CROSS-LINKED ENZYME AGGREGATES OF CATALASE FROM BOVINE LIVER

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ABSTRACT – Stabilization of multimeric enzymes is one of the major challenges in biocatalysis, because dissociation of subunits can inactivate the enzyme. Catalase (CAT; EC 1.11.16) is a homotetramer containing Fe-protoporphyrin IX in its active site. CAT breaks down hydrogen peroxide into water and molecular oxygen. In this study, cross-linked enzyme aggregates of bovine liver CAT (CAT-CLEAs) were prepared. The effects of precipitation and cross-linking on enzyme activity were studied. Thermal stability of free and immobilized enzyme were also evaluated at 40 °C and pH 7 (200 h). CAT-CLEAs were successfully prepared using ammonium sulfate and glutaraldehyde (50 mM) as the precipitant and cross-linking agent, respectively. The best recovered activity obtained was 62 %. The derivative retained high activity along the stability test. The kinetic parameters values v_{max} and K_m were estimated as 11,350 U/mg and 66.7±10 mM for the free CAT and 2,000 U/mg and 392±22 mM for the CAT-CLEAs, respectively.

1. INTRODUCTION

Enzymes are proteins with catalytic functions, which play an important role in various biological and industrial chemical reactions. Enzymes are used in chemical conversions, biosensing, and bioremediation (Fagain, 2003). The great interest in enzymes by the industry is due to several factors. They act on a large variety of substrates and catalyzes a variety of complex reactions, on routes where the generation of waste and by-products is reduced (Brady and Jordaan, 2000). However, its use in some processes is often limited by its low operational stability and high cost. In the case of multimeric enzymes, where dissociation of the subunits often leads to enzyme inactivation and product contamination, it is necessary to stabilize its quaternary structure (Wilson *et al*., 2004; Fernandez-Lafuente, 2009).

Catalase (CAT; EC 1.11.16) is a homotetramer containing Fe-protoporphyrin IX in its active site (Fita and Rossman, 1985). CAT is usually 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 (Kiseler *et al.*, 1967). CAT catalyzes the H_2O_2 (hydrogen peroxide) decomposition through Bi-Bi Ping-Pong mechanism. 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 ($O\text{-}Fe^{4+}$), generating Fe^{3+} and releasing H_2O and O_2 (Scandalios *et al*., 1997; Switala and Loewen, 2002; Adányi *et al.*, 2007).

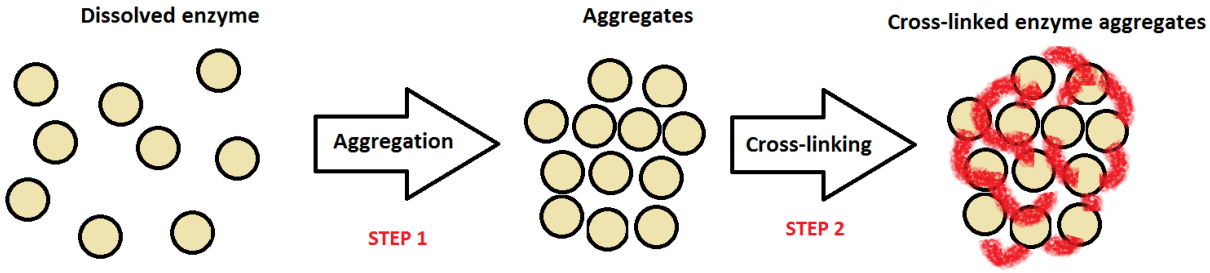

Figure 1 – Schematic representation of the preparation of cross-linked enzyme aggregates. In the first step, the soluble enzyme is aggregated by the action of a precipitant agent. In second step occurs the cross-linking by the addition of a cross-linker to the reaction medium

During the early 1960s, Doscher and Richards (1963) studied the cross-linking of dissolved enzymes through the reaction of amino groups with a bifunctional cross-linker, like glutaraldehyde, generating the cross-liked enzymes (CLE). However, CLE showed several drawbacks like poor reproducibility, low mechanical stability, low recovered activity, low volumetric activity and difficulties in handling. In a subsequent study, Quiocho and Richards (1964) investigated the cross-linking of a crystallized enzyme obtaining the cross-liked enzyme crystals (CLEC). Nevertheless, an enzyme to crystalize need to be highly pure, which greatly increases the process cost. Thereafter, Cao *et al.* (2000) developed a new way to immobilize enzymes, the cross-linked enzyme aggregates (CLEAs) (Schoevaart *et al.*, 2004; Sheldon, 2007a), showed in Figure 1.

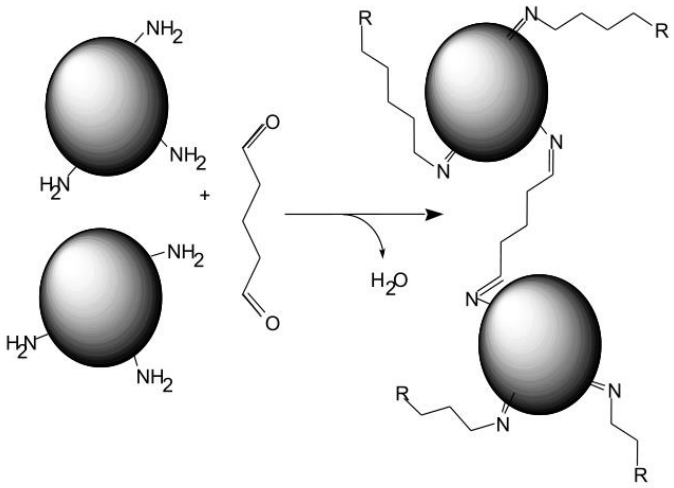

Figure 2 – Schiff base formation from the reaction of amino group of enzymes and carbonyl group of glutaraldehyde, during the cross-linking of aggregates

This methodology consists in the addition of salts, water-miscible organic solvents or non-ionic polymers to an aqueous solution containing proteins. The proteins are precipitate as physical aggregates, without denaturation or perturbation of the tertiary structure. In order to keep the aggregates physically linked is necessary to perform the cross-linking (Cao *et al.*, 2000).

Glutaraldehyde is often used as low cost cross-linking agent. The cross-linking reaction occurs with a Schiff base formation between the free amino groups of lysine residues on the enzyme and the carbonyl group of glutaraldehyde, as showed in Figure 2. The concentration of the cross-linking agent is a sensitive parameter because an excessive concentration of the cross-linker can lead to the enzyme inactivation and a low concentration may result in an inefficient cross-linking (Schoevaart *et al*., 2004; Sheldon *et al*., 2007b).

Adsorption, encapsulation, entrapment and covalent binding (Kondo *et al*., 1993; Alptekin *et al*., 2008, 2009 and 2010; Itoh *et al*., 2009) have been used to immobilize CAT. However, Wilson *et al*. (2004) reported that these methods are difficult to stabilize the CAT quaternary structure. The authors have showed that the CLEAs methodology can stabilize CAT from bovine liver and from *Micrococcus lysodeikticus*.

In this context, the aim of this work was to investigate and optimize the preparation of CAT-CLEAs from bovine liver. Different precipitation (organic solvents and inorganic salts) and cross-linking agents were tested. CAT recovered activity was evaluated for all obtained derivatives. Kinetics of soluble CAT and CAT-CLEAs were estimated. Thermal stability of the biocatalyst was also addressed.

2. MATERIAL AND METHODS

2.1. Material

Bovine liver catalase (5,140 U/mg protein) was obtained from Sigma-Aldrich (St. Louis, MO). Glutaraldehyde solution (25% w/v) in H_2O, hydrogen peroxide (H_2O_2), ammonium sulfate solution (AS), tert-butyl alcohol (TBA), polyethylene glycol (PEG), dimethoxyethane (DME) and acetone (A) were obtained from Sigma-Aldrich (St. Louis, MO). Other reagents were of analytical grade.

2.2. CAT assay

CAT-CLEAs and soluble CAT activities were determined spectrophotometrically at 240 nm, following the decomposition of a H_2O_2 solution (35 mM H_2O_2 solution in 0.05 M phosphate buffer, pH 7.5) at 25 °C. One CAT unit was defined as the amount of enzyme that catalyzes the decomposition of 1 μmol of H_2O_2 per minute.

2.3. Protein concentration

Lowry method was used to measure protein concentration in the enzyme preparations (LOWRY, 1951). The protein content (mg) was spectrophotometrically quantified at 750 nm using bovine serum albumin (BSA) as protein standard.

2.4. Preparation of CLEAs

CAT-CLEAs derivatives were prepared by dissolving CAT in sodium phosphate buffer (100 mM, pH 7.0) to a final protein concentration of 40 mg/mL. Protein aggregation was induced by mixing 1 mL of the enzymatic solution and 1 mL of precipitant (saturate solution of ammonium sulphate, tert-butyl alcohol, polyethylene glycol, dimethoxyethane and acetone). After 1 min of

mixing, glutaraldehyde was added slowly to the final concentration of 50, 100 or 200 mM. After 3 h of cross-linking reaction at 10 ^{o}C, the suspension was centrifuged at 10,000×g for 10 min at 4 ^{o}C. CLEAs were recovered as pellet and washed with 100 mM sodium phosphate buffer (pH 7.0). After preparation the enzyme was kept in the same buffer (2 mL) at 4 ^{o}C until use.

The recovered activity in CLEAs was calculated as follows:

Activity recovery (%) = (Total activity of CLEAs in units/Total crude enzyme activity used for CLEAs Production in units) × 100.

2.5. Thermal stability study

Thermal stability of soluble CAT and CAT-CLEAs were evaluated at 40 ^{o}C and pH 7.0 (100 mM sodium phosphate buffer) for 200 h. In all assays, the initial activity was taken as 100 %.

2.6. Kinetic analysis

Kinetic parameters of soluble CAT and CAT-CLEAs were estimated by measuring initial reaction rates using different H_2O_2 concentrations ranging from 35 mM to 280 mM, in sodium phosphate buffer (0.1 M, pH 7) at 25 ^{o}C. Michaelis-Menten kinetic parameters (K_m and v_{max}) of soluble CAT and CAT-CLEAs were calculated from nonlinear regression fitting using experimental data of initial reaction rates *versus* H_2O_2 concentrations.

3. RESULTS AND DISCUSSION

3.1. Preparation of CAT-CLEAs

Aiming to precipitate all protein in the reaction medium, several protein precipitants, organic solvents and an inorganic salt, were tested: tert-butyl alcohol (TBA); polyethylene glycol (PEG); dimethoxyethane (DME); acetone (A); and, ammonium sulfate (AS) were tested. At the same time, the effect of the cross-linker concentration (50, 100 and 200 mM of glutaraldehyde) in the CAT-CLEAs final properties was investigated. Figure 3-A shows the recovered activity results to CAT-CLEAs prepared using five different precipitant agents and three different glutaraldehyde concentrations. Figure 3-B presents the residual activities to CAT-CLEAs after 200 h of at 40 ^{o}C.

Using TBA as precipitant agent, the CAT-CLEAs retained around 40 % of the offered activity. The best thermal stability (60 % of residual activity after 200 h) was obtained by using 100 mM of glutaraldehyde. CAT-CLEAs prepared using DME as precipitant agent and 50, 100, 200 mM of glutaraldehyde retained almost 45 % of the offered activity. Interestingly, for the lowest concentration of glutaraldehyde the biocatalyst showed the best result in the thermal stability (almost 50 % of the initial activity was retained after 200 h at 40 ^{o}C). For CAT-CLEAs prepared with PEG as precipitant agent, only the CAT-CLEAs prepared using 200 mM of glutaraldehyde presented a good recovered activity, around 35 %. However, these CAT-CLEAs did not show good thermal stability. The highest recovered activity in CAT-CLEAs prepared with acetone as precipitant agent (around 30 %) was obtained by using 100 mM of glutaraldehyde. These CAT-CLEAs presented residual activity of 70 % after 200 h at 40 ^{o}C. All CAT-CLEAs precipitated with ammonium sulfate present high stability at 40 ^{o}C. The lowest glutaraldehyde concentration (50 mM) was the best condition to preserve CAT activity during the CLEAs

preparation (62 ± 0.2 %). The CAT-CLEAs prepared using AS as precipitant was very stable, maintaining above 90 % of the initial activity after 200 h at 40 ºC.

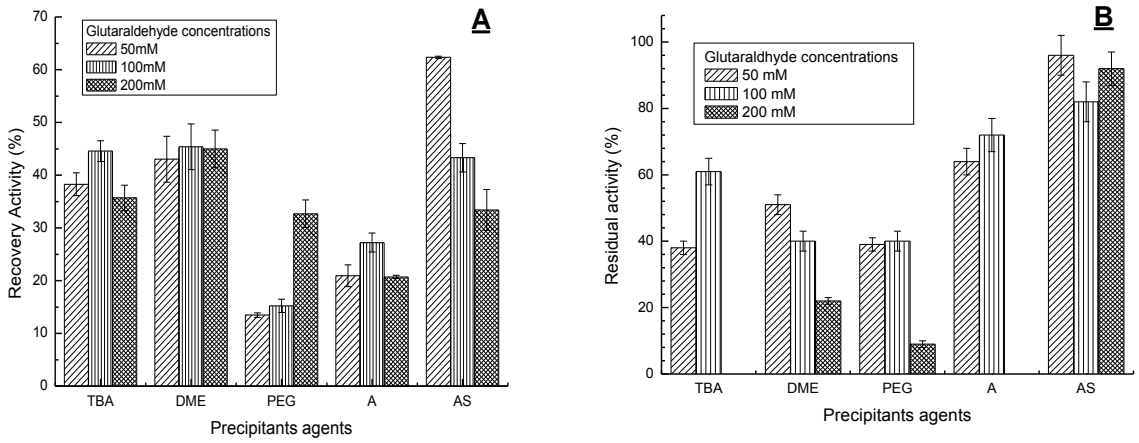

Figure 3 – (A) Recovered activity of CAT-CLEAs prepared by different precipitant agents: ammonium sulfate solution (AS), tert-butyl alcohol (TBA), polyethylene glycol (PEG), dimethoxymethane (DME), acetone (A) and different glutaraldehyde concentrations (50, 100, 200 mM). (B) Residual activity of CAT-CLEAs after 200 h at 40 ^{o}C

The best derivative prepared in the present work showed to be more active than the reported by Wilson *et al*. (2004). The authors immobilized CAT from bovine liver and from *Micrococcus lysodeikticus* by CLEAs technique using diethylene-glycol-dimethyl ether as precipitant agent and glutaraldehyde (5 % v/v) as cross-linking agent. Their derivatives retained 45 and 40 % of activity, respectively. To our knowledge, this is the only work previously reported in the literature addressing immobilization of CAT by CLEAs technique that show recovered activity.

3.2. Determination of kinetic parameters

The best CAT-CLEAs prepared in this work was kinetically characterized. According to Chelikani *et al*. (2004) there are almost 16 types of CAT. These enzymes do not follow the classic model of Michaelis-Menten and they are inactivated at high concentration of H_2O_2. The classic kinetic model of Michaelis-Menten was fitted to the experimental data of initial reaction rate (in the range where inactivation by H_2O_2 is neglected) versus substrate concentration at 25 ^{o}C and pH 7.5 for soluble CAT and CAT-CLEAs, as shown in Figure 4. The values of kinetic parameters are presented in Table 1.

Table 1 – Kinetic parameters of free and immobilized CAT.

CAT	v_{max} (U/mg $_{protein}$	K_m (mM)
Free	11,350 ± 175	66.7 ± 18
Immobilized	2,000 ± 200	392 ± 22

K_m: Michaelis-Menten coefficient
v_{max}: maximum reaction rate

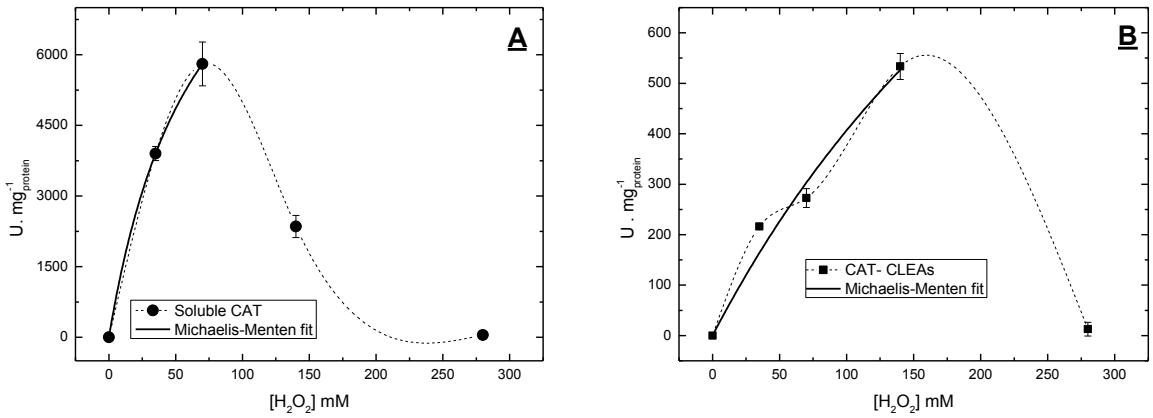

Figure 4 – Effect of H_2O_2 concentration on activities of : (A) soluble CAT and (B) CAT-CLEAs, at 25 ^oC and pH 7.5. The kinetic model of classic Michaelis-Menten was fitted to the experimental data of initial reaction rate in concentration range 0-70 mM for soluble CAT and 0-40 mM for immobilized CAT

The maximum velocity (v_{max}) obtained for the CAT-CLEAs were only 16 % of v_{max} of soluble CAT. Reduction on v_{max} parameter is commonly observed for immobilized enzymes. This reduction is mainly attributed to diffusional delays, steric hindrances, and conformational changes in the tridimensional structure. In fact, v_{max} for CAT immobilized onto pore glass (Alptekin *et al.*, 2009), cross-linked CAT in presence of bovine serum albumin, BSA (Tukel *et al.*, 2013), and CAT immobilized on pHEMA-based membrane (Hidalgo *et al.*, 2003) decreased to only 14 %, 0.2 % and 50 % compared to the value of the free CAT, respectively. Tukel *et al.* (2013) investigated the effect of BSA as a feeder protein on the CAT-CLEAs activity. At the ratio of 10 mg/mL of CAT / 10 mg/mL of BSA the authors observed the highest activity of CAT-CLEAs. K_m and v_{max} values of CAT-BSA-CLEAs derivative were estimated from Lineweaver–Burk plots obtaining 43 mM and 41 U/mg $_{protein}$, respectively.

K_m values of free CAT and CAT-CLEAs were estimated as 66.7 ± 18 mM and 392 ± 22 mM H_2O_2, respectively. The large increase in the apparent K_m may be due to diffusional delays. According to Sheldon (2007a), CLEAs form aggregates in a small volume that can be considered as a barrier to substrate diffusion. On the other hand, Ozyilmaz *et al.* (2007) immobilized bovine liver CAT covalently onto florisil activated with glutaraldehyde and they observed a K_m value of 1722.0 mM H_2O_2. These authors also reported that a high K_m value might be due to the production of oxygen gas into the pores of florisil, which might have hindered the reaction between CAT and H_2O_2 in aqueous phase. In this way, it is possible that this phenomenon has contributed to the high K_m value observed in this work.

4. CONCLUSIONS

CAT was immobilized without carrier via cross-linking enzyme aggregates and the optimal immobilization conditions were determined. The precipitant agent and glutaraldehyde concentration were determined as ammonium sulfate and 50 mM glutaraldehyde, respectively. Under optimal conditions, the CAT-CLEAs recovered 62 % of the offered activity and showed

high stability after 200 h at 40 ^{o}C. CAT-CLEAs and free CAT were kinetically characterized. CAT-CLEAs showed low maximum reaction rate (v_{max}) compared to the free enzyme. The catalyst prepared in this work was tolerant at high H_2O_2 concentrations and could be designed to be used in continuous process or fed-batch.

5. ACKNOWLEDGMENT

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