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Preparation and use of cross-linked enzyme aggregates (CLEAs) of laccases

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ABSTRACT

Cross-linked enzyme aggregates (CLEA®) were prepared from laccases from three different sources: *Trametes versicolor*, *Trametes villosa* and *Agaricus bisporus*. The effect of the various parameters – nature of the precipitant, pH, temperature, glutaraldehyde concentration and cross-linking time – on the activity recovery and storage and operational stability of the resulting CLEAs was different. The laccase CLEAs exhibited the expected increased stability compared to the free enzyme but there was no direct correlation with the number of surface lysine residues in the latter. It is clearly not the only parameter influencing the properties of the CLEA. Co-aggregation with albumin did not improve the stability. The laccase CLEAs, in combination with the stable N-oxy radical, TEMPO, were shown to be active and stable catalysts for the aerobic oxidation of linear C_5–C_{10} aliphatic alcohols, to the corresponding aldehydes, in aqueous buffer (pH 4). Rates were an order of magnitude higher than those observed with the corresponding free enzyme and the CLEAs could be recycled several times without appreciable loss of activity. The addition of water immiscible or water miscible solvents showed no further improvement in rate compared with reactions in aqueous buffer alone.

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1. Introduction

The application of biocatalysts, as whole cells or isolated enzymes, has increased considerably over the years, not only on a laboratory but also on an industrial scale [1]. Enzymes catalyze chemical conversions with high chemo-, regio- and stereoselectivities under mild conditions (ambient temperature, aqueous media). However, the commercialization of enzymatic processes is often hampered by a lack of availability, high price and/or limited stability under operational conditions of the enzyme in question. These drawbacks can be overcome by immobilization of the enzyme thereby rendering it more stable and easy to recover and recycle [2].

Conceptually, there are three types of methodology for immobilization: binding to a carrier, inclusion/entrapment or encapsulation in an organic or inorganic polymer, and cross-linking of protein molecules. There is no universal method of enzyme immobilization. Many factors effect successful immobilization: a suitable carrier, immobilization conditions (pH, temperature, nature of medium) and the enzyme itself (source, purity, nature of protein). All the methodologies mentioned above have advantages and disadvantages. A major disadvantage of many carrier-bound or encapsulated enzymes is lack of stability towards leaching in aqueous media. Moreover, a high enzyme loading is needed for industrial viability and a compromise has to be made between the pore size and surface area, to reach high enzyme loading and activity retention [3].

Biocatalysts with high volumetric activities and catalyst productivities can be obtained by simply cross-linking the enzyme, thereby avoiding the need for the extra inactive mass of a carrier. The technique of protein cross-linking, via the reaction of a bifunctional reagent such as glutaraldehyde with reactive NH_2 groups on the protein surface, was initially developed by Doscher in the 1960s [4]. However, this method of producing cross-linked enzymes (CLEs), by reaction of the dissolved enzyme with glutaraldehyde has several drawbacks, such as low activity retention, low mechanical stability and difficulties in handling the gelatinous immobilizates. In the early 1990s cross-linked enzyme crystals, CLECs®, prepared by cross-linking the crystalline enzyme, were shown to have excellent activities and operational stabilities in a variety of biotransformations [5]. However, a major drawback of CLECs is the high purity needed to crystallize the enzyme, which translates to high prices. In contrast, the preparation of Cross-Linked Enzyme Aggregates CLEAs®, by precipitation of the enzyme from aqueous buffer and cross-linking of the resulting physical aggregates with, for example, glutaraldehyde, can be successfully performed with crude enzyme preparations. Indeed, since precipitation is often used to purify enzymes, the preparation of CLEAs essentially combines purification and immobilization into a single operation. CLEAs essentially combine the excellent operational

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performance of the CLECs with ease of preparation and relatively low price. The CLEA methodology is applicable to a wide variety of enzymes: hydrolases (lipases, esterases, amidases, proteases, nitrilases and glycosidases), lyases (nitrile hydratases, oxynitrilases and aldolases) and oxidoreductases (galactose oxidase, glucose oxidase and laccase) [5,6].

We envisaged the preparation of a laccase CLEA, that is stable in organic solvents, as a means for improving the performance of the laccase under operating conditions, e.g. in combination with TEMPO as a catalyst, for the aerobic oxidation of alcohols [7]. Herein we report the preparation of laccase CLEAs, from three different laccases, and their application in alcohol oxidations.

2. Experimental

2.1. General

The substrates and reagents were of analytical grade and obtained from Fluka and Sigma–Aldrich. Laccase [E.C. 1.10.3.2] from *Trametes versicolor* and *Agaricus bisporus* were purchased from Fluka. Laccase from *Trametes villosa* was kindly donated by Givaudan Nederland B.V., Naarden. 2,2,6,6-Tetramethylpiperidinyl-1-oxy (TEMPO) was received from Acros. The reactions were carried out in a stationary Omni Reaction Station (Screening Devices B.V.) with comprehensive heating/cooling/stirring/aeration control. Open air New Brunswick Innova®2100 shaker was applied for precipitation and cross-linking procedures. Shimadzu UV-2450/2550 spectrophotometer was used for optical absorbance measurements. All experiments were performed in triplicate.

2.2. Metol activity assay for laccase

Laccase activity was measured spectroscopically using metol (N-methyl-p-aminophenol hydrosulfate) as a substrate [8]. Metol was chosen as the substrate for activity measurements based on its superior air stability, lower price and ease of handling compared to other commonly used substrates. The reaction mixture contained 1 ml 0.05 M metol solution in water. The reaction was initiated by addition of 0.5 ml enzyme solution in 0.1 M acetate buffer (pH 4.5) and the increase in absorbance was monitored at 540 nm (ε = 2000 M^{-1} cm^{-1}). One unit (U) of the laccase was defined as 1 μmol metol oxidized per min. under the stated assay conditions [8].

2.3. Catechol activity assay for laccase

The metol assay can be applied only for soluble enzyme as solid particles interfere with the optical density measurement. Consequently, a catechol activity assay was used for insoluble cross-linked enzyme aggregates. When the reaction was stopped, by addition of a strong acid, the aggregates were easily removed by centrifugation.

A catechol solution (ε = 1260 M^{-1} cm^{-1} [9]) was prepared in 0.05 M citrate/phosphate buffer at pH 4.5. For the activity assay 1 ml of catechol of 5 mM concentration solution and 1 ml of total enzyme solution (100 μl of enzyme and 900 μl buffer) were incubated at 30 °C in a water bath for 90 s. The reaction was stopped by adding 10% H_2SO_4 solution and the absorbance measured by a UV–Vis spectrophotometer at 400 nm wavelength.

2.4. Protein concentration assay

The protein concentration was determined by measuring the absorbance of the protein assay reagent, bicinchoninic acid (BCA) after 30 min incubation with the enzyme (www.piercenet.com/products). Bovine serum albumin was used as a standard for the calibration curve.

2.5. Precipitation procedure

One ml of enzyme solution was added drop-wise to 2.5 ml of the chilled precipitant (ethanol, 2-propanol, acetone, acetonitrile, tetrahydrofuran, dioxane, dimethyl sulfoxide, polyethylene glycol 1500, polyethylene glycol 3400, ammonium sulfate or 1,2-dimethoxyethane) and pH of the solution was adjusted to the desired pH. After shaking for 1 h at 200 rpm and the given temperature, the mixture was centrifuged and the pellets were well washed with 0.1 M phosphate buffer (pH 4). The pellets were then redissolved in buffer and activity was measured using the metol assay (see Section 2.2) and protein concentration by the BCA method (see Section 2.4). For pH screening experiments the initial enzyme solution was adjusted to the given pH.

2.6. Cross-linking procedure

After precipitation the given amount of glutaraldehyde was added drop-wise to the mixture to afford a concentration in the range of 2.4–40 mM and shaking at 200 rpm was continued for 1–27 h at 20 °C. The mixture was centrifuged and the supernatant decanted from the pellets. The latter were washed with 0.1 M phosphate buffer (pH 4) and centrifuged again. The washing procedure was repeated until no traces of activity were determined in the supernatant. The activity of the resulting CLEAs was measured using the catechol assay (see Section 2.3).

2.7. Scale-up procedure for CLEA preparation

One g of laccase was dissolved in 50 ml 0.1 M phosphate buffer pH 4. The pH was adjusted to pH 6 and 100 μl of 200 mM phenyl methyl sulfonyl fluoride (PMSF) solution was added. This solution was added drop-wise to the 100 ml chilled precipitant, dimethoxyethane (DME) or dioxane, and shaken for 1 h at 200 rpm and room temperature. Glutaraldehyde solution (200 mM) was added until the concentration was 5–20 mM and the mixture shaken for a further 19 h at the same speed and temperature. The solutions were centrifuged at 3000 rpm for 20 min at 4 °C. The pellets were washed with 0.1 M phosphate buffer (pH 4), centrifuged and the activity was measured using the catechol assay (see Section 2.3).

2.8. Scale-up procedure for CLEA preparation by co-aggregation with albumin

One g of laccase was dissolved in 50 ml 0.1 M phosphate buffer pH 4 (the corresponding initial activity was measured by the catechol assay). The pH was adjusted to pH 6 and 100 μl of 200 mM PMSF solution was added. Egg albumin (0.66 g) was added, corresponding to a 1:1 molar ratio to laccase, and the mixture was shaken for 15 min (200 rpm, room temperature). Subsequently, 5 ml of 100 mM pentaethylene-hexamine (PEHA) was added and the mixture shaken for a further 30 min. PEHA was added to increase the number of available amino groups for cross-linking. Then 100 ml of chilled DME was added drop-wise and the mixture shaken for 1 h at 200 rpm and room temperature. After 1 h glutaraldehyde solution was added until the concentration reached 5–20 mM and the mixture shaken for a further 19 h. The mixtures were centrifuged at 3000 rpm for 20 min at 4 °C. The pellets were washed with 0.1 M phosphate buffer (pH 4), centrifuged and the activity was measured using the catechol assay (see Section 2.3).

2.9. Alcohol oxidations

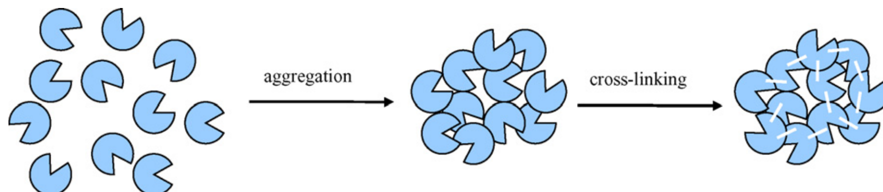
In the standard protocol 1.6 mmol alcohol, 0.15 mmol TEMPO, 0.25 mmol anisole (internal standard) were mixed with 10 U of free laccase or CLEA in 10 ml of 0.1 M phosphate buffer pH 4. The vials were aerated using a steel filter stone. The reactions were carried out in an Omni Reaction Station at 30 °C for the given time. After reaction, the reaction product and unreacted substrate were extracted with ethyl acetate (3× 10 ml), dried over anhydrous magnesium sulfate and analysed with GC. The blank reactions were performed by omitting one of the components mentioned above.

2.10. Analytical methods

Alcohol conversions were determined by GC analysis using a WAX 52 CB column (50 m × 0.53 mm). The column was programmed as follows: 80 °C for 5 min, then at the rate of 7 °C per minute to 235 °C. Anisole was used as internal standard.

3. Results and discussion

As mentioned above, CLEA preparation consists of two steps: aggregation by precipitation and cross-linking:



Precipitation, by the addition of salts, organic solvents or non-ionic polymers to aqueous solutions of proteins, is a commonly used method for protein purification [10]. The resulting physical aggregates of enzyme molecules are supramolecular structures that are held together by non-covalent bonding and can be easily redissolved in water. Cross-linking produces insoluble CLEAs in which the structural properties and catalytic activities of the protein are maintained. Due to the different biochemical and structural properties of proteins the best precipitant and cross-linker can vary from one enzyme to another. Laccase is a highly glycosylated enzyme, which has 4 copper atoms in the active site. We chose laccases from three different sources for our initial studies of CLEA preparation. Laccase from *T. versicolor* is commercially available. It is a monomeric enzyme and has 5 lysine and 15 arginine residues which are potential sites for cross-linking [11]. Laccase from *T. villosa* (*Polyporus pinsitus*) is a dimeric protein consisting of two subunits each of which is approximately 61 kDa in size and has 5 lysine and 12 arginine residues [12]. Laccase from *A. bisporus* is also a dimeric enzyme, but has almost twice the number of lysine (11) and arginine (27) residues [13]. Our first goal was to optimize the conditions for the preparation of CLEAs from the three different laccases.

3.1. Selection of the optimum precipitant

The three laccases were precipitated at three different temperatures: 4 °C and 8 °C or 20 °C with *T. versicolor* laccase (see Table 1). Organic solvents such as ethanol, isopropanol, acetone, acetonitrile, dioxane, and dimethylformamide, saturated ammonium sulfate solution, and water miscible polymers with two different molecular weights were investigated as precipitants. A ratio of 1:2.5 (v/v) of enzyme solution to precipitant was used for protein precipitation. The specific activity is the activity of laccase in Units per mg of total protein. The purification factor (P.F.) refers to the specific activity of redissolved aggregates obtained using a particular precipitant divided by the specific activity of the sample of free enzyme that they were derived from. It is a measure of how effective the precipitation was for a given enzyme sample protein using different precipitants. This contrasts with activity recovery (see later) which is defined as Total Units in divided by Total Units out. 2-Propanol, dioxane, dimethoxyethane (DME), ammonium sulfate ($(NH_4)_2SO_4$) and polyethylene glycol (PEG 3400) were effective precipitants for laccase from *T. versicolor* at 20 °C. At 4 °C the observed specific activities were lower. In contrast, the laccase from *T. villosa* showed higher activities with these precipitants at 4 °C. With ethanol, acetone and dioxane higher activities were obtained at 20 °C. Similarly, the laccase from *A. bisporus* showed higher activities when DME, $(NH_4)_2SO_4$, dioxane and PEG 3400 were used as precipitant. Polyethylene imine (PEI) and polyethylene glycol polyacrylamide (PEGA) were also tested as precipitants but no aggregate formation was observed. Although laccases from *T. villosa* and *A. bisporus* gave better precipitation at lower temperatures, in comparison with the laccase from *T. versicolor*, it was decided to perform further preparations at 20 °C to streamline the precipitation procedure. The choice of the optimum precipitant is different for laccases from different sources. Nevertheless, a compromise between the best activities and ease of handling of the resulting laccase aggregates led to a choice of four precipitants –

Table 1
Selection of optimum precipitant[a].

Precipitant	*Trametes versicolor*				*Trametes villosa*				*Agaricus bisporus*			
	20 °C		8 °C		20 °C		4 °C		20 °C		4 °C	
	U/mg	P.F.	U/mg	P.F.	U/mg	P.F.	U/mg	P.F.	U/mg	P.F	U/mg	P.F.
Control, buffer	1.04	1	6.8	1	4.5	1	4.9	1	1.12	1	1.4	1
EtOH	2.06	2	2.3	0.3	5.6	1.2	3.2	0.7	0.47	0.4	1.5	1.
2-Propanol	16.5	17	9.4	1.4	1.8	0.4	6.4	1.3	0.4	0.7	1.2	0.9
Acetone	4.1	4	1.3	0.2	4.2	0.9	6.5	1.3	0.54	0.5	1.5	1.0
Acetonitrile	4.5	4	1.5	0.2	3.7	0.8	0.5	0.1	0.45	0.4	0.5	0.4
THF	7.6	7	1.5	0.2	–	–	0.9	0.2	–	–	0.5	0.4
Dioxane	11.1	11	8.1	1.2	5.2	1.2	2.9	0.6	0.58	0.5	1.9	1.4
DMF	–	–	–	–	0.4	0.1	0.3	0.1	–	–	–	–
PEG 1500	5.0	5	1.9	0.3	4.7	1.0	2.4	0.5	–	–	1.4	0.9
PEG 3400	17.6	17	5.5	0.8	–	–	2.5	0.5	1.2	1.1	2.8	1.9
$(NH_4)_2SO_4$	13.6	13	–	–	–	–	9.6	2.0	0.65	0.6	3.6	2.5
DME	7.4	7.1	2.1	0.3	6.4	1.4	2.7	0.6	1.9	1.7	3.6	2.6

[a] For conditions and procedure see Section 2.5; P.F.—purification factor.

2-propanol, acetone, dioxane and dimethoxyethane – for further investigation of the effect of pH.

3.2. The effect of pH on precipitation

Addition of water miscible solvents to an aqueous solution causes proteins to precipitate. The solvation layer around the protein decreases as a result of organic solvent gradually displacing water from the protein surface. The resulting protein molecules, which now contain less hydration layers, can aggregate by attractive electrostatic and dipole forces. The isoelectric point of proteins (p*I*) is the pH of a solution at which the net charge of the protein becomes zero. As a result of changes in pH the enzyme can be subjected to repulsive forces (both electrostatic and dispersive). When pH ≈ p*I* the electrostatic forces are reduced and dispersive forces dominate causing the protein to aggregate and precipitate. Although, the p*I* values for the laccases used are known and are in the range 4–6, we performed precipitation at pH values in the range 4–8 due to interest in the application of the CLEAs in processes where a higher pH is required. The control shows the activity of soluble enzyme, with no addition of precipitant, at pH 4, which corresponds to the conditions usually applied in oxidation reactions. The laccase solution was mixed with organic solvent in the v/v ratio 1:2.5 and stirred for 1 h leading to the formation of a precipitate in the form of pellets. After centrifugation and several washings with the buffer, the pellets were redissolved and activity was determined using the metol assay. The results are presented in Fig. 1. Good to excellent activity recoveries were observed with the laccase from *T. versicolor* with all four solvents at pH 4–7. A dramatic decrease in activity was observed at pH 8. The laccase from *T. villosa*, showed a rather similar pattern with good activity recoveries between pH 4 and 7. Interestingly, an excellent recovery was also observed with 2-propanol at pH 8. In the case of the *A. bisporus* laccase the activity of the precipitated enzyme on re-dissolution in aqueous buffer was substantially higher (300–350%) than that of the control, i.e. the original enzyme sample that it was derived from (see Fig. 1). A plausible explanation for this observation is that natural inhibitors or additives contained in the original enzyme sample are removed during precipitation. The *A. bisporus* laccase gave high activity recoveries at higher pH (pH 8), perhaps owing to it having more of the strongly basic Arg and Lys residues compared with the other two laccases. A second set of experiments revealed essentially the same results confirming that the precipitation behaviour of laccases from different sources is different.

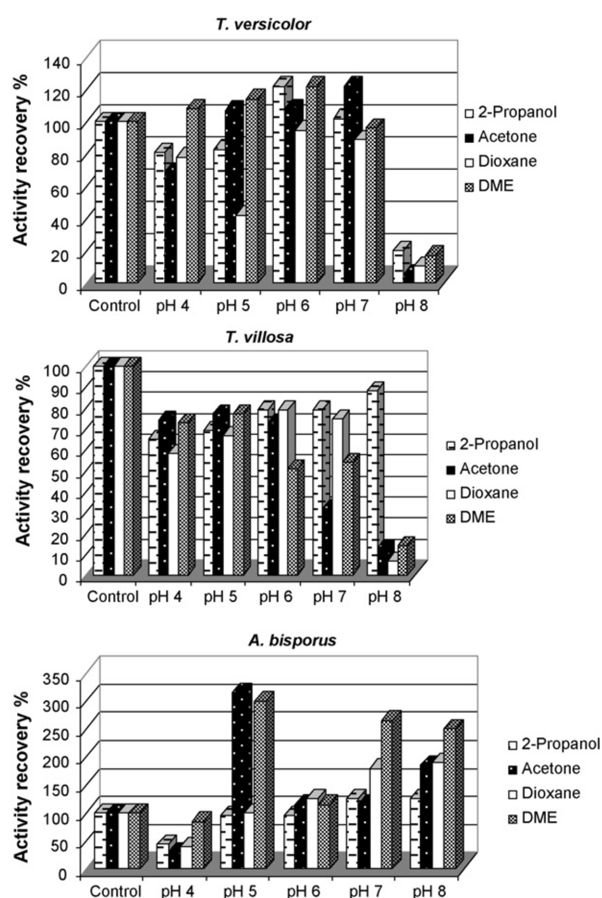


Fig. 1. Precipitation of laccases at different pH (the experiments were done in triplicate and the percentage error in each set of reading was within 5%).

3.3. Effect of glutaraldehyde concentration

A priori, one might expect that the precipitant affording the highest activity recovery will give the best CLEA. However, we note that the activity of redissolved aggregates provides only an indication for the best precipitant. The aggregates may contain the enzyme in a less favourable conformation which reverts to a favourable one on re-dissolution. On cross-linking, in contrast, the enzyme will be "locked" in this less favourable conformation. For this reason, the three best precipitants were included in further experiments. Glutaraldehyde is generally the cross-linking agent of choice as it is inexpensive and readily available in commercial quantities. It is worth mentioning, however, that not all enzymes give optimum results with glutaraldehyde as cross-linking agent [5,14,15]. Glutaraldehyde is a small, reactive molecule which could penetrate the internal structure of the protein and react with amino residues that are crucial for enzyme catalytic activity. In such a case other, more bulky cross-linkers can be used, such as dextran polyaldehyde. Optimization of the CLEA procedure also involves optimization of the glutaraldehyde/enzyme ratio. If too little cross-linker is used the enzyme molecule may still be too flexible while too much cross-linker can result in a loss of the minimum flexibility needed for the activity of enzyme. The influence of the amount of glutaraldehyde on the activity recovery is shown in Fig. 2 for laccases from the three different sources and three precipitants: DME, dioxane and 2-propanol. Laccases from *T. versicolor* and *T. villosa* were used at pH 6 with the exception of the latter in DME at pH 5. Laccase from *A. bisporus* was used at pH 7 except for DME at pH 5. The activity assay is based on catechol oxidation (see Section 2) in which the reaction is stopped by adding 10% sulfuric acid. After 1 h glutaraldehyde in the concentration range 2.4–40 mM was added and the mixture stirred for 24 h at 20 °C to complete the cross-linking process. Subsequently the laccase CLEAs were centrifuged, and washed with buffer. The washing procedure was repeated three times. When no activity was observed in the supernatant, the pellets were suspended in buffer and the recovered activity was measured. The best results for laccase from *T. versicolor* were obtained with DME and 2-propanol using 5 and 10 mM glutaraldehyde concentration, respectively (Fig. 2). For laccase from *T. villosa* the best activity recovery was found using 20 and 5 mM glutaraldehyde concentration with dioxane and DME, respectively. Surprisingly, the activity recovery for the laccase from *A. bisporus*, which has more lysine and arginine residues compared with the other two, was low: 40% activity recovery was observed with 10 mM glutaraldehyde in DME and 30% with 20 mM glutaraldehyde in dioxane. A possible explanation is that there is an optimum number of free amino groups on the surface of an enzyme. If there are too few there is not enough cross-linking and CLEA formation is not successful. If there are too many extensive cross-linking occurs and the enzyme loses the flexibility that is necessary for its activity. Similarly, for a given number

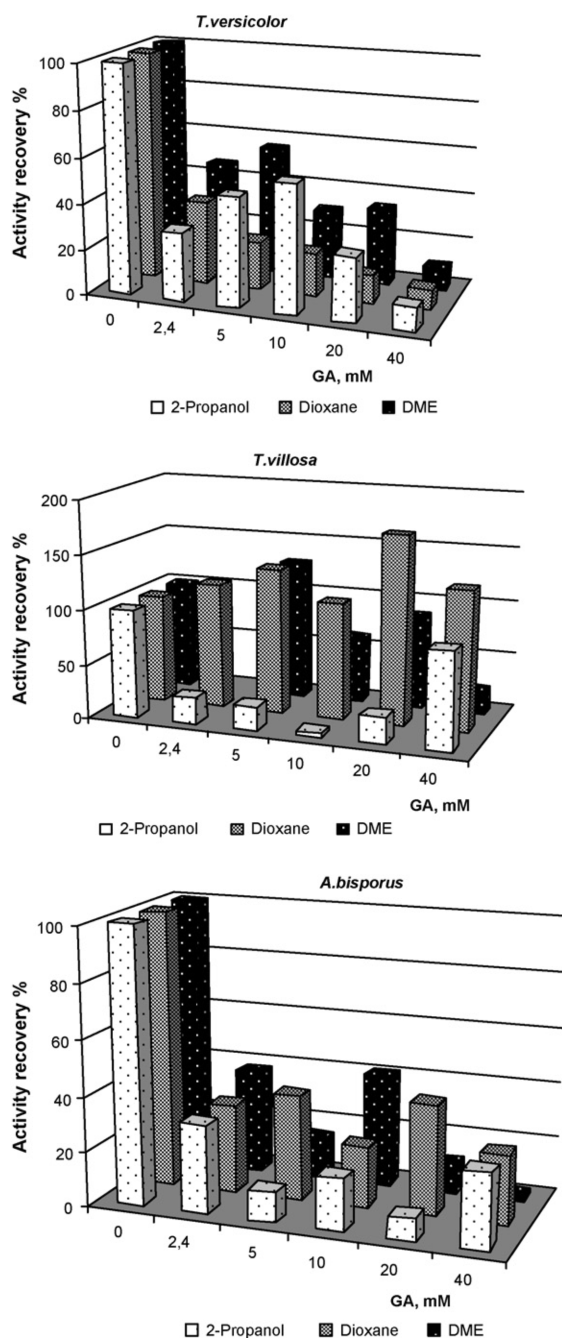


Fig. 2. Preparation of CLEAs with different glutaraldehyde (GA) concentration (the experiments were done in triplicate and the percentage error in each set of reading was within 5%).

of free amino groups one would expect there to be an optimum ratio of glutaraldehyde to enzyme and that higher ratios result in a loss of the minimum flexibility needed for activity. We conclude from the data that the concentration of glutaraldehyde is important, but that other factors such as the choice of precipitant and enzyme source are important too and can influence the performance of CLEAs.

3.4. Activity recovery and cross-linking time

The initial cross-linking experiments were performed over 24 h. The optimum time for cross-linking can involve a compromise between efficient cross-linking and enzyme stability during the procedure. Therefore, the cross-linking was performed from 1 to 27 h with the same three precipitants and laccases used in the above experiments. The leaching of enzyme was checked by determining the laccase activity in the supernatant. The pH and GA concentration in combination with a particular precipitant indicated in previous experiments were used. The data indicated that the cross-linking time for laccase from *T. versicolor* should not be shorter than 19 h (Table 2). In contrast, no leaching was observed for laccases from *T. villosa* and *A. bisporus* after 3 h of cross-linking when dioxane was the precipitant. However, activity recoveries, measured in catechol oxidation, were generally low, which could mean that the CLEA is subject to diffusion limitations in this rapid reaction.

Table 2
Activity recovery *vs* cross-linking time.

Source of laccase	Precipitant	Activity recovery %			
		1 h	3 h	19 h	27 h
T. versicolor	Dioxane	21	18	32	16
	2-Propanol	21	15	32	42
	DME	21	22	47	15
T. villosa	Dioxane	85	148	128	90
	2-Propanol	28	31	9	9
	DME	104	25	119	116
A. bisporus	Dioxane	14	31	23	172
	2-Propanol	9	24	24	21
	DME	16	20	11	38

For conditions and procedure see Section 2.5.

From Table 2 it can be concluded that the best activity recovery from laccase *T. versicolor* was obtained in 3 h using DME or 27 h using 2-propanol. The laccase from *T. villosa* gave the best results in dioxane after 3 h while with the laccase from *A. bisporus* the optimum recovery of 70% was reached after 27 h in dioxane. These results substantiate previous observations that the optimum conditions of precipitation and cross-linking (pH, precipitant, and concentration of precipitant, amount of cross-linking agent, cross-linking time, and temperature) are different for laccases from different sources.

3.5. Scale-up procedures for CLEA preparation

To test the validity of the parameters found in the small-scale CLEA preparations, we subjected the procedure to a 100-fold scale up. A compromise was made between the optimum activity recovery and easy handling of the formed aggregates. For the laccase from *T. versicolor* a 5 mM glutaraldehyde concentration was used with DME as precipitant and for laccase from *T. villosa* 20 mM of glutaraldehyde with dioxane. The laccase from *A. bisporus* was no longer commercially available at this point. Additionally, phenyl methyl sulfonyl fluoride (PMSF) was used as a protease inhibitor in the scale-up procedure. 58% activity recovery was obtained for laccase from *T. versicolor* when 100 μl of 200 mM PMSF was used and 22% activity recovery when PMSF was not used. Hence, we conclude that PMSF addition is very important. Surprisingly, for laccase from *T. villosa* the activity recovery was only 16%, while in a small-scale preparation procedure it gave better results in comparison with other laccases. It is also known that the addition of albumins like bovine serum or egg albumins which can form co-aggregates with the enzyme can form CLEAs with higher residual activity [16,17]. Therefore, we prepared CLEA samples of laccase with addition of egg albumin (see Section 2.8). However, contrary to expectations, the residual activities were even lower. In the case of laccase from *T. versicolor* 52% residual activity was obtained and even lower for laccase from *T. villosa* – 8%.

The storage stability of these CLEAs suspended in buffer, compared to the free enzyme, was determined at 5 °C (Fig. 3). The

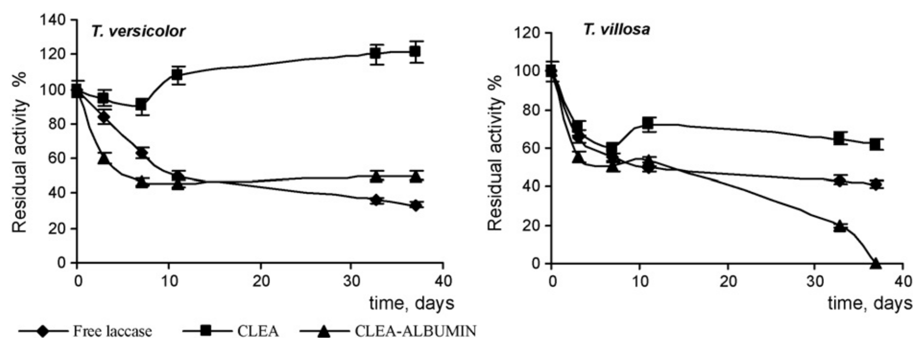


Fig. 3. Stability of laccase CLEAs at 5 °C.

activity of the CLEA just after preparation was taken as the initial 100% activity. The samples were suspended in buffer kept in the solution state, not under nitrogen. The results from Fig. 3 show that the activity of the free enzyme decreases dramatically in the first 10 days of storage. Almost 50% of initial activity is lost in 10 days with the laccase from *T. villosa*. The CLEAs show the expected increase in stability and the laccase CLEA from *T. versicolor* even showed an increase in activity on storage. The addition of albumin does not improve the stability of the resulting CLEA; the activity decreases in the same range as for free enzyme. The CLEA from *T. villosa* laccase showed an initial sharp decrease in activity, either with co-aggregation with albumin or without.

3.6. The performance and recyclability of laccase CLEAs in oxidation reactions

We subsequently studied the performance and recyclability of the *T. versicolor* laccase CLEA in aerobic oxidations of alcohols using TEMPO as a mediator. 1.6 mmol of 1-octanol was allowed to react with oxygen for 3 h in buffer solution at 30 °C in the presence of 50 U of laccase CLEA and 0.15 mmol TEMPO. The CLEA was recycled three times with a new portion of alcohol and mediator. In the first run 30% octanol conversion was obtained. The CLEA was separated from the reaction mixture by centrifugation, washed three times and used again in the second run. Surprisingly, after the second run the observed conversion was 10% higher (40%) in comparison with the first run. The conversions in the third and fourth run were in the range of 20–25%. These results indicate that the CLEA can be reused without appreciable loss of activity. The lower conversions obtained in runs 3 and 4 could be due to mechanical losses of CLEAs during the washing and centrifugation procedures. The oxidation of 1-octanol catalyzed by *T. versicolor* laccase/TEMPO was performed using 1 U/ml of free laccase and CLEA. The units for free enzyme and CLEA were determined by the catechol assay. The conversion after 8 h was 5% for the free enzyme and 45% for the CLEA (Fig. 4). These results were quite unexpected but could possibly be explained by instability of the free enzyme in the reaction mixture, especially at these low enzyme concentrations. Hence, we checked the activity of the free enzyme and the CLEA after the reaction. Indeed, a drastic activity decrease (almost 80% in first 2 h) was observed for the free enzyme, while for the CLEA only 20% of activity was lost (Fig. 4).

The long chain alcohols (1-hexanol, 1-heptanol, and 1-octanol) are more soluble in organic solvents but laccase prefers an aqueous environment. Initially, we presumed that the observed low conversions could be due to the formed biphasic system during the oxidation. Since we expected the laccase CLEAs to be more stable in organic solvents we tried to combine better solubility of aliphatic alcohols and CLEA stability in organic media. However, results obtained with *T. versicolor* laccase CLEA in the oxidation of 1-hexanol, 1-heptanol and 1-octanol in biphasic systems consisting of toluene or ethyl acetate and aqueous buffer (1:1) were disappointing. Less than 6% conversion was observed in 6 h. Similarly, experiments using water miscible organic co-solvents such as dioxane and acetonitrile (1% of organic solvent from total reaction volume) also gave disappointing results: 1-hexanol and 1-octanol conversions of ca. 15% in 4 h compared to 30–35% in aqueous buffer. Based on these results we decided to perform further reactions in buffer.

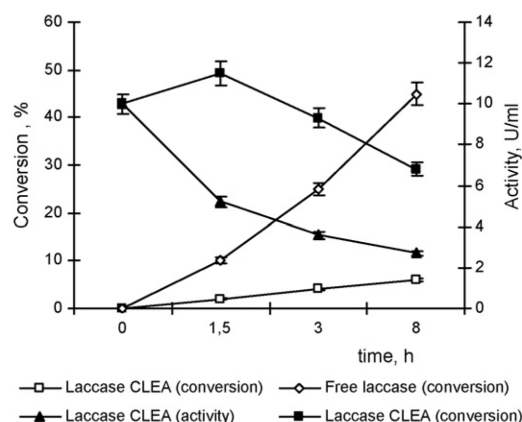


Fig. 4. Activity and conversion profile of free enzyme and CLEA from *Trametes versicolor* during 1-octanol oxidation reaction.

The oxidation of a range of aliphatic alcohols was performed with 10 U of CLEA, 0.15 mmol of TEMPO and 1.6 mmol of alcohol substrate in a total reaction volume of 10 ml of buffer (see Table 3). As expected the oxidation of a benzyl alcohol was faster (80% conversion in 5 h). The oxidation of the aliphatic alcohols was significantly slower. Nonetheless, 1-heptanol, for example, underwent 55% conversion in 20 h using just 1 U/ml of laccase CLEA compared to the 10 U/ml of free laccase needed for the same conversion. The differences observed with aliphatic alcohols of varying chain length can probably be explained by considering that the rate is influenced by both the size and hydrophobicity of the aliphatic

Table 3
Oxidation of aliphatic alcohols by CLEA of laccase from *T. versicolor*.

Alcohol	Reaction time	Conversion %
Benzyl alcohol		81
1-Pentanol	5 h	7
1-Hexanol		15
1-Heptanol		55
1-Octanol	20 h	34
1-Nonanol		13
1-Decanol		21

Reaction conditions: 1.6 mmol alcohol, 0.15 mmol TEMPO, 10 U of CLEA (see Section 2.9).

alcohols. Moreover, the observed good storage stability and recyclability give promising expectations for further applications.

4. Conclusions

CLEAs were prepared from three sources of laccase, *T. versicolor*, *T. villosa* and *A. bisporus*, and the effects of various parameters – the nature of the precipitant, pH, temperature, glutaraldehyde concentration, and cross-linking time – on the activity and the storage and operational stability of the resulting CLEAs were investigated. The CLEAs exhibited the expected increase in stability compared to the free enzyme and the laccase CLEA from *T. versicolor* even showed an increase in activity on storage. The addition of albumin did not improve the stability of the resulting CLEA.

As would be expected, the optimum conditions for precipitation and cross-linking were different for laccases from different sources. We could not draw any definite conclusions regarding the influence of the numbers of surface lysine and arginine residues on the activity recovery in CLEA formation and the storage and operational stability of the resulting CLEA. It is clearly not the only factor influencing the properties of the CLEA.

The aerobic oxidation of a range of linear aliphatic alcohols in the presence of the laccase CLEA and TEMPO, in aqueous buffer (pH 4), produced high conversions to the corresponding aldehydes using just 1 U/ml of laccase CLEA. In contrast, 10 U/ml of the free enzyme was needed to obtain similar conversions in the same time. Moreover, the results of recycling experiments demonstrated that the CLEAs can be reused several times without appreciable loss of activity. Hence, we conclude that laccase CLEAs clearly have practical utility in the aerobic oxidation of primary alcohols to the corresponding aldehydes. The addition of water immiscible or water miscible solvents showed no improvement in rate compared with reactions in aqueous buffer alone.

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