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A novel immobilized chloroperoxidase biocatalyst with improved stability for the oxidation of amino alcohols to amino aldehydes

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ABSTRACT

Chloroperoxidase from *Caldariomyces fumago* (CPO, EC 1.11.1.10) is one of the most promising of the heme peroxidase enzymes for synthetic applications. Since the synthetic use of CPO suffers severely from its rapid deactivation in the presence of peroxides, the immobilization of this enzyme was studied as a possibility for stability improvement. Three methods of immobilization were considered using monoaminoethyl-N-aminoethyl (MANA) agarose gels: ionic adsorption, covalent attachment via carbodiimide coupled activation and covalent attachment of oxidized CPO. The most successful results led to almost complete immobilization with retained activities of around 51% for the two methods of covalent attachment and 77% for the ionic adsorption of CPO on MANA. Besides, all of the immobilized enzyme systems showed drastically improved stability toward presence of peroxide; CPO immobilized on MANA through carbodiimide coupled method resulted to be the most stable one with an increase in apparent half-life time of more than 500-fold that of the soluble enzyme. CPO immobilized by this method was compared to the soluble enzyme as catalyst for Cbz-ethanolamine oxidation to Cbz-glycinal using *tert*-butyl hydroperoxide (t-BuOOH) as an oxidant. Despite the lower reaction rate, the reaction catalyzed by immobilized CPO reached higher Cbz-glycinal yield with almost three-fold lower activity loss.

1. Introduction

Immobilization of enzymes has been developed rapidly in the past 40 years since its benefits were evaluated and utilized with success in biotechnological, medical and analytical applications. The chemical, biochemical, mechanical and kinetic properties of the immobilized enzyme depend both on the properties of the enzyme and the support used for immobilization, while the apparent activity retention upon the process depends mostly on the support characteristics, the enzyme-support interaction and the amount of soluble enzyme used. The measured enzymatic activity after immobilization may be reduced by the binding procedure, and also by mass transfer effect as a result of diminished availability of enzyme molecules within pores or from slowly diffusing substrate molecules. Nevertheless, this drawback can be compensated by a stability improvement of the immobilized enzymes under working conditions compared to the soluble enzyme, resulting in overall benefit [1,2].

The methods of immobilization via carboxyl groups on the protein surface may be very suitable since the aspartic and glutamic acids usually constitute one of the major fractions of surface groups on proteins [3]. Monoaminoethyl-N-aminoethyl (MANA) agarose gels containing very low p*K* primary amino groups are very suitable supports for immobilization of enzymes at low pH values [3]. The method proposed consists in ionic adsorption of the enzymes to the support in a low ionic-strength media. Further, by applying a mild coupling method for the activation of carboxyl groups of the enzyme using carbodiimide, ionic adsorption is transformed to covalent attachment.

Another possibility for the successful and simple application of these primary amino supports is the immobilization of glycoproteins via their sugar moieties, previously oxidized with periodate resulting in a high density of aldehyde groups. These aldehydes can further react with amino groups from the resins, yielding Schiff base bonds [4,5].

Haloperoxidases are peroxidases capable to halogenate substrates in the presence of halide and peroxide [6,7]. Heme-thiolate haloperoxidases are the most versatile biocatalysts of the heme-protein family and for a long time, only one enzyme of this type – the chloroperoxidase (CPO; EC 1.11.1.10.) from the ascomycete *Caldariomyces fumago* – was known [8,9]. CPO was firstly isolated and characterized over 40 years ago [10]. Molecular weight of this enzyme is 42,000 Da. CPO is rich in aspartic acid, glutamic acid, serine and proline; these four amino acids constitute about 45%

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of the total amino acid content. It has 321 amino acids with predominantly acidic residues and a pI in a range of 3.2–4.0. CPO is a glycoprotein; 25–30% of the molecule is carbohydrate, in which major constituents are glucosamine and arabinose.

CPO uses peroxides as electron acceptors and does not require any cofactor for its activity. Nevertheless, a main shortcoming of CPO and all heme-containing peroxidases is their low operational stability, which is caused by the rapid deactivation by peroxides, despite its catalase activity. Deactivation involves destruction of the heme group, but its precise mechanism is not clear yet [11].

CPO exists in two active forms: the acidic form (pH < 3) which catalyses halide-dependent reactions, and the neutral form (pH 5–6) which catalyses halide-independent reactions [7,12,13]. Within the group of halide-independent reactions CPO has been demonstrated to be able to catalyze the oxidation of primary alcohols to the corresponding aldehydes [14,15]. Among these compounds, amino aldehydes have special interest as they can act as substrates of aldolases in the aldol addition of dihydroxyacetone phosphate (DHAP), obtaining aminopolyols. In turn, these aminopolyols can be directly cyclated or isomerized to obtain iminocyclitols, which are inhibitors of glycosidases and glycosyltransferases with a great therapeutic potential [16].

Several methods for CPO immobilization were reported. Immobilization of CPO on silica-based materials was demonstrated to increase its pH, oxidizing agent and temperature tolerance [17–19]. Besides, CPO was immobilized on mesoporous materials by adsorption [20,21] or covalent attachment [22,23], on aminopropyl-glass beads [24], talc [25] and celite [26] by adsorption, and on magnetic beads [27] and chitosan membranes [28] by covalent attachment. Generally, the main disadvantage of all the adsorption methods is the leaching of the enzyme from the support. All of the reported methods based on covalent attachment involved the formation of the bonds between various functional groups on the support surface and amino groups from the lysine residues of CPO. The presence of only four lysine residues in the CPO amino acid sequence is the most probable reason for the relatively low immobilization yields achieved using this kind of supports.

In the present work, CPO was proposed to be immobilized on MANA-gels applying three different methods: ionic adsorption, covalent attachment using carbodiimide and covalent attachment of oxidized enzyme. Application of these immobilization methods allowed the formation of covalent bonds between the amino groups of the support and either the most abundant amino acid residues of CPO (Asp and Glu) or its sugar moiety. Furthermore, reaction of oxidation of amino alcohols catalyzed by immobilized and soluble CPO was performed. Since it was previously shown that branched-chain alcohols were not substrates for CPO [14], ethanolamine containing a protective carbobenzoxy (Cbz) group was selected as substrate for the synthesis of the corresponding protected amino aldehyde Cbz-glycinal.

2. Materials and methods

2.1. Materials

CPO from *C. fumago* was obtained from Chirazyme Labs (Greenville, NC, USA) as a solution of partially purified enzyme (11.6 mg protein/mL), with a specific activity of 1400 U/mg. Agarose 10% crosslinked beads (10 BCL) was purchased from Iberagar (Coina, Portugal). Monochlorodimedone (1,1-dimethyl-4-chloro-3,5-cyclohexanedione), Cbz-ethanolamine (benzyl-N-(2-hydroxyethyl)-carbamate), Cbz-glycine, tert-butyl hydroperoxide (70% (w/w) in water) and N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Cbz-glycinal was obtained from Sunshine Chemlab, Inc (Downingtown, PA, USA). All the other reagents and solvents used were obtained from diverse commercial suppliers and were of the highest available purity and of analytical grade.

2.2. Chloroperoxidase activity assay

The spectroscopic assay for the measurement of CPO activity was based on the decrease in absorbance of a solution of monochlorodimedone, MCD ($\varepsilon_{278\,nm}$ = 12,200 M^{-1} cm^{-1}) as it was converted to dichlorodimedone, DCD ($\varepsilon_{278\,nm}$ = 120 M^{-1} cm^{-1}), by CPO according to the method described by Hager et al. [29]. The assay mixture contained 100 mM potassium phosphate buffer pH 2.75, 20 mM KCl, 2 mM hydrogen peroxide and 0.16 mM MCD. The absorbance was measured at 25 °C at a wavelength of 278 nm in UV-Visible Spectrophotometer Cary 50 (Varian, Palo Alto, CA, USA) using quartz cuvettes. One unit of CPO was defined as the enzyme activity required to catalyze the conversion of 1 μmol of MCD to DCD per minute at 25 °C. In order to measure the immobilized enzyme activity in the suspension samples, magnetic stirring was applied to the spectrophotometric cuvette. No interference of the resin was observed in the assay.

2.3. Stability of soluble CPO

Effects of pH on CPO stability were studied over the pH range 5.0–10.0. The reaction media (10 mL of selected buffer – sodium phosphate or bicarbonate, depending on the pH – in a concentration of 50 mM) containing 20 U of enzyme were incubated at room temperature on a roller. Effect of sodium cyanoborohydride on CPO stability was analyzed using molar excesses of 1:10, 1:100, 1:1000 and 1:10,000 (CPO:cyanoborohydride). The reaction media containing 2 U/mL of enzyme and 100 mM phosphate buffer pH 6.0 were incubated at 4 °C in a roller. In both cases, after different times of incubation aliquots of enzyme solution were withdrawn and enzymatic activity was measured using the previously described assay.

2.4. Preparation of MANA-agarose support

The amino-agarose support was prepared from glyoxyl-agarose gels as previously described by Fernandez-Lafuente et al. [3]. Glyoxyl-agarose gels were prepared by etherification of 10% crosslinked agarose gels with glycidol, and further periodate oxidation of the resulting glyceril-agarose [30]. For that, 15 mL of agarose gels were washed thoroughly with distilled water and the gel was suspended in 11.6 mL of a solution that contained NaOH, $NaBH_4$ and glycidol, in final concentrations of 0.32 N, 5.38 mg/mL and 2.04 M, respectively. The reaction was left on mild agitation in the rotary evaporator for approximately 19 h at room temperature (20–25 °C). Once the reaction was finished, agarose was vacuum-filtered and washed with distilled water (around 10 successive washings) in order to eliminate the rest of reagents. The next step was the oxidation of the resulting glyceryl-agarose with an excess of sodium periodate (300 μmol $NaIO_4$/mL agarose), during approximately 30–45 min. The formation of aldehyde groups was calculated from the consumed $NaIO_4$, which was evaluated by colorimetry, detecting the iodine liberated from KI by oxidation with remained $NaIO_4$ in the presence of $NaHCO_3$. The values of absorbances were measured using Spectrophotometer Cary 50 (Varian, Palo Alto, CA, USA). This support presented 264 μmol of aldehyde groups per mL of agarose. After the oxidation, glyoxyl-agarose was vacuum-filtered and washed with distilled water.

Then, 15 g of glyoxyl-agarose were suspended in 135 mL of 0.1 M bicarbonate buffer at pH 10.0 with 2 M ethylenediamine. The suspension was gently stirred on a roller for 2 h. Finally, 1.5 g of $NaBH_4$