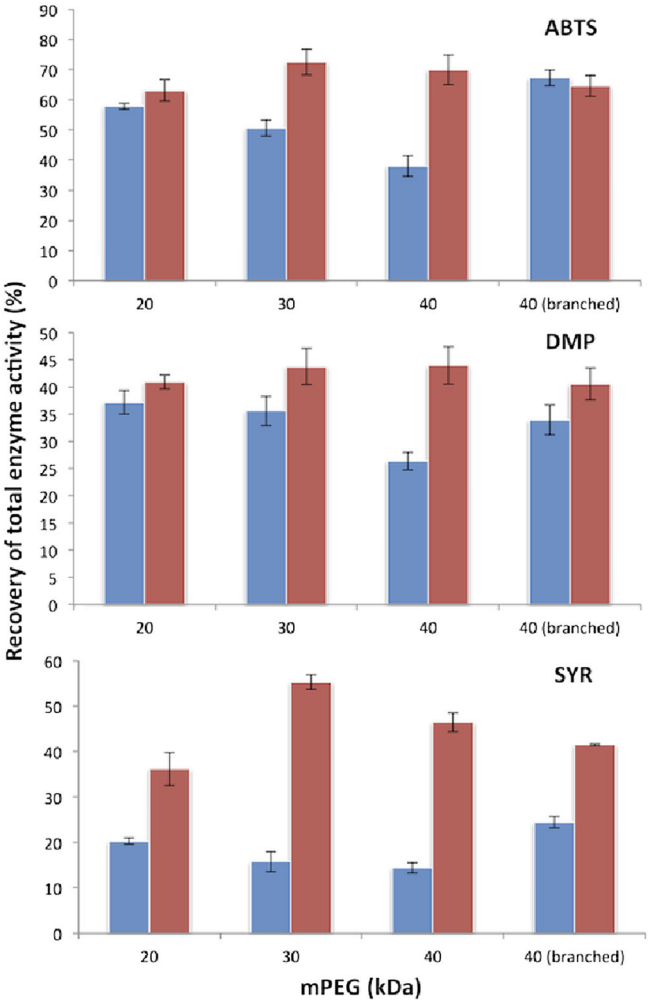


Figure 2. Effect of molecular weight and type of mPEG using three substrates. Molar ratio = 1:4; time reaction = 17 h; mPEG = methoxy poly(ethylene glycol); ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DMP = 2,6-dimethoxyphenol; SYR = syringaldazine. Controls are in blue and PEGylated reactions are in red.

Laccase enzymatic activity determination

The activity was measured by monitoring the oxidation of three substrates: ABTS $\varepsilon^M = 29.3 \times 10^3\ M^{-1}\ cm^{-1}$ at 436 nm (1 mM in ethanol); DMP $\varepsilon^M = 27.5 \times 10^3\ M^{-1}\ cm^{-1}$ at 468 nm (6 mM in ethanol) and syringaldazine $\varepsilon^M = 6.5 \times 10^4\ M^{-1}\ cm^{-1}$ at 526 nm (1 mM in ethanol) (Flores *et al*., 2009). The reaction mixture consisted of 0.222 ml of acetate buffer (0.1 M; pH 4.0 for ABTS, pH 5.0 for DMP and syringaldazine), 0.025 ml of substrate, and 0.003 ml of sample. One unit of enzyme activity was defined as the amount of enzyme catalizing the formation of 1.0 μM of the reaction product per minute (Mayolo-Deloisa *et al*., 2009). Results reported are the average of at least three independent experiments.

Separation of PEGylated laccase

The reaction mixture of laccase (6.0 ml) was analyzed via size-exclusion chromatography with an Äkta Explorer 100 system (GE Healthcare, Uppsala, Sweden) using a Sephacryl S-300 column (2.6 cm inner diameter, 60 cm length, Amersham Pharmacia, Uppsala, Sweden) with an isocratic mobile phase of 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM potassium chloride at a flow rate of 1.0 ml/min. The column was pre-equilibrated with one-half column volume of distilled water and two column volumes of mobile phase (Cisneros-Ruiz *et al*., 2009).

RESULTS AND DISCUSSION

The location where the mPEG attaches to the protein surface has a large effect on the bioactivity of the PEG-protein conjugate. A common approach to the site-direct PEGylation is the attachment of the mPEG to the N-terminal of a protein (Payne *et al*., 2010). The reaction products depend on the reaction conditions: pH, molar ratio, protein concentration, reaction time, and temperature. Thus, in this work, an N-terminal PEGylation reaction previously reported by the group, with 20 kDa mPEG and a reaction time of 17 h, was used (González-Valdez *et al*., 2011; Mayolo-Deloisa *et al*., 2012). The reaction was conducted at pH 5.10. This strategy is based on the fact that primary amine residues in protein have different pKa values: pKa 7.8 for N-terminal α-amino groups and 10.1 for ε-amino groups in lysine residues (Lee *et al*., 2003).

Molar ratio

The first step was to evaluate the effect of the molar ratio (protein : 20 kDa mPEG) on the total enzymatic activity recovery with ABTS as substrate. For this, three molar ratios were used: 1:4, 1:8, and 1:12. The results are shown in Table 1. The highest

enzymatic activity recovery was observed with a molar ratio of 1:8 (58.65%), when the molar ratio was increased up to 1:12 (52.91%) the recovery decreased. The typical recommended ratio for this type of reaction is 1:4; thus, that proportion was challenged by doubling the concentration of enzyme and 20 kDa mPEG. This approach was pursued in an attempt to increase the percentage recovery of activity obtained with the ratio of 1:8. Additionally, DMP and syringaldazine were used as substrate. As a result, a positive effect in the recovery of activity with all substrates was observed (Table 2). When ABTS was used, the recovery increased from 43.39% to 65.92%, thus obtaining a higher percentage compared with the molar ratio of 1:8 (Table 1). Furthermore, when the concentration of laccase and mPEG was doubled, the recovery was also higher for DMP and syringaldazine. However, a greater loss of enzymatic activity was obtained with the DMP and syringaldazine; apparently, the PEGylation reaction has a negative effect with these two substrates. This decrease in activity may be attributed to the large mPEG 20 kDa used, which could mask the active site (Huang *et al*., 2009). Usually, PEGylation process affects the activity of a protein but increases its stability. This phenomenon has been extensively studied with therapeutic proteins, which show a reduction of their *in vitro* biological activity after PEGylation. Nevertheless, the *in vivo* pharmacological effects are usually enhanced (Jevsevar *et al*., 2010). From the results obtained, reaction conditions involving molar ratio of 1:4 with 12 mg/ml of laccase and 15 mg/ml of 20 kDa mPEG were selected.

In order to confirm the presence of PEGylated proteins, the products from the reactions were analyzed using SDS-PAGE. The same gel was stained with silver for protein detection and I_2-$BaCl_2$ for mPEG detection. The reactions at 0 and 17 h are shown in Figure 1. The silver staining shows the presence of PEGylated laccase at about 100 kDa (Figure 1A, lanes 3–8), the bands are more intense at 17 h, but PEGylated proteins can

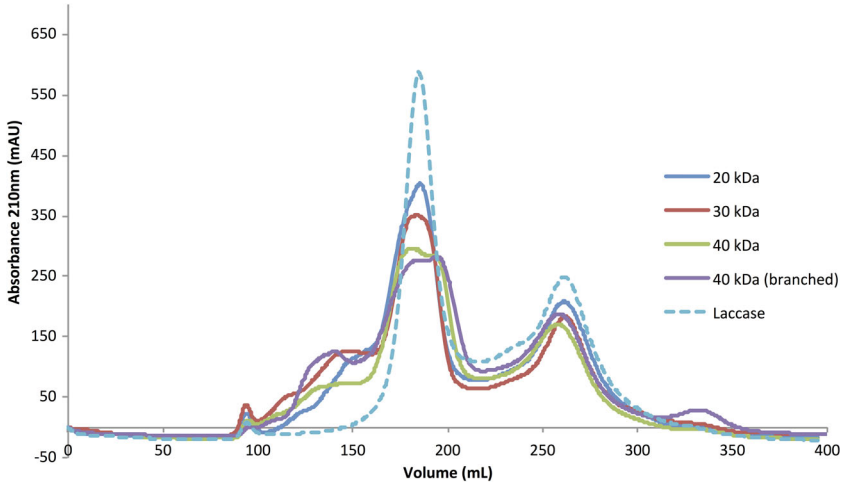
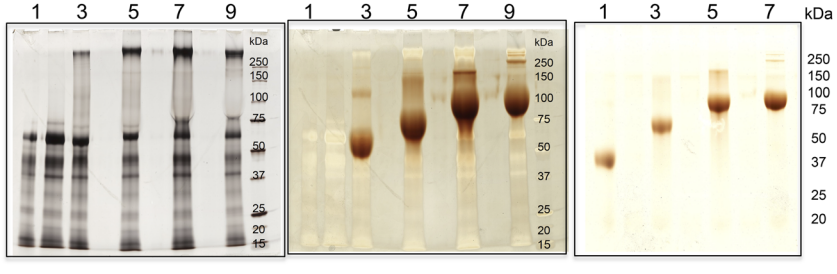


Figure 3. Size exclusion chromatography profile of PEGylation reactions with different molecular weights of mPEG and laccase standard. Molar ratio: 1:4; reaction time: 17 h.



Lane	Part A and B	Part C
1	Laccase standard	Standard mPEG 20 kDa
2	Reaction without PEG	
3	Laccase reaction with 20 kDa mPEG	Standard mPEG 30 kDa
5	Laccase reaction with 30 kDa mPEG	Standard mPEG lineal 40 kDa
7	Laccase reaction with lineal 40 kDa mPEG	Standard mPEG branched 40 kDa
9	Laccase reaction with branched 40 kDa mPEG	
10	Molecular weight marker Precision Plus Protein All Blue Standards	

mPEG = methoxy poly(ethylene glycol)

Figure 4. SDS-PAGE analysis of the laccase PEGylation reaction with mPEGs of different molecular weights (A and B) and mPEGs standards (C). Silver staining for protein detection (A) and I_2-$BaCl_2$ staining for PEG detection (B and C).

Journal of Molecular Recognition

also be observed at 0 h, which means that the PEGylation reaction is performed promptly. It is important to note that time 0 is a PEGylation reaction aliquot after 10 min of agitation to guarantee that all the reaction components are completely dissolved. The molecular weight corresponding to the PEGylated laccase seemed larger than the theoretical molecular size (approximately 86 kDa). This phenomenon has been explained by the lower mobility of the PEGylated proteins during electrophoresis on SDS-PAGE (Kurfürst, 1992; Huang *et al*., 2009). The standard of 20 kDa mPEG (Figure 1B, lane 2) appears at 50 kDa. It is important to mention that the molecular weight of the bands of mPEG read with respect to the Precision Plus Protein Kaleidoscope (lane 9) will not correspond to the real weight of the mPEG (González-González *et al*., 2012). Based upon these results, it is clear that at least one monoPEGylated protein is being produced.

Effect of molecular weight and type of mPEG

The effect of molecular weight and type of mPEG on the enzymatic activity is reported in Figure 2. For this purpose, mPEG of 20, 30, 40 kDa and 40 kDa Y-shaped (branched) were used. The concentration of laccase (12 mg/ml) and the 1:4 molar ratio (protein : mPEG) were kept constant, and enzymatic activity was measured using the three selected substrates. In general, the behavior of enzymatic activity was the same as shown in the Table 2; the highest recovery was with ABTS, and it decreases with DMP and syringaldazine. The recovery with ABTS varied between 63.16 and 72.51% (maximum value was achieved using 30 kDa mPEG). In the case of DMP, the enzymatic activity recovery was almost constant (40.89–43.97%). For syringaldazine, a positive effect was observed using a molecular weight of 30 kDa mPEG,

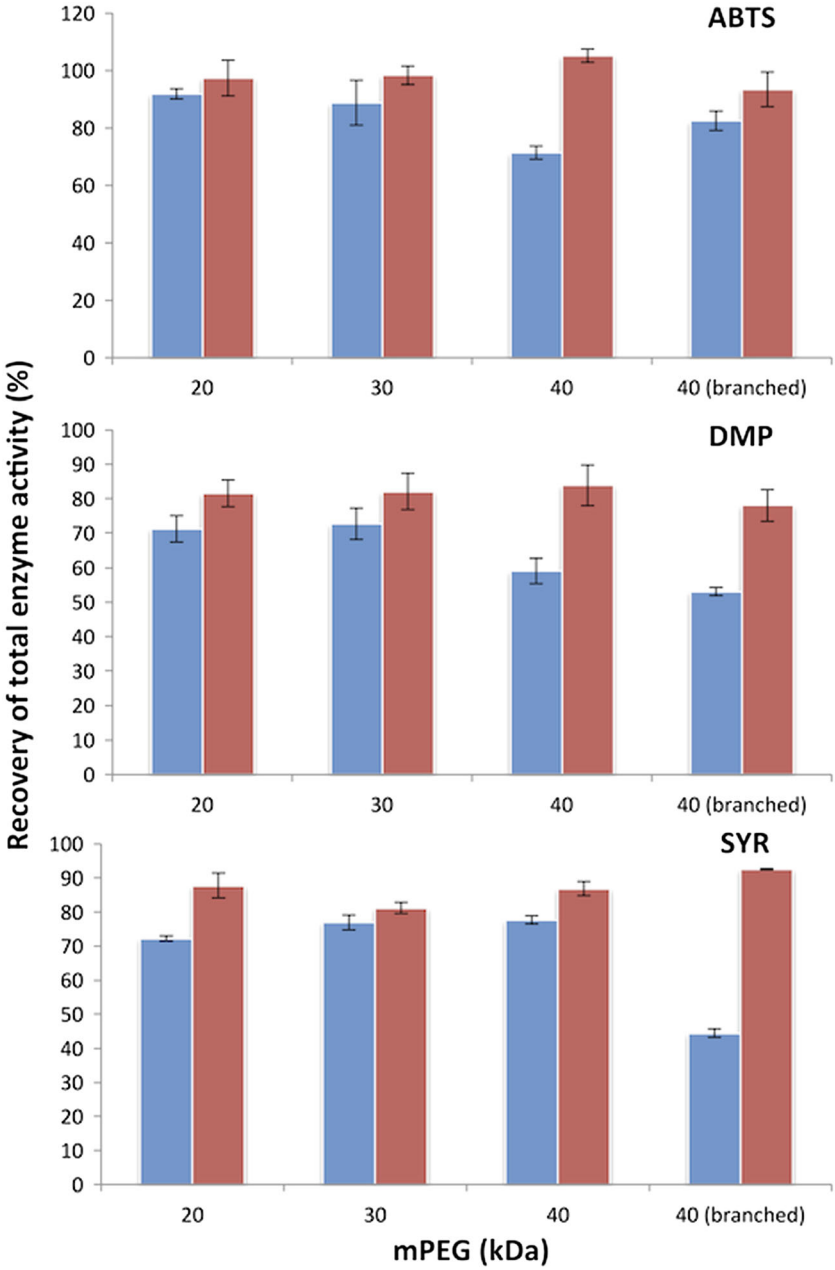


Figure 5. Effect of time of reaction on enzyme activity. Molar ratio = 1:4; time of reaction = 4 h; mPEG = methoxy poly(ethylene glycol); ABTS = 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); DMP = 2,6-dimethoxyphenol; SYR = syringaldazine. Controls are in blue and PEGylated reactions are in red.

where the percentage of recovery reached a value of 55.28%. When comparing the type of mPEG with linear or branched 40 kDa mPEG, no marked effect on the enzyme activity was observed. This last result is interesting because the branched mPEG was expected to cause a steric effect, which would significantly decrease the activity of the laccase. There are no rules for selecting the most suitable molecular weight for linking to a protein, or a ratio of protein molecular weight with respect to the molecular weight of mPEG. But it is important to mention that mPEG has different properties as high solubility in water and in many organic solvents, high hydration and flexibility of the chain (Pasut and Veronese, 2007). From the results reported in Figure 2, it can be concluded that the recovery of activity is higher using 30 kDa mPEG for all substrates.

Size exclusion chromatography (SEC) has been widely used for separation of PEG-protein conjugates. This particular type of chromatography exploits the increase in molecular weight of the protein caused by PEGylation (Jevsevar *et al*., 2010). In this work, SEC was used to confirm the formation of PEGylated laccase and to explore its potential as a purification method. Figure 3 illustrates the SEC profile of the products of the PEGylation reactions and standard laccase. Peaks between 100 and 150 ml can be observed in the chromatography profile of the samples from the PEGylated reactions; nevertheless, the laccase standard is free of any sign of absorbance in this same zone. Noticeably, the resolution of PEGylated proteins is very poor, leaving an area of opportunity to explore alternative purification techniques, which could be more effective. However, the distinctions in the signal due to the different molecular weights of mPEG may be noted and PEGylated proteins can be separated from the unreacted laccase; but the retention times cannot be accurately determined. The presence of PEGylated proteins with different molecular weights of mPEG was confirmed by SDS-PAGE (Figure 4). In this gel, a reaction control (without mPEG) was included (Part A and B, lane 2); the control profile is almost equal to the standard laccase (Part A and B, lane 1). In part B, it can be seen that there is no band indicating the presence of mPEG in lanes 1 and 2. However, it can be clearly seen the presence of mPEG in excess and the PEGylated laccase (Figure 4A and B; lanes 3, 5, 7, and 9). Figure 4C shows the mPEG controls.

The results showed that 72.51% was the best recovery percentage of enzyme activity, which was obtained using 30 kDa mPEG and ABTS as substrate. Reports on the amount of activity lost by the laccase during the N-terminal PEGylation reaction are not known, but there are reports for other proteins in which the reduction of activity can reach up to 60% (Huang *et al*., 2009). It is noteworthy that in all PEGylation reaction experiments, a control without mPEG was considered. The reduction of the activity observed from this control was higher than that obtained from the PEGylation reactions. Thus, the loss of activity can be attributed to the presence of sodium cyanoborohydride in the reaction buffer.

Effect of time of reaction

In an attempt to further optimize the conditions for the PEGylation of laccase, which minimizes the loss of its activity, it was decided to reduce the PEGylation reaction time to 4 h. The experiments were performed using the different molecular weights of mPEG previously tested (Figure 5). In general, a reduction in reaction time resulted in an increase in enzymatic activity recovery. The percentages of recovery varied between 93.45% and 105% with ABTS. The behavior is similar to preliminary experiments (Table 2); laccase shows more affinity for ABTS. However, the recovery of activity with DMP (78.08–83.90%) and syringaldazine (81.22–92.53%) was also higher than the ones obtained at 17 h. The reactions were analyzed by SEC to confirm evidence of the PEGylation (Figure 6). PEGylated proteins can be observed between 100 and 150 ml (as in Figure 3). In these chromatograms, the peaks are sharper; thus, they have higher resolution than those obtained at 17 h. This may be because the short reaction time increases the production of monoPEGylated protein. Prolonging the reaction time usually leads to the production of other PEGylated proteins (di- or tri-PEGylated).

Until now, PEGylation processes have been focused on the modification of therapeutic proteins because of their high value. Nevertheless, the application of this type of process to multifunctional proteins as laccase is very interesting. In this study, an attempt to reduce the impact of PEGylation on the enzymatic activity of laccase was pursued. However, although the reaction conditions under which the enzymatic activity was

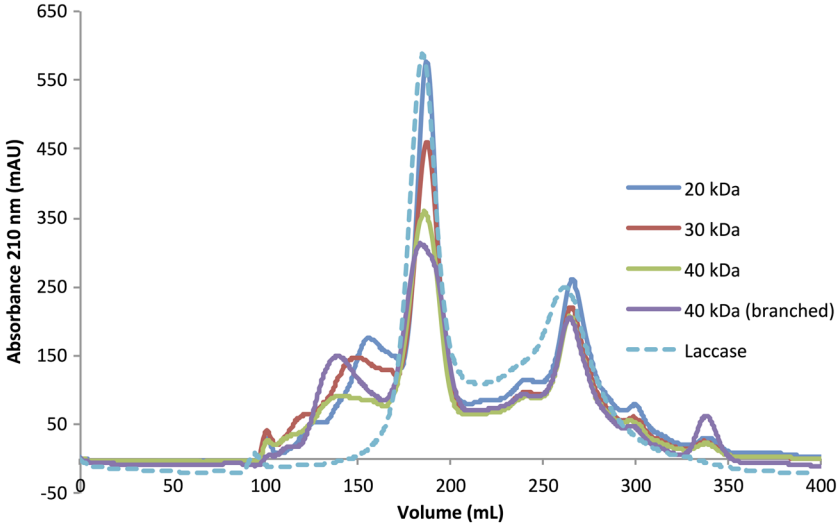


Figure 6. Size exclusion chromatography profile of PEGylation reactions with different molecular weights of mPEG and standard laccase. Molar ratio = 1:4; reaction time = 4 h.

preserved were obtained, important questions were raised. Such questions involve the potential role of PEGylation to change the affinity of the enzyme for a specific substrate. It is clear that this is an issue that needs to be addressed in future research.

The modification of enzymes has been used for a long time; the interest in preserving and/or increasing the enzyme activity is a subject that will be relevant in any process. PEGylation has been little explored to modify nontherapeutic enzymes. However, this work shows that it is possible to produce 'improved enzymes' without affecting enzyme activity. The potential generic application of this approach to other enzymes of industrial interest opens a line of research that can be widely exploited.

CONCLUSIONS

Selecting the laccase PEGylation reaction conditions is not a trivial task. Site-specific modification of N-terminal was selected in order to preserve the activity of the native protein in the conjugate. The effects of molar ratio (laccase : mPEG), concentration, time of reaction, molecular weight, and type of mPEG were evaluated. The factors that had more impact on the recovery of activity were the concentration of laccase and the time of reaction. The molecular weight of mPEG had not significant impact in the laccase activity. The best conditions for laccase PEGylation were 12 mg/ml of laccase, molar ratio 1:4, and 4 h of time of reaction. Under these conditions, the enzyme is able to maintain nearly 100% of its enzymatic activity with ABTS; whereas with DMP and syringaldazine, the loss of activity is on average 19% and 13%, respectively. In all experiments, the recovery of activity increased with ABTS. As it can be observed in controls, sodium cyanoborohydride affects the laccase activity. However, mPEG provides stability to the protein during aldehyde PEGylation reaction. Determine the reaction conditions is the first stage in PEGylation process design. Identifying at early stage the parameters that affect the product activity is crucial. Because once the conditions are optimized, PEGylated protein can be obtained and the degree of PEGylation determined. If it is necessary, the purification process can be designed.

Acknowledgements

The authors wish to acknowledge the financial support of Tecnologico de Monterrey, Bioprocess research chair (grant CAT-161). Karla Mayolo-Deloisa thanks the posdoctoral program of Tecnológico de Monterrey.

REFERENCES

Baldrian P. 2006. Fungal laccases—occurrence and properties. *FEMS Microbiol. Rev.* **30**: 215–242. DOI: 10.1111/j.1574-4976.2005.00010.x

Cisneros-Ruiz M, Mayolo-Deloisa K, Przybycien TM, Rito-Palomares M. 2009. Separation of PEGylated from unmodified ribonuclease A using sepharose media. *Sep. Purif. Technol.* **65**: 105–109. DOI: 10.1016/j.seppur.2008.10.038

Daly SM, Przybycien TM, Tilton RD. 2005. Adsorption of poly(ethylene glycol)-modified ribonuclease A to a poly(lactide-co-glycolide) surface. *Biotechnol. Bioeng.* **90**: 856–868. DOI: 10.1002/bit.20481

Fee CJ, Van Alstine JA. 2006. PEG-proteins: reaction engineering and separation issues. *Chem. Eng. Sci.* **61**: 924–939. DOI: 10.1016/j.ces.2005.04.040

Flores C, Vidal C, Trejo-Hernández MR, Galindo E, Serrano-Carreón L. 2009. Selection of *Trichoderma* strains capable of increasing laccase production by *Pleurotus ostreatus* and *Agaricus bisporus* in dual cultures. *J. Appl. Microbiol.* **106**: 249–257. DOI: 10.1111/j.1365-2672.2008.03998.x

Gaberc-Porekar V, Zore I, Podobnik B, Menart V. 2008. Obstacles and pitfalls in the PEGylation of therapeutic proteins. *Curr. Opin. Drug Disc.* **11**: 242–250.

González-González M, Mayolo-Deloisa K, Rito-Palomares M. 2012. PEGylation, detection and chromatographic purification of site-specific PEGylated CD133-Biotin antibody in route to stem cell separation. *J. Chromatogr. B* **893–894**: 182–186. DOI: 10.1016/j.jchromb.2012.03.002

González-Valdez J, Cueto LF, Benavides J, Rito-Palomares M. 2011. Potential application of aqueous two-phase systems for the fractionation of RNase A and α-Lactalbumin from their PEGylated conjugates. *J. Chem. Tech. Biotechnol.* **86**: 26–33. DOI: 10.1002/jctb.2507

Huang Z, Ni C, Chu Y, Wang S, Yang S, Wu X, Wang X, Li X, Feng W, Lin S. 2009. Chemical modification of recombinant human keratinocyte growth factor 2 with polyethylene glycol improves biostability and reduces animal immunogenicity. *J. Biotechnol.* **142**: 242–249. DOI: 10.1016/j.jbiotec.2009.05.004

Jevsevar S, Kunstelj M, Gaberc-Porekar V. 2010. PEGylation of therapeutic proteins. *Biotechnol. J.* **5**: 113–128. DOI: 10.1002/biot.200900218

Kim T, Choi D, Yoon K-H, Kim K, Lee S. 2013. Application of mixture rule to determine arrhenius activation energy of time temperature integrator using mixture of laccase from *Pleurotus ostreatus* and PEGylated laccase from *Trametes versicolor*. *J. Korean Soc. Appl. Bi.* **56**: 419–425. DOI: 10.1007/s13765-013-3093-x

Kurfürst MM. 1992. Detection and molecular weight determination of polyethylene glycol-modified hirudin by staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **200**: 244–248. DOI: 10.1016/0003-2697(92)90460-O

Lee H, Jang IH, Ryu SH, Park TG. 2003. N-terminal site-specific mono-PEGylation of epidermal growth factor. *Pharm. Res-Dordr* **20**: 818–825. DOI: 10.1023/A:1023402123119

Lopez-Cruz JI, Viniegra-Gonzalez G, Hernandez-Arana A. 2006. Thermostability of native and pegylated *Myceliophthora thermophila* laccase in aqueous and mixed solvents. *Bioconjugate Chem.* **17**: 1093–1098. DOI: 10.1021/bc0503465

Madhavi V, Lele SS. 2009. Laccase: properties and applications. *Bioresources* **4**: 1694–1717.

Mayolo-Deloisa K, Trejo-Hernandez MD, Rito-Palomares M. 2009. Recovery of laccase from the residual compost of *Agaricus bisporus* in aqueous two-phase systems. *Process Biochem.* **44**: 435–439. DOI: 10.1016/j.procbio.2008.12.010

Mayolo-Deloisa K, Lienqueo ME, Andrews B, Rito-Palomares M, Asenjo JA. 2012. Hydrophobic interaction chromatography for purification of monoPEGylated RNase A. *J. Chromatogr. A* **1242**: 11–16. DOI: 10.1016/j.chroma.2012.03.079

Morozova OV, Shumakovich GP, Shleev SV, Yaropolov YI. 2007. Laccase-mediator systems and their applications: a review. *Appl. Biochem. Microbiol.* **43**: 523–535. DOI: 10.1134/S0003683807050055

Pasut G, Veronese FM. 2007. Polymer–drug conjugation, recent achievements and general strategies. *Prog. Polym. Sci.* **32**: 933–961. DOI: 10.1016/j.progpolymsci.2007.05.008

Payne R, Murphy B, Cornell-Manning M. 2010. Product development issues for PEGylated proteins. *Pharm. Dev. Technol.* **16**: 423–440. DOI: 10.3109/10837450.2010.513990

Rodríguez-Couto S, Toca-Herrera JL. 2006. Industrial and biotechnological applications of laccases: a review. *Biotechnol. Adv.* **24**: 500–513. DOI: 10.1016/j.biotechadv.2006.04.003

Schroeder M, Heumann S, Silva CJSM, Cavaco-Paulo A, Guebitz GM. 2006. Specificities of a chemically modified laccase from *Trametes hirsuta* on soluble and cellulose-bound substrates. *Biotechnol. Lett.* **28**: 741–747. DOI: 10.1007/s10529-006-9052-4

Vandertol-Vanier HA, Vazquez-Duhalt R, Tinoco R, Pickard MA. 2002. Enhanced activity by poly(ethylene glycol) modification of *Coriolopsis gallica* laccase. *J. Ind. Microbiol. Biotechnol.* **29**: 214–220. DOI: 10.1038/sj.jim.7000308

Veronese FM, Mero A. 2008. The impact of PEGylation on biological therapies. *BioDrugs* **22**: 315–329. DOI: 10.2165/00063030-200822050-00004

Veronese FM, Pasut G. 2005. PEGylation, successful approach to drug delivery. *Drug Discov. Today* **10**: 1451–1458. DOI: 10.1016/S1359-6446(05)03575-0

Zhang G-Q, Tian T, Liu Y-P, Wang H-X, Chen Q-J. 2011. A laccase with antiproliferative activity against tumor cells from a white root fungus *Abortiporus biennis*. *Process Biochem.* **46**: 2336–2340. DOI: 10.1016/j.procbio.2011.09.020

Zou Y-J, Wang H-X, Ng T-B, Huang C-Y, Zhang J-X. 2012. Purification and characterization of a novel laccase from the edible mushroom *Hericium coralloides*. *J. Microbiol.* **50**: 72–78. DOI: 10.1007/s12275-012-1372-6