Journal of Molecular Catalysis B: Enzymatic 133 (2016) 107–116

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Diffusion effects of bovine serum albumin on cross-linked aggregates of catalase

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ARTICLE INFO

Article history:
Received 5 May 2016
Received in revised form 20 July 2016
Accepted 5 August 2016
Available online 6 August 2016

Keywords:
Catalase
CLEA
BSA
Kinetic model
Diffusional delay

ABSTRACT

Stabilization of multimeric enzymes is one of the major challenges in biocatalysis since dissociation of subunits can inactivate the enzyme. Particularly, catalase that breaks down hydrogen peroxide in water and molecular oxygen is an enzyme difficult to stabilize by conventional immobilization techniques, because it is a tetrameric structure containing Fe-protoporphyrin IX in its active site. Cross-linking of enzyme aggregates is a methodology that can overcome this bottleneck, but diffusional delay of mass transport within the particles is a recurrent drawback. In this work, cross-linked aggregates of catalase from bovine liver were prepared, evaluating the influence of precipitant and cross-linking agents, as well as bovine serum albumin (BSA) as feeder protein on the catalytic properties, thermal stability, and mass transport resistance of the derivatives. The most active derivatives were prepared using ammonium sulfate as precipitant agent, 50 mM glutaraldehyde as cross-linker, and mass ratio BSA/catalase of 3.0. These derivatives in the absence of diffusive effects showed recovered activity of 98 ± 1.7% and high stability at 40 °C and pH 7.0 (~80% of the initial activity was recovery after 200 h under these conditions). The co-precipitation of BSA together with catalase reduced the size of clusters suggesting a decrease of diffusive effects within the biocatalyst. Empirical kinetic model was fitted to the experimental data of initial rate vs. substrate concentration and used to make a comparative analysis of mass transfer into derivatives with and without BSA. Results suggested that the main effect that differentiates the free enzyme and the two derivatives analyzed was of diffusive nature. In fact, the effectiveness factor of the cross-linked aggregates of catalase with BSA increased around 4 times. Statistical design of experiments and the analysis of the response surface methodology showed that the immobilization did not alter the conditions of maximum activity of the catalase, which were found to be 30 °C and pH ~7.0 for all biocatalysts.

1. Introduction

Catalase (CAT; EC 1.11.16) is a homotetramer containing Fe-protoporphyrin IX in its active site [1], which is normally obtained from bovine liver or from microbial sources. CAT from bovine liver has a molecular mass of 250 kDa and each subunit has a molecular weight above 65 kDa [2].

CAT catalyzes the hydrogen peroxide (H_2O_2) decomposition through Bi–Bi Ping-Pong mechanism [3–5]. The reaction of CAT with H_2O_2 follows two steps. In the first step of the reaction, a molecule of H_2O_2 oxidizes the ion Fe^{3+} in the prosthetic group, with the condensation of one molecule of water. In the second step, a second molecule of H_2O_2 reduces the prosthetic group, which was oxidized in the first step (O–Fe^{4+}), generating Fe^{3+} and releasing H_2O and O_2 [3–5].

Commercially, CAT is used to remove H_2O_2 from milk before cheese processes [6]. It can also be found in disinfectants and food containers to prevent oxidation [6], keeping the food fresh for longer periods of time, and as an oxygenator for skin rejuvenation [7].

Enzymes play an important role in industrial chemical reactions. However, low operational stability and high costs may limit their

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http://dx.doi.org/10.1016/j.molcatb.2016.08.002
1381-1177/

usage in some processes. In the case of multimeric enzymes, as CAT, dissociation of the subunits often leads to enzyme inactivation and product contamination. Thus, stabilization of the quaternary structure of the enzyme is necessary [8,9].

An alternative to improve the performance of enzymes in industrial processes is the enzyme immobilization [10]. Adsorption, encapsulation, entrapment and covalent binding on insoluble carriers [11–16] have been used to immobilize CAT. However, having four subunits to stabilize, CAT immobilization using these methods has shown troublesome [8]. On the other hand, CLEAs methodology showed to be efficient to stabilize its quaternnary structure [8].

CLEAs methodology [17–19] was developed by Sheldon et al. [20] and consists in precipitating the enzyme from an aqueous solution then linking the enzymes together. First, a precipitant agent (e.g., salts, water-miscible organic solvents or non-ionic polymers) is added to an aqueous solution containing proteins. The proteins are precipitated as physical aggregates, and to keep the aggregates insoluble once the precipitant is removed, cross-linking is performed between the adjacent proteins in the CLEAs supramolecular structure [21]. Glutaraldehyde is often chosen as cross-linking agent since it is available commercially at lower prices.

One of the main advantages of the CLEAs, compared to immobilization on insoluble carriers, is its high volumetric activity. Unfortunately, high volumetric activity can also cause severe mass transfer limitations [17–19,22,23]. Talekar et al. [18] proposed a porous CLEA where starch was co-aggregated with the enzyme. The starch was subsequently removed by α-amylase action, resulting in large pores in the CLEA structure. This technique however (using an additional enzyme), may significantly increase the costs of the biocatalyst preparation. Bovine serum albumin (BSA) has also been used to dilute the enzyme in the CLEA, reducing its volumetric activity. Tukel et al. [24] and Shah et al. [25] showed that the addition of BSA could increase the recovery activity of CLEAs, which may be an evidence of a reduction on diffusional resistance. On the other hand, Cabana et al. [19] obtained low activity for CLEAs of laccase (EC 1.10.3.2) when BSA was added. The authors attributed the activity reduction to diffusional problems. The role that BSA plays in CLEAs may be of several natures. BSA may dilute the enzyme (reducing diffusional resistance) or make the biocatalyst denser (increasing diffusional effects). Free amino groups of the BSA compete with the amino groups of the enzyme reducing enzyme cross-linking. This competition may avoid excessive cross-linking of the enzyme reducing enzyme denaturation. On the other hand, it may prevent the necessary cross-linking of the molecules of enzymes.

The aim of this work was to evaluate and optimize the preparation of CLEAs of catalase from bovine liver (henceforth CAT-CLEAs). First, the effects of different precipitant agents (organic and inorganic salts) and cross-linking agent concentration (Glutaraldehyde) were evaluated on the recovered activity of CAT-CLEAs. Then, the amount of a feeder protein (BSA) was also evaluated. Soluble CAT and the best CAT-CLEAs without and with BSA (henceforth CAT-BSA-CLEAs) were characterized in terms of pH and temperature by using response surface methodology. The thermostability of the enzyme at 40 °C was measured. Empirical models were proposed to describe the kinetic of free and immobilized CAT until complete inactivation in the presence of high concentration of substrate. Finally, generalized Thiele modulus was used to verify if diffusional limitations could explain the behavior of the biocatalysts.

2. Material and methods

2.1. Material

Bovine liver catalase (CAT; EC:1.11.1.6) (30 U/mg protein) and bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO). For CAT-CLEAs and CAT-BSA-CLEAs syntheses and for the evaluation of their properties, dimethoxyethane (DME) (Fluka), *tert*-butyl alcohol (TBA) (Vetec, Brazil), acetone (A) (Vetec, Brazil), polyethylene glycol 600 (PEG 600) (Synth, Brazil), ammonium sulfate salt (AS) (Vetec, Brazil), glutaraldehyde 25% (w/w) aqueous solution (Vetec, Brazil) and hydrogen peroxide (H_2O_2) (Sinth) were used.

2.2. CAT activity assay

Activities of soluble CAT and CAT-CLEAs were determined spectrophotometrically at 240 nm, following the decomposition of a H_2O_2 solution (0.35 mM H_2O_2 in 0.05 M phosphate buffer, pH 7.5) at 25 °C. Briefly, 10 μL of a solution of CAT or suspension of CAT-CLEAs were added to 25 mL of substrate in a stirred and thermostatically controlled reactor. Samples of 1 mL at intervals of 1 min were withdrawn (free of solids) up to 3 min of reaction for reading the absorbance at 240 nm. The initial rate of the peroxide decomposition was calculated from the slope of the curve absorbance versus time, where absorbance was converted to H_2O_2 concentration from a standard curve. One CAT unit was defined as the amount of enzyme that catalyzes the decomposition of 1 μmol of H_2O_2 per minute.

The low substrate concentration used in this work to evaluate CAT activity was chosen to maintain a low reaction rate. High reaction rates would lead to an accumulation of bubbles inside the biocatalyst, such as reported by Ozilmaz et al. [12]. Presence of bubbles inside the biocatalyst could impair the analysis of the diffusive effects inside the particles because of the complexity of the phenomenon. However, the reaction rate is very sensitive to substrate concentration, when its value is low. Therefore, during the activity measurements, extreme care was taken to assure that the rate remained constant, at initial velocity. This was accomplished by assuring a linear increase of absorbance in time and low consumption (∼5%) of H_2O_2.

2.3. Protein concentration

Lowry method was used to measure protein concentration in the enzyme preparations [26]. The protein content was spectrophotometrically quantified at 750 nm using BSA as standard protein.

2.4. Preparation of CAT-CLEAs and CAT-BSA-CLEAs

40 mg or 20 mg of CAT powder was dissolved in 1 mL of sodium phosphate buffer (100 mM, pH 7.0). Protein aggregation was induced by mixing 1 mL of the enzymatic solution and 1 mL of precipitant (saturate solution of ammonium sulfate, *tert*-butyl alcohol, polyethylene glycol, dimethoxyethane or acetone). After 1 min of mixing, glutaraldehyde was slowly added to the final concentration of 0, 25, 50, 100 or 200 mM.

For the CAT-BSA-CLEAs preparation, BSA (20, 40, and 60 mg) was added as a feeder protein into the enzyme solution. Then, the same protocol was used in precipitation and cross-linking. However, in this case, only saturated ammonium sulfate was used as precipitant and glutaraldehyde was added only to the final concentration of 50 mM. All the reactions were performed at 4 °C.

After 3 h of cross-linking at 4 °C, the biocatalyst solution was centrifuged at 10,000 × *g* for 10 min at 4 °C. CLEAs were recovered as pellets and washed twice with 100 mM sodium phosphate buffer (pH 7.0). After preparation, the enzyme was stored in the same buffer (2 mL) at 4 °C at concentrations of 20 mg/mL and 10 mg/mL.