Supporting information

Oxidation of phenolic compounds catalyzed by immobilized multi-enzyme systems with integrated hydrogen peroxide production.

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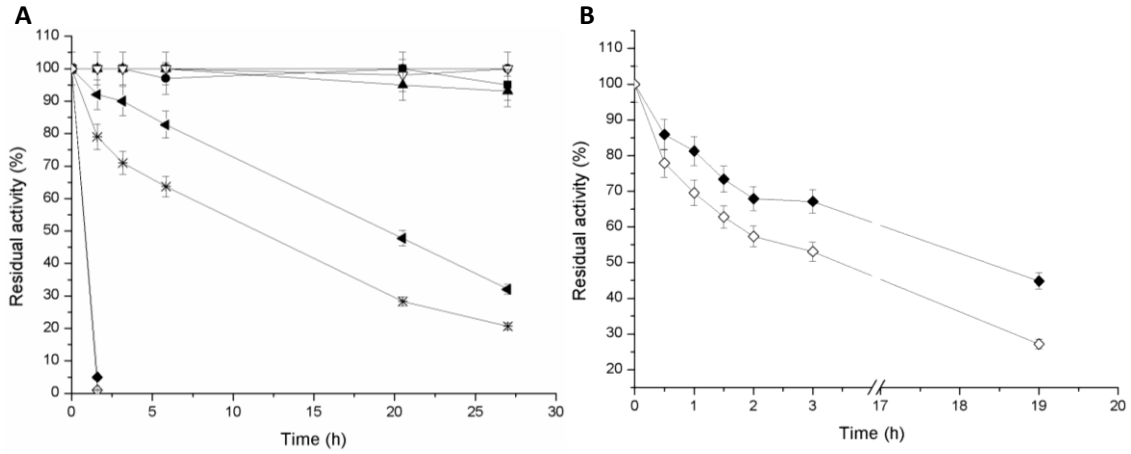
Stability of different immobilized preparation of the multi-enzyme system

Different immobilized preparations of NOX, FDH and HRP were thermally inactivated under different temperatures and compared to their soluble counterparts (Figure S1). Soluble and solid enzyme preparations dissolved in 0.1 M sodium phosphate at pH 6 were incubated at different temperatures. Samples were withdrawn at different times and then NOX, FDH and HRP activity were spectrophotometrically analyzed.

As expected, immobilized enzymes were more stable than soluble ones except for HRP where both immobilized and soluble preparation showed similar thermal stabilities. Figure S1 shows that NOX and FDH immobilized preparations were far more stable than HRP preparation, suggesting that production of H_2O_2 will not be the limiting step of the cascade reaction. Moreover, thermal stability of both NOX and FDH was exactly the same when they were co-immobilized or separately immobilized. Immobilized preparations of NOX retained 100% of their initial activity after 27 hours at 60 ºC and pH 6, while soluble NOX lost more than 60% of its activity after the same time period. Similar results were found with FDH the soluble form of which lost more than 70% activity after 27 hours under the mentioned conditions, while the immobilization preparation retained 100% of its initial activity under the same incubation period. In contrast, for HRP the immobilization chemistry was not optimal in terms of thermal stability, but it was in terms of residual activity after the immobilization protocol.

we have demonstrated that co-immobilizing enzymes stabilizes them, as well as when both enzyme are separately immobilized on two different carriers, but only when the immobilization chemistry is exactly the same in both co-immobilization and individual immobilization protocol.

Figure S1. Inactivation courses of different preparations. (A) 60 ºC and pH 6. (B) 40ºC and pH 6. Symbols: (◆) Soluble HRP; (◇) HRP immobilized on Ag-B; (●) activity FDH co-immobilized with NOX on Ag-G; (■) activity NOX co-immobilized with FDH on Ag-G; (▲) NOX immobilized on Ag-G; (▽) FDH immobilized on Ag-G; (◂) Soluble NOX; (✳) Soluble FDH. Experiments carried out with 1.4 U of NOX, 2.8 U of FDH and 5.6 U of HRP in a total volume of 5 mL. Each preparation was incubated in 25 mM sodium phosphate buffer pH 6 at temperature indicated



Effect of NADH on HRP activity.

Higher NADH concentrations decreased the phenol removal efficiency in terms of both rate and yield. This negative effect would be dramatically enhanced when NADH would be clearly in excess relative to H_2O_2. This negative effect triggered by NADH needs to be further investigated in order to shed light on this unexpected effect, we measured the HRP activity in the presence of different concentrations of NADH and H_2O_2 (Figure S2). At high H_2O_2 concentration, NADH had no effect on HRP activity at any concentration. Nevertheless, when the H_2O_2 concentration was very low (80 µM) -as would be the scenario for *in situ* H_2O_2 production systems- HRP activity decreased in the presence of high NADH concentrations. These data may suggest a competition between H_2O_2 and NADH that would decrease the oxidative activity of HRP. This negative effect would be dramatically enhanced when NADH would be clearly in excess relative to H_2O_2

Figures S2. Effect of NADH concentration on the immobilized HRP activity under different concetration of co-substrate (H_2O_2). The activity was measured by oxidation of ABTS as substrate. Reactions were triggered by the addition of HRP immobilized on Ag-B.