Improved chemical and physical stability of laccase after spherezyme immobilisation

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ABSTRACT

Laccase was successfully self-immobilised into particles using spherezyme methodology, with a retention of 53.9% of original enzyme activity. Post-treatment of the spheres with ethanolamine or a combination of polyethlyeneimine and ethanolamine reduced this to 49.4% and 23.3%, respectively. However, polyethyleneimine coated spherezymes displayed improved stability towards oxidative denaturation by mediators with improvements of 5.61-, 6.95- and 15.55-fold for 2-hydroxyphtalimide, N-hydroxybenzotriazole and 2,2,6,6-tetramethylpiperidinooxy free radical, respectively. The immobilised preparation displayed enhanced stability during temperature incubation at 60–70 °C with an improvement of 2.04- and 4.03-fold respectively for ethanolamine post-treated spherezymes and polyethyleneimine coated spherezymes. Furthermore, enhanced stability at acidic pH was observed, with the most pronounced enhancement of 2.49-fold obtained at pH 2, and 1.69 and 1.38 at pH 3 and 4, respectively.

1. Introduction

Enzyme immobilisation is advantageous as it simplifies enzyme recovery and therefore recyclability for applications such as biocatalysis [1,2]. Immobilisation has been shown to significantly improve the mechanical and chemical stability of enzymes [1,3,4]. It is thought that these improvements are acquired through multipoint attachment of the enzyme during immobilisation, which enhances the rigidity of the enzyme by stabilising the tertiary structure of the protein [4]. Enhancing the stability of enzymes is crucial for realising their industrial applications [5].

Spherezyme (SZ) is a novel protein self-immobilisation method where proteins are crosslinked in an emulsion [6,7]. The resulting stabilised spherical protein particles have the benefit of having a high specific activity and narrow particle size distribution due to this method of manufacture. Enhanced activity retention can be achieved through the addition of an active site protectant, such as an enzyme substrate or analog, during preparation [6,7].

Laccases (EC 1.10.3.2) are receiving attention due to their potential applications; including biosensors [8], biofuel cells [9], biocatalysis [10], biobleaching and bioremediation [11–14]. These oxidoreductases are capable of oxidising a broad range of phenolic substrates [14,15]. The substrate range of this enzyme may be extended to non-usual substrates through the use of mediators [15,16], chemicals that when oxidised by laccase may themselves oxidise secondary substrate. Typically mediators may be subsequently re-oxidised by laccase to perform further oxidations of the secondary substrate, and hence are only required in catalytic quantities. This indirect catalytic oxidation of a secondary substrate is known as mediation [15–17]. Mediation has resulted in a broadening of the application range of laccases, particularly for bioremediation [18] and biocatalysis applications [19]. However, the benefits incurred by the use of mediators may be negated by their disadvantages, including instability of laccase in the presence of mediators, which has been attributed to their ability to oxidise laccase, toxicity of the mediator, and their expense [16,20,21].

Due to the variety of potential applications that have developed for laccases, their immobilisation to help realise these applications is under investigation. Several studies on the immobilisation and characterisation of laccases for potential applications such as dye decolourisation have been published [22–24]. However, investigation of stability in the presence of mediators has largely been neglected.

This article outlines a process for the immobilisation of laccase using the spherezyme method [7]. Improvements to pH, temperature and chemical stability in the presence of mediators are quantified. Enhancement in the presence of mediators is a significant step towards the realisation of laccase applications requiring mediation.

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2. Materials and methods

2.1. Chemicals and reagents

Denilite® II Base laccase and Denilite® II Assist (methyl syringate) were generously donated by Novozymes SA. Polyethyleneimine (PEI), N-hydroxybenzotriazole (HOBT), 2-hydroxyphtalimide (HPT), 2,2,6,6-tetramethylpiperidinooxy free radical (TEMPO), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), Trizma® base, ethanolamine, bovine serum albumin fraction V (BSA), lactic acid and succinic acid were purchased from Sigma–Aldrich. Glutaraldehyde was obtained as a 25% aqueous solution from Acros Organics. Mineral oil (white oil medicinal) was obtained from Castrol, and Nonoxynol-4 was a gift from the CHC Group (South Africa).

2.2. Laccase assay

The activity of laccase was followed by the oxidation of ABTS at 420 nm (1 mM) in 100 mM succinate-lactate buffer, pH 4.5 [25]. Assays were performed by addition of 20 μl of the laccase preparation to 180 μl of the buffer substrate solution. Activity was followed spectrophotometrically at 420 nm using a PowerWave HT (Biotek Instruments) with the instrument parameters set at rapid read, incubation at 25 °C and medium shaking (intensity 3, for 1 s). One unit of enzyme activity was defined as the amount of enzyme required to catalyse the oxidation of 1 μmol of substrate to product per minute at 25 °C. These methods were used for both free and immobilised enzyme preparations, blanks containing immobilised enzyme particles but without substrate were used as controls. The interference caused by the particles was insignificant compared to the oxidation rate of ABTS. All values are represented as means of triplicate assays and error as standard deviation.

2.3. Partial laccase purification

Commercial Denilite® II Base (Novozymes) (10 g), a laccase-based denim bleaching product, was dissolved in 200 ml of 20 mM Tris–Cl buffer (pH 8.0). This slurry was magnetically stirred for 2 h at 200 rpm (4 °C). The slurry was clarified by centrifugation for 15 min at 15,330 × g and 4 °C. The solution was concentrated to 50 ml using an Amicon ultrafiltration unit (Millipore) fitted with a 10 kDa NMWL polyethersulphone membrane (PALL filtration). This concentrate was dialyzed against Milli-Q water at 4 °C, three washes of 3 h in Snakeskin® dialysis tubing (Pierce) with 10 kDa NMWL. This preparation was subsequently lyophilised with a temperature gradient from −20 to 15 °C over 24 h. The lyophilised protein preparation was stored at 4 °C until required for spherezyme preparation.

2.4. Laccase spherezyme preparation

The aqueous phase of the emulsion consisted of a 1 ml solution containing 200 mg ml^{-1} partially purified laccase premixed with 50 mg ml^{-1} BSA in 100 mM acetate-phosphate-borate buffer pH 7.5 (Britton-Robinson universal buffer, pH 7.5). The laccase was co-immobilised with BSA since the laccase did not crosslink adequately without a lysine rich protein [6]. The minimum stoichiometric quantity of BSA required for crosslinking was determined empirically in a 96-well microtitre plate. The active site protectant solution for the crosslinking procedure was prepared by dissolving Denilite® II Assist in Milli-Q water (100 mg ml^{-1}), which was subsequently clarified by centrifugation at 16,060 × g for 15 min. The oil phase for the emulsion was prepared by dissolving the detergent Nonoxynol-4 (250 μl) in mineral oil (50 ml) using magnetic stirring for 10 min. The protectant solution, 250 μl, was added to the enzyme solution. This solution was allowed to react for 5 min while concurrently reacting the crosslinking reagent [6,7] consisting of glutaraldehyde (25% aqueous solution) with 0.33 M ethylenediamine (pH 6.5) in a 1:1 volumetric ratio in a separate vessel (24 °C); this forms a polymer of alternating monomers of glutaraldehyde and ethylenediamine terminated with glutaraldehyde moieties due to their molar excess. The crosslinking of the aqueous phase proteins was initiated by the addition of 150 μl of the crosslinking solution to the aqueous phase. These solutions were mixed thoroughly and immediately emulsified in the oil phase with magnetic stirring for 1 min at 700 rpm. This emulsion was maintained at 4 °C with intermittent stirring for 1 min every 30 min over a period of 3 h to prevent the emulsion from breaking during crosslinking.

The immobilised enzyme preparation was recovered from the oil phase by centrifugation at 837 × g for 15 min. The spheres were washed five times for 10 min with 50 ml of 40 mM Tris–Cl (pH 8.0) containing 5 mM ethanolamine (for excess glutaraldehyde quenching) and a final wash with 50 ml of 40 mM Tris–Cl to remove unreacted ethanolamine. This preparation was termed SZ EA. PEI coated spheres (SZ PEI) were prepared similarly except that the first wash solution was replaced with 50 ml of 1% PEI in 40 mM Tris–Cl buffer (pH 8.0). A third preparation (SZ) without the addition of PEI or ethanolamine was prepared as a control to compare the effect of post-treatment on activity maintenance of the immobilised laccase preparations. The washed particles were resuspended to 20 ml in 40 mM Tris–Cl (pH 8.0) and stored at 4 °C.

2.5. Stability in the presence of mediators

Laccase and immobilised laccase preparations were incubated in the presence of 2 mM mediator, either 2-hydroxyphtalamide (HPT), N-hydroxybenzotriazole (HOBT) or 2,2,6,6-tetramethylpiperidinooxy free radical (TEMPO) at pH 6.0 in 50 mM Britton-Robinson buffer at 30 °C. Samples from these preparations were taken at regular time intervals (4 h) and assayed for residual enzyme activity. Controls using non-immobilised laccase and BSA with an equivalent protein content (mass) were also incubated as mentioned above.

The possible interference of these mediators with the ABTS assay was foreseen, and hence we investigated the influence of reduced and oxidised mediators on the assays. Oxidised mediators were prepared by incubation of the mediator (2 mM in Britton-Robinson buffer, pH 6.5) with laccase (1 mg) for 40 min. The laccase was subsequently removed from the oxidised products by retention on a 10 kDa ultrafiltration membrane. Fresh laccase was added to the filtrate and assayed. These controls indicated no interference of the mediators (native or oxidised) on the assay with ABTS as a substrate.

2.6. pH stability

The pH stability of the free and immobilised Denilite® laccase was compared by incubation of the enzyme and spherezyme preparations in 100 mM Britton-Robinson universal buffer from pH 2 to 12 at 1 pH unit intervals. The activities of the preparations were assayed periodically to determine residual enzyme activity.

2.7. Optimum temperature profiling

The temperature profiles of free and immobilised laccase preparations were performed using 1 mM ABTS as the substrate in 100 mM succinate-lactate buffer (pH 4.5). Assays were performed using a DU800 spectrophotometer (Beckman-Coulter) fitted with a Peltier temperature controller. The Peltier was set to the temperature of interest and cuvettes were allowed to equilibrate for 5 min prior to addition of reagent pre-equilibrated to the same temperature over 10 min in a dry-bath.

2.8. Temperature stability

Temperature stability assays were performed by incubation of free laccase and immobilised laccase preparations in 20 mM Tris–Cl buffer (pH 8.0) at temperatures of 50, 60 and 70 °C. Samples were taken at 30 min intervals and assayed for residual activity by kinetic assay at 420 nm using 1 mM ABTS as the substrate in 100 mM succinate-lactate buffer (pH 4.5) using a DU800 spectrophotometer (Beckman-Coulter) set to 25 °C.

3. Results and discussion

3.1. Spherezyme preparation and enzyme activity maintenance

Laccase spherezymes were prepared incorporating BSA to improve the crosslinking efficiency since initial attempts to crosslink the partially purified enzyme using the spherezyme method were unsuccessful. BSA may be used as a co-crosslinking agent or 'proteic-feeder' [26] to improve the crosslinking of many other proteins since it contains a relatively higher quantity of lysine residues.

Activity maintenance was calculated by comparing the specific activity of immobilised enzyme preparations to that of the starting enzyme preparation (16.2 U mg^{-1} protein). The immobilisation of Denilite® laccase using the spherezyme method resulted in an appreciable activity recovery of 54.9% (8.8 U mg^{-1}) as compared to the initial enzyme activity. This activity yield was reduced by post-treatment with aldehyde quenching agents, such as ethanolamine and polyethyleneimine to 49.4% (8.1 U mg^{-1}) and 23.3% (3.8 U mg^{-1}), respectively. In general, colorimetric assays for the immobilised enzyme particles exhibited more error than the free enzyme samples. This is likely due to the interference of the immobilised enzyme preparation with the light path of the spectrophotometer and was compensated for by a substrate free control.

3.2. Stability in the presence of mediators

Although mediators may be used to extend the substrate range of laccases, a major hindrance to mediated applications is the instability of laccase in the presence of mediators. Thus, possi-

Table 1
Stability (half-life) and fold stability improvement of free and immobilised laccase preparations in the presence of mediators at pH 6 and 30 °C; ethanolamine post-treated spherezymes (SZ EA) and polyethyleneimine coated spherezymes (SZ PEI).

| Mediator	Sample	$t_{50\%}$ (min)	Fold improvement
HPT	Free laccase	38.6	
	SZ EA	45.4	1.17
	SZ PEI	216.7	5.61
HOBT	Free laccase	28.9	
	SZ EA	40.1	1.39
	SZ PEI	200.4	6.95
TEMPO	Free laccase	17.1	
	SZ EA	31.7	1.86
	SZ PEI	265.6	15.55

ble improvements to laccase stability were assessed. Free laccase and immobilised laccase preparations were incubated in the presence of mediators at 30 °C. The results obtained indicated a general improvement in the stability of the enzyme after immobilisation. Coating spheres with PEI resulted in the most pronounced stability improvement in this experiment (Table 1). Assay controls with mediators indicated that ABTS activity of laccase was unaffected by the presence of the various mediators in the reduced or oxidised form.

Improvements were quantified in terms of fold improvements with respect to the free enzyme. Improvements of 5.61-, 6.95- and 15.55-fold were observed for HPT, HOBT and TEMPO respectively with PEI coated spherezymes.
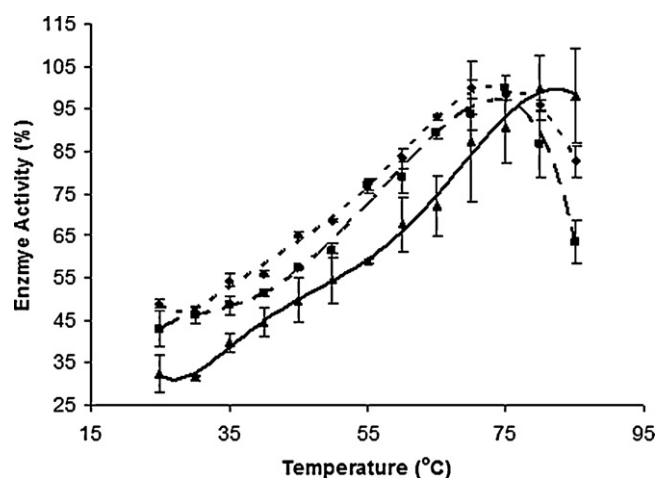
3.3. pH stability

The non-immobilised Denilite® laccase was stable from neutral to alkaline pH during the time course of the experiment (not shown), while limited stability was observed at acidic pH (Fig. 1).

Difficulties in assays at pH greater than 6 were encountered for the spherezyme preparations as the particles demonstrated agglomeration under these conditions, and this resulted in non-homogenous oxidation of the substrates. This agglomeration appeared to be time dependant and may be due to electrostatic
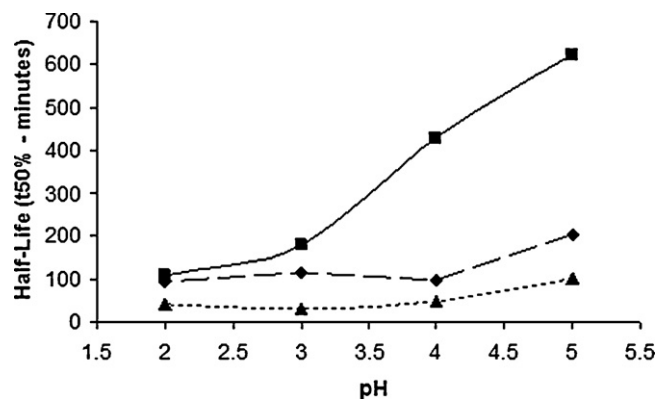
Fig. 1. pH stability data indicating half-life of free enzyme and immobilised enzyme preparations. Polyethyeleneimine coated spherezymes (SZ PEI; ■) and ethanolamine post-treated spherezymes (SZ EA; ♦) compared to free enzyme (▲) at acidic pH.

Fig. 2. Temperature profiles for free enzyme (♦), spherezyme immobilised (■) and PEI post-treated spherezyme (▲).
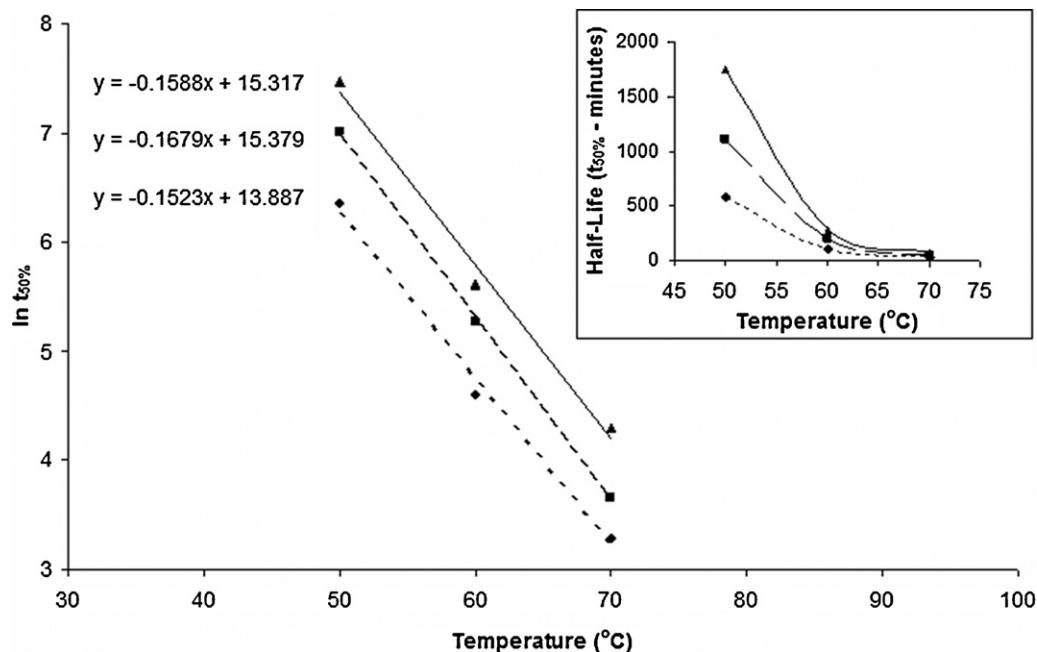
Fig. 3. Temperature stability of free enzyme (♦), spherezyme immobilised (■) and PEI post-treated (▲). Preparations during incubation at 50, 60 and 70 °C. (Insert) Half-life plots of preparations.

bridging by the buffer used for the assay. The improved physical mixing of the solutions possible at larger volumes (10 ml) alleviated this problem.

Polyethyleneimine coated spherezymes (SZ PEI) displayed vastly improved acidic pH stability over that of the free enzyme. The ethanolamine quenched spherezymes indicated limited stability improvements under the same conditions (Fig. 1). The SZ PEI preparation showed a 6.14-, 8.69-, and 5.40-fold improvement in stability for pH 5, 4 and 3, respectively, while at pH 2 this improvement was reduced to 2.61-fold.

3.4. Temperature profiling

The temperature profiles indicated an increase in the optimum temperature for laccase activity from ~70 to ~80 °C (Fig. 2) for the polyethyleneimine post-treated spherezymes, while no improvement was noted for ethanolamine treated spherezymes. This optimum temperature increase may be a function of the increase in thermostability with immobilisation (see below).

3.5. Temperature stability

Improved stability was observed for both spherezyme preparations over that of the free laccase (Fig. 3). The most pronounced improvement in thermal stability was noted for PEI coated spherezymes. The half-life of the polyethyleneimine coated laccase spherezymes improved 3.07-, 2.73- and 2.70-fold for 50, 60 and 70 °C, respectively ($t_{50\%}$; Fig. 3, insert). Spherezyme immobilised laccase indicated enhanced temperature tolerance with lower enzyme loss in the same time periods (Fig. 3).

4. Conclusions

Enhancements to the stability in the presence of mediators and in an acidic environment was observed for laccase immobilised using the spherezyme method. Instability in the presence of mediators has previously been noted as a hindrance to applications using laccase-mediated systems [16,20]. The enhanced stability properties of the spherezyme immobilised laccase indicates a substantial improvement towards overcoming this drawback and is a significant step towards realising these applications. Treatment of spherezymes with PEI resulted in the most pronounced improvements for all the properties investigated.

Acknowledgements

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