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Preparation of an immobilized–stabilized catalase derivative from *Aspergillus niger* having its multimeric structure stabilized: The effect of Zn^{2+} on enzyme stability

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ABSTRACT

Multimeric catalase from *Aspergillus niger* was immobilized on CNBr activated agarose, increasing the enzyme stability. However, it was found that some enzyme subunits could be desorbed to the supernatant after boiling the enzyme preparation in the presence of SDS or during thermal inactivation. Moreover, a positive enzyme concentration-enzyme stability correlation was detected in the immobilized preparation. This suggested the existence of some dissociation mechanism as a first step in the enzyme inactivation. The treatment of the immobilized enzyme with aldehyde–dextran permitted to fully stabilize its multimeric structure, but even this preparation exhibited an enzyme concentration-stability correlation. The presence of EDTA reduced the enzyme stability, suggesting that some cation could be involved in enzyme stability. It was found that 10 mM Zn^{2+} increased the enzyme stability of this immobilized–stabilized preparation. Now, the dilution of the biocatalyst did not produce a reduction in the enzyme stability.

Thus, we have prepared an immobilized enzyme that does not release any subunit to the medium even after inactivation, and found that Zn^{2+} has a very positive effect on the stability of this immobilized–stabilized enzyme.

1. Introduction

Catalases (EC 1.11.1.6) are complex enzymes with a high technological relevance (bleaching in textile industries [1], coupling to oxidases to prevent enzyme inactivation by H_2O_2 [2,3], biosensors [4–6], etc.). Most of these enzymes have a tetrameric structure [7–9]. According to the nature of the catalytic group, they may be divided in hemo-catalases or manganese catalases [7,10].

Stabilization of multimeric enzymes is a quite complex task. Inactivation of these enzymes normally starts by the dissociation or the promotion of a wrong assembly of the enzyme subunits [11,12]. If the subunits dissociation is the first step of the inactivation, this produces an enzyme concentration–stability relation and the stability of the enzyme under continuous use will be severely reduced. However, this phenomenon may be solved via immobilization (multisubunit immobilization) and post-immobilization techniques (crosslinking of the subunits not directly bound to the support with some subunits that were already attached to the support) [11,13–17]. In any case, the attachment of all the enzyme subunits to the support will be a requirement to prevent the contamination of the medium by the enzyme.

In many instances, there are some cations implicated in the maintenance of this correct assembly of the multimeric structure, not essential for enzyme activity but very important for enzyme stability [18,19]. If this is the case, depending on the dissociation constant of the protein–ion complex, the dilution of the enzymes may give a reduction of the enzyme stability even if all subunits are covalently bound to the support, because of the dilution of this cation. This may be solved by protein engineering, e.g., increasing the binding strength of this cation to the right position.

However, in some instances, if the dissociation of the subunits is the first step in the inactivation, may be very complex to discover the implication of an ion involved in the protein stability. The use of enzyme molecules with the multimeric structure fully stabilized may permit to detect this effect by avoiding the enzyme subunit dissociation. Thus, stabilization of the quaternary structure of

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multimeric enzymes may be not only interesting from an applied point of view, but also to study other properties of the enzyme.

In this paper, we report results in the stability and stabilization of the catalase from *Aspergillus niger* by immobilization and post-immobilization techniques, and the effect of different compounds on enzyme thermal stability This large tetrameric enzyme (MW around 360 kDa) is very interesting because is an enzyme from a GRAS microorganism used in many industrial processes as well as at laboratory level [20].

2. Materials and methods

2.1. Materials

Cyanogen bromide (CNBr) activated Sepharose beads 4B were supplied by Amersham Bioscience (Uppsala, Sweden). A powder of lyophilized catalase from *Aspergillus niger* (CATAn) was kindly donated by Puleva Biotech S.A. (Granada, Spain). Dextrans of 10,000 Da were supplied by Sigma–Aldrich (St. Louis MO, EEUU). All other reagents were of analytical grade.

2.2. Methods

All results presented here are calculated from averaging at least three different experiments. In all cases, experimental error was found to be smaller than 10%.

2.3. Determination of catalase activity

Catalase activity was determined spectrophotometrically by monitoring the decomposition of H_2O_2, through the measurement of the decrease in the absorbance at 240 nm [21]. 2.9 mL of H_2O_2, 0.14% (w/v) in 50 mM sodium phosphate, pH 7, were incubated with 0.2 mL of enzyme solution or suspension. All the measurements were carried out at 25 °C. The spectrophotometer had a themostatized cell with magnetic stirring, able to keep a homogeneous suspension of that concentration of agarose during the reaction. Under this condition, agarose beads only produces some light scattering and the experimental error was lower than 5%.

One catalase unit was defined as the amount of enzyme that decomposes 1 μM of H_2O_2 per minute under the previously described conditions. The specific activity of the sample used was 2035 U/mg.

2.4. Protein concentration determination

Protein concentration was determined according to Bradford's method [22] using bovine albumin as standard.

2.5. Preparation of CNBr-activated sepharose 4B

A sample of 2 g of the vacuum dried CNBr-activated Sepharose 4B (keeping the intra-particle water) was incubated with 7 mL of a 1 mM HCl during 15 min and then washed with 0.1 M sodium bicarbonate at pH 8.4, 0.5 M NaCl.

2.6. Immobilization of catalase on the CNBr-activated sepharose 4B

A sample of 1 g of washed CNBr-activated Sepharose 4B prepared as previously described was incubated with 5 mL of a catalase solution (4 mg of CATAn in 5 mL of 25 mM sodium phosphate at pH 7) during 1 h at 25 °C under gentle stirring. After that, the agarose beads were filtered and washed with 25 mM sodium phosphate at pH 7, the gel was blocked with 10 mL of 0.1 Tris–HCl at pH 8 during 2 h under gentle stirring, and washed with 25 mM potassium phosphate at pH 7 and storage at 4 °C. This derivative was called CNBr–CATAn.

2.7. Chemical treatment of the immobilized enzymes with aldehyde–dextran

Dextran (MW = 10,000 Da) at 20% oxidation degree (using sodium periodate) [16] was used. One gram of BrCN–CATAn (containing 4 mg of catalase/wet g) was incubated with 10 mL of 15 mg/mL dextran in 0.2 M sodium phosphate pH 7.5 at 4 °C. At different times, samples were withdrawn and the enzyme activity was measured. To stop the reaction, 2 mg/mL of sodium borohydride were added to the suspension (after adjusting the pH value at 10). After 30 min, the immobilized enzyme was washed with 5 mM sodium phosphate pH 7.

2.8. SDS-PAGE of the enzyme preparations

Samples of the different enzyme preparations were boiled in the presence of SDS and β-mercaptoethanol to desorb any protein subunit that was not covalently immobilized to the support [23]. The samples of the supernatant thus obtained were withdrawn and analysed by SDS-PAGE.

2.9. Thermal stability studies

Different preparations of CATAn (prepared under identical conditions as described above) were incubated at different concentrations of biocatalyst at 55 or 60 °C in 25 mM sodium acetate pH5 in the absence or the presence of different cationic metals at different concentration. Each sample was analyzed at different times by measuring its enzymatic activity as described above. Stability and stabilization refers to the half-lives of the different enzyme preparations.

3. Results

3.1. Immobilization of the enzyme in BrCN–agarose: effects on stability

The immobilization of the enzyme on BrCN–agarose permitted to immobilize 100% of the enzyme, keeping 75% of the enzyme activity, producing a stabilization of a 30-fold factor when compared to the soluble enzyme (Fig. 1). However, there is certain

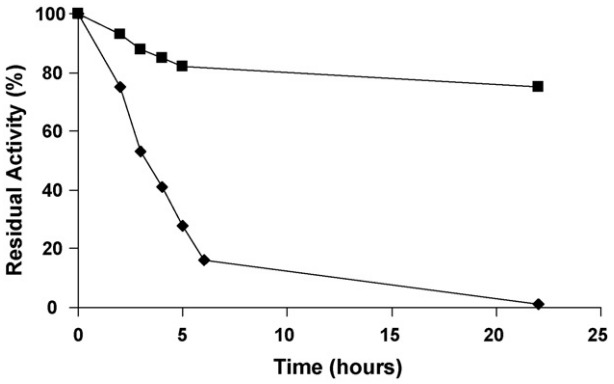

Fig. 1. Thermal inactivation course of soluble CATAn (♦) and CNBr–CATAn (■). The experiments were carried out at pH 5, 55 °C. Further details were described in Section 2.2.

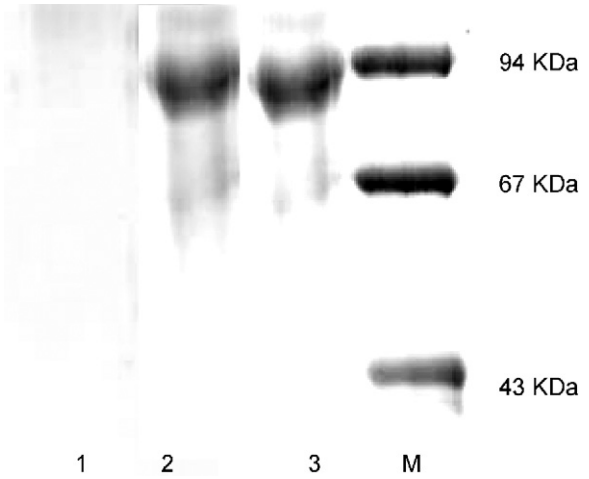

Fig. 2. SDS-PAGE analysis from different CATAn preparations. The derivatives were re-suspended in denaturation buffer as described in Section 2.2, in the case of soluble enzyme using 1 mg/mL, using suspension the concentration was 2 mg of catalase/mL. The supernatants of suspensions of different enzyme preparations—Lane (1): CNBr derivative further cross-linked with dextran–aldehyde in the conditions previously described in Section 2.2; Lane (2): CNBr derivative; Lane (3): soluble CATAn; Lane M: markers.

enzyme concentration-enzyme stability dependence (results not shown).

To check if there are any enzyme subunits not bound to the support, the preparations were boiled in the presence of SDS for 3 min, and the supernatants obtained submitted to SDS-PAGE. Fig. 2 (Lane 2) shows that there are some enzyme subunits that can be released from the support (a similar treatment on BSA–BrCN–agarose immobilized enzyme did not produce any release of protein to the medium). Therefore, based on the intensity of the bands of the gel, it may be assumed that around two of the four subunits of the enzyme are not bound to the support and may still dissociate. Therefore, the enzyme concentration-stability dependence could come from the enzyme dissociation or some other dissociation phenomena.

In fact, during thermal inactivation experiments, it was possible to detect the presence of protein in the supernatant that correlate with the inactivation of the immobilized preparation (result not shown). That way, even if the dissociation of the enzyme is not the first reason for enzyme inactivation, the release of the enzyme to the medium after enzyme inactivation would contaminate the product.

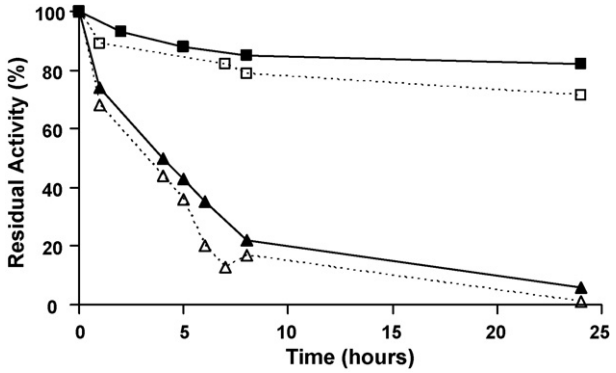

Fig. 3. Effect of dextran–aldehyde cross-linking in the thermal inactivation of CNBr–CATAn at different assay concentrations. The CNBr derivatives (dotted lines) and the CNBr derivatives cross-linked with dextran–aldehyde (full lines) were thermally inactivated at 55 °C and pH 5, at two different biocatalyst dilutions 1/50 (w/v) (triangles) and 1/5 (w/v) (squares).

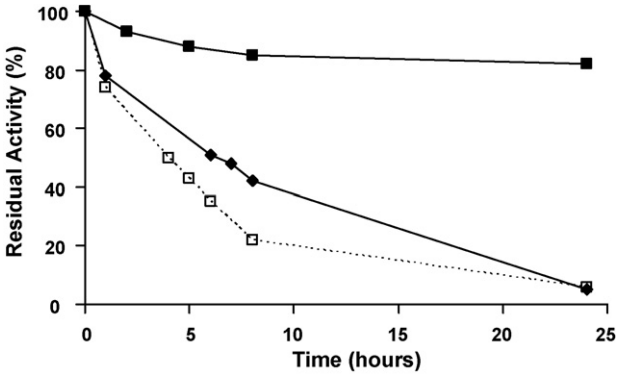

Fig. 4. Effect of EDTA on the thermal inactivation of CNBr–CATAn cross-linked with dextran–aldehyde. (♦), dilution 1/50 (w/v) without EDTA (solid lines); (■), 1/5 (w/v) without EDTA (solid lines) and (□, dotted lines) 1/5 and 1/50 (w/v) with 10 mM EDTA (both dilutions have identical inactivation courses and only one line is used). The different enzymatic preparations were thermally inactivated at 55 °C and pH 5.

3.2. Chemical crosslink of the immobilized enzyme by dextran–aldehyde

To fully stabilize its multimeric structure, the enzyme was submitted to crosslinking with aldehyde–dextran. This treatment decreased the enzyme activity (by 33%), and the SDS-PAGE showed that there was no release of any enzyme subunit to the medium (Fig. 2, Lane 1).

However, the diluted suspension of the immobilized biocatalyst was less stable than the concentrated one at high temperature. Considering that the entire enzyme subunits are attached to the support or crosslinked to one subunit that is attached to the support, and that the enzyme molecules are dispersed because it is immobilized using low loading, this result suggested that there are still some additional dissociation phenomena causing this dependence (Fig. 3), implying some small molecule no covalently attached to the enzyme after the dextran treatment and that can reversible interact with it.

3.3. Effect of different cations on enzyme stability

The incubation of the enzyme at 25 °C in the presence of EDTA for 24 h did not have any significant effect on the enzyme activity (results not shown). However, when the incubation was performed at 55 °C, this compound greatly decreased the enzyme stability. In fact, in the presence of 10 mM EDTA, the dilution of the suspension of immobilized and crosslinked enzyme did not present a significant effect on enzyme thermostability (Fig. 4). Thus, this suggests that the enzyme stability at high temperature may depend on anion.

We found that 1 mM of different cations (Cu^{2+}, Ni^{2+}, Zn^{2+}) have no effect on the enzyme activity (neither in soluble or immobilized form). Then, we studied the effect of these cations on stability of the immobilized enzyme at high temperature. To this purpose, the diluted immobilized enzyme was incubated at 55 and 60 °C (Fig. 5A) in the presence of different ions, and it was found that while Cu^{2+} slightly reduced the enzyme stability, Zn^{2+} slightly increased it.

Finally, the effect of the concentration of Zn^{2+} on the thermal stability of the immobilized and crosslinked enzyme was analyzed. It was found that at 10 mM $ZnCl_2$ the stability reached a maximum, the stability even decreasing at 30 mM $ZnCl_2$ (Fig. 5B). At 10 mM $ZnCl_2$, the thermal stability of this preparation did not longer significantly depend on the dilution of the biocatalyst suspension (results not shown). When this cation was added to the concentrated enzyme, the effect was not significant (results not shown).

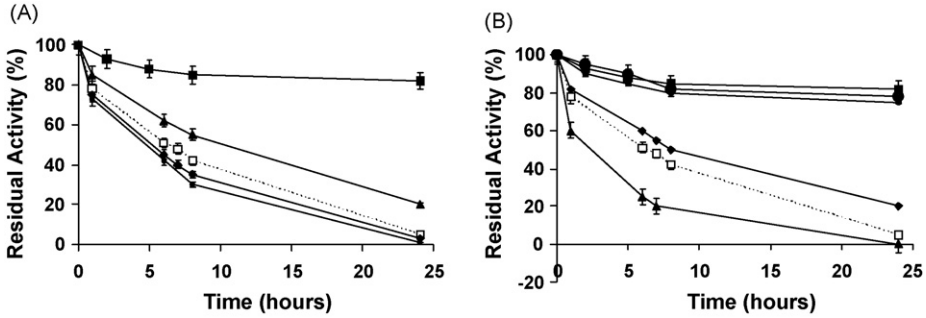

Fig. 5. Effect of cations in the thermal inactivation course of CNBr–CATAn cross-linked with dextran–aldehyde. The CNBr–CATAn and cross-linked with dextran–aldehyde derivative was inactivated in 10 mM sodium acetate pH5 at 55 °C in the presence of different cations at different enzyme concentrations: (A) 1/5 without cations (w/v) (■) or 1/50 (dotted lines without cations (□) and 1/50 in the presence of 1 mM of $CuSO_4$ (●), $NiCl_2$ (♦) or $ZnCl_2$ (▲). Moreover, the effect of the $ZnCl_2$ was studied. (B) (1/5) without cations (w/v) (■), 1/50 (w/v) 0 mM (dotted line). (□), 1 mM (♦), 5 mM (×), 10 mM (●), 30 mM (▲).

4. Conclusions

Catalase from *A. niger* has been greatly stabilized by using immobilization and post-immobilization techniques, and by determining the role of Zn^{2+} on the immobilized enzyme thermal stability. Thus, the multisubunit immobilization of the enzyme permitted to increase the enzyme thermal stability, but still some subunits are not covalently attached to the supports and may be released to the medium. The crosslinking of the immobilized enzyme with aldehyde–dextran fully prevented the enzyme subunits dissociation, permitting an additional improvement in the enzyme thermal stability. However, it was unable to prevent the enzyme-concentration/stability dependence. The addition of Zn^{2+} to the medium permitted to increase the enzyme stability, and also reduce the dilution effect on enzyme stability.

Thus, the aldehyde–dextran crosslinked immobilized preparation did not release subunits to the reaction media (avoiding contamination of the medium by the enzyme) and greatly increased the enzyme stability; however, the enzyme stability may be further increased by just adding 10 mM Zn^{2+} to the reaction media. It seems that this cation is relevant for the enzyme stability of this biocatalyst, although it did not seem to be directly relevant for the enzyme activity.

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