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Stabilization of enzymes (D-amino acid oxidase) against hydrogen peroxide via immobilization and post-immobilization techniques

Roberto Fernández-Lafuente, Verónica Rodríguez, César Mateo, Gloria Fernández-Lorente, Pilar Arminsen, Pilar Sabuquillo, José M. Guisán *

Departamento de Biocatálisis, Instituto de Catálisis, CSIC, Lab. de Tecnologia Enzimatica, Campus Universidad Autonoma, Madrid 28049, Spain

Abstract

Three different approaches are proposed to increase the resistance of enzymes against hydrogen peroxide. (a) *Multipoint covalent immobilization.* Through this technique, enzyme rigidity would be greatly increased and hence, any conformational change on the enzyme structure involved before or after oxidation with hydrogen peroxide becomes greatly prevented. (b) *Oriented immobilization on supports having large internal surfaces*. The immobilization of enzymes, through different areas of their surface on solid supports with internal morphology composed by large surfaces, promotes a certain masking of the enzyme areas that are very close to the support surface. In this way, the accessibility of hydrogen peroxide to such protein areas becomes greatly restricted. (c) *Additional chemical modification of immobilized enzyme derivatives with polymers*. By adding thick barriers surrounding the whole enzyme molecule, the effective concentration of hydrogen peroxide in the proximity of the most sensitive residues may be strongly reduced. Multipoint covalently immobilized D-amino acid oxidase (DAAO) from *Rhodotorula gracilis* on glyoxyl-agarose is 11-fold more stable than native enzyme against the deleterious effect of hydrogen peroxide. On the other hand, DAAO from *Trigonopsis variabilis* was not stabilized by rigidification but it could be highly stabilized by an adequate combination of the best orientation on the support plus an additional modification with poly-aldehyde polymers.

Keywords: Hydrogen peroxide; D-Amino acid oxidase; Enzyme stabilization; Enzyme immobilization; Chemical modification of enzymes; Dextrans in chemistry of proteins

1. Introduction

Hydrogen peroxide is a substrate or product of many interesting industrial enzymes (oxidases, peroxidadses, catalases, etc.). Under physiological conditions, these enzymes are exposed to very low concentrations of this reagent but they may have to act in the presence of high or moderate concentrations of such a product during analytical or industrial applications. Under these conditions, these enzymes may be very sensitive to hydrogen peroxide [1–3] because it can attack some residues of the proteins (methionine, cysteines, etc.) [1] and hence, the increase of their resistance to this chemical reagent has a major practical relevance. This

* Corresponding author. Tel.: +34-91-5854800; Fax: +34-91-5854760; E-mail: jmguisan@icp.csic.es

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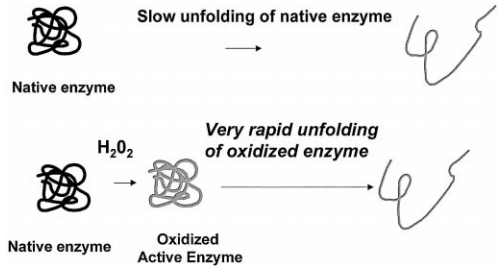

Fig. 1.

may be an example of a more general problem; other enzyme substrates or products may have a very deleterious effect on enzyme activity (oxygen, superoxides, chemical radicals, etc.).

Two different strategies are proposed to increase the stability of enzymes against chemical reagents.

1.1. Rigidification of enzyme structure

Deleterious effects of chemical reagents may be highly related to conformational changes on enzyme structure. On one hand, chemical modification may affect a protein residue that is not critical for the enzyme function but which plays a key role in the promotion of more rapid conformational changes (Fig. 1). On the other hand, some sensitive residues may be partially buried inside the protein structure and they only become accessible to chemical modification during thermal vibrations of enzyme structure (Fig. 2). In both cases, an increase in enzyme rigidity should promote a great increase in the resistance of rigidified enzyme molecules towards chemical reagents (namely hydrogen peroxide). A high increase in enzyme rigidity may be achieved through *multipoint covalent immobilization* on highly activated glyoxyl-agarose [4–6].

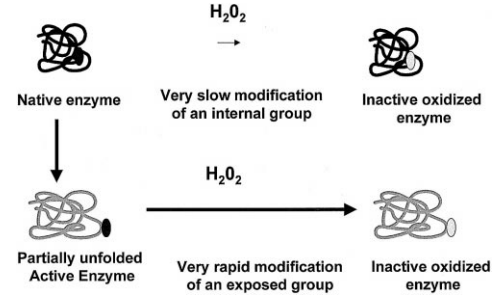

Fig. 2.

1.2. Masking of sensitive enzyme residues

The promotion of physical hindrances to the contact between chemical reagents and enzyme surfaces should also promote an interesting increase in stability of enzymes against chemical modification promoted by chemical reagents, whatever the mechanism of inactivation. This may be the only option to stabilize an enzyme against hydrogen peroxide when the action of this reactive attacks a critical catalytic group of the enzyme that is fully exposed to the medium (Fig. 3). Masking of enzyme surface can be intended trough two different experimental approaches.

(i) Immobilization on supports having large internal surfaces (e.g., agarose [7]. In this way, the area of the enzyme in close contact with such large support surface may become less accessible to deleterious reagents.

(ii) Additional chemical modification of immobilized derivatives with poly-functional macromolecules. In this way, new physical barriers placed all around the whole enzyme surface should protect the enzyme surface from interaction with chemicals.

At first glance, these physical barriers could also hinder the accessibility of substrates to the enzyme active center. However, industrial application of enzymes is usually performed on very

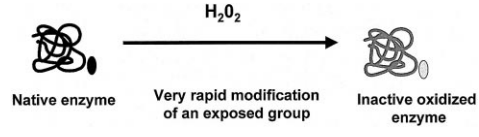

Fig. 3.

high substrate concentrations where enzyme kinetics may be zero-order ones. On the contrary, inactivation by chemicals may follow a first-order kinetics. In this way, profitable effects of physical barriers should be much greater than the possible negative ones.

In this paper, the three approaches to increase enzyme stability against chemicals have been tested by checking the stabilization of two D-amino acid oxidases (DAAOs) (from *Rhodotorula gracilis* and from *Trigonopsis variabilis*) against hydrogen peroxide, enzymes with a great interest as catalysts of different processes [8–11].

2. Materials and methods

2.1. Materials

D-Amino acid oxidases from *T. variabilis* and from *R. gracilis* and cephalosporin C were gifts from Antibioticos S.A. (Leon, Spain). Glutaraldehyde was from Merck (25% V/V). Cross-linked 6% agarose-amino (MANA) or glyoxyl-agarose beads [activation degree 15 (poorly activated supports) or 75 (highly activated supports) μmol/ml] were gifts from Hispanagar (Burgos, Spain). Both of them were produced as previously described [4,12].

2.2. Enzymatic activity assays

D-Amino acid oxidase activity was checked spectrophotometrically, using cephalosporin C as substrate. We follow the increase of the absorbance at 420 nm promoted by coupling of the oxidative deamination of cephalosporin C catalyzed by DAAO with the reaction between the hydrogen peroxide and *O*-phenylendiamine catalyzed by peroxidase that produced a colored compound [13,14]. The reaction mixture consisted of 1.5 ml of 0.1 M phosphate buffer/6.5 mM cephalosporin C, pH 7.5, 0.5 ml of 1.85 mM *O*-phenylendiamine in distilled water and 0.1 ml of 2 mg/ml peroxidase in 0.1 M sodium phosphate, pH 7.5. This reaction solution was pre-incubated at 25°C. The reaction was initialized by adding a maximum of 0.1 DAO units. The assays were carried out in a Shimadzu UV-V 160 thermostated at 25°C and with magnetic stirring.

One DAAO unit is defined as the amount of enzyme that produced 1 mmol of hydrogen peroxide (or oxide 1 μmol of cephalosporin C) per minute under the previously described conditions.

2.3. Preparation of agarose-glutaraldehyde

Ten milliliters of MANA-agarose (activated with 15 or 75 μmol/ml support) were suspended in 50 ml of 200 mM sodium phosphate, pH 7. Then, 40 ml of commercial glutaraldehyde (25% V/V) were added. After 24 h of gentle stirring at room temperature, the gel was washed with a great excess of distilled water. The activated gels were used immediately after activation. The activation of the MANE-agarose determines the final activation of glutaraldehyde groups: all amino groups were modified [14].

2.4. Immobilization of DAAOs on glutaraldehyde-agarose

A 90-ml suspension containing 40 mg of protein in 0.1 M sodium phosphate/0.25 M NaCl at pH 7 and 20°C was prepared. Then, 10 ml (packed bed volume) of agarose-glutaraldehyde were added and the suspension was very gently stirred. As an end point to the enzyme-support reaction, the pH was increased at 8.5 and 200 mg of solid sodium borohydride were added [15–17]. After 30 min, the enzyme derivative was washed with 0.1 M phosphate and distilled water.

2.5. Immobilization of DAAOs on glyoxyl-agarose

A 20-ml suspension containing 40 mg of protein in 0.1 M sodium bicarbonate at 20°C

and pH 10 was prepared, then 10 ml (packed bed volume) of glyoxyl-agarose (15 or 75 μmol, 7 ml of support) were added. As an end point to the support-enzyme reaction, 70 ml of 0.1 M sodium bicarbonate pH 10 containing 100 mg of solid sodium borohydride were added [18] and, after 30 min, the gel was washed with 0.1 M sodium phosphate, pH 7, and distilled water.

2.6. Preparation of aldehyde dextran

A 100-ml solution containing 3.33 g of dextran (MW 6000 Da) in distilled water was prepared. Then, 8 g of solid sodium periodate were added (this permitted the full oxidation of the dextran molecule) and this solution was stirred for 3 h. After, this solution was four times dialyzed against 50 volumes of distilled water to eliminate the formaldehyde produced during the oxidation [19].

2.7. Modification of the immobilized enzymes with dextran-aldehyde

Ten milliliters of immobilized DAAO derivative were suspended in 60 ml of sodium phosphate at pH 7 and 4°C. Then, 30 ml of aldehyde dextran (prepared as previously described) were added to the suspension. This suspension was very gently stirred for 12 h. Then, 900 ml of 0.1 M sodium borate at 4°C and pH 8.5 containing 2 g of sodium borohydride were added to reduce the remaining aldehyde groups as well as the aldehyde–amine bonds [19].

2.8. Stability assays in the presence of hydrogen peroxide

Enzyme derivatives were incubated in 200 mM phosphate, pH 7.5, at 25°C containing hydrogen peroxide 10 mM. Periodically, samples of these inactivation solutions or suspensions were withdrawn, washed with water, resuspended in 25 mM sodium phosphate buffer and assayed as previously described.

3. Results

3.1. Inactivation of DAAOs by hydrogen peroxide

Fig. 4 shows that both DAAOs immobilized on a poorly activated glyoxyl support were very sensitive to the presence of 10 mM hydrogen peroxide. Thus, the half-life of DAAO from *R. gracilis* was around 15–20 h under conditions where, in absence of hydrogen peroxide, the enzyme was fully stable. The enzyme from *T. variabilis* was even less stable, having a half-life of around 10 h. This concentration of hydrogen peroxide was similar to that accumulated during the oxidation of D-amino acids by these enzymes [3]. Therefore, the interest in increasing the stability of the enzymes against this reagent is evident.

3.2. Stabilization of DAAOs against inactivation by hydrogen peroxide via multipoint covalent attachment

Both enzymes were immobilized on agarose highly activated with glyoxyl groups (75 μmol/

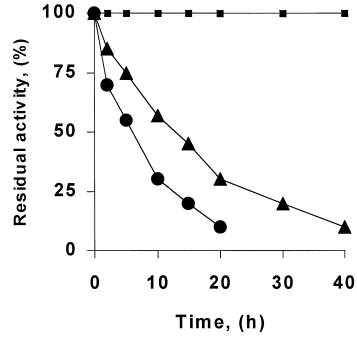

Fig. 4. Effect of hydrogen peroxide on DAAO stability. Experiments were carried out at pH 7 and 25°C. Squares: non-stabilized derivatives of DAAO from *Trigonopsis* and *Rhodotorula* in the absence of hydrogen peroxide. Triangles: non-stabilized derivative from *Rhodotorula* in the presence of 10 mM hydrogen peroxide. Circles: non-stabilized derivative from *Trigonopsis* in the presence of 10 mM hydrogen peroxide.

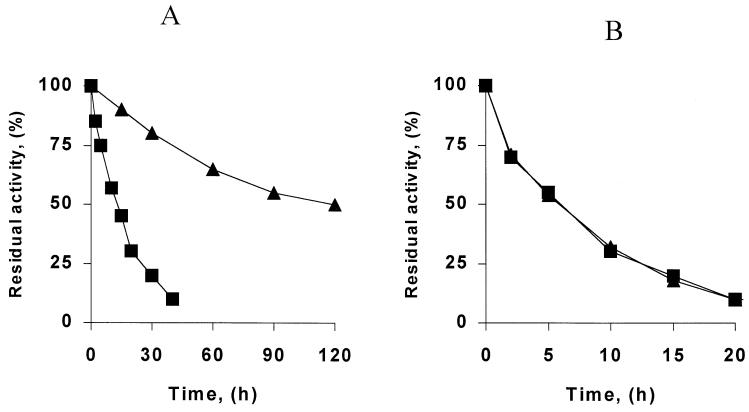

Fig. 5. Effect of enzyme rigidity on the DAAO stability in the presence of hydrogen peroxide. Inactivations were carried out at pH 7 and 25°C in the presence of 10 mM hydrogen peroxide. Squares: derivatives prepared on lowly activated glyoxyl-agarose (15 μmol/ml of support). Triangles: derivatives prepared on highly activated glyoxyl supports (75 μmol/m of support) of DAAOs from *Rhodotorula* (A) and *Trigonopsis* (B).

ml) for 24 h in order to get a intense multipoint covalent attachment between the enzyme and the support [20]. The recovered activity was, in both cases, over 85%. Although both enzymes increased significantly its thermostability (results nor shown), only the enzyme from *R. gracilis* showed a significant increment in the stability against hydrogen peroxide (Fig. 5). The half-life of this enzyme in the presence of hydrogen peroxide was increased by an 11-fold factor. These results suggested that the inactivation of the enzyme from *R. gracilis* by hydrogen peroxide required the movement of the enzyme structure before or after the inactivation (see Section 1).

However, the enzyme from *T. variabilis* exhibited exactly the same stability in the presence of hydrogen peroxide than the one-point covalently attached derivative (Fig. 5). This suggested that the inactivation promoted by hydrogen peroxide proceeds via the modification of a group critical for the catalytic function and exposed to the medium.

3.3. Stabilization of DAAOs against hydrogen peroxide chemical modification via oriented immobilization

D-Amino acid oxidase from *T. variabilis* was immobilized on two different supports, glutaraldehyde and glyoxyl-agarose, activated with only 15 μmol of reactive groups per milliliter of support. Fig. 6 shows that the enzyme immobilized on glutaraldehyde was 4-fold less stable than the DAAO immobilized on glyoxyl-agarose. This suggested that when DAAO was immobilized on glyoxyl supports (i.e., via the richest area in Lys residues [15]), the most sensitive residues to the modification with hydrogen peroxide might be 'hidden' by the support surface and, in this way, the concentration of hydrogen peroxide in the environment of the

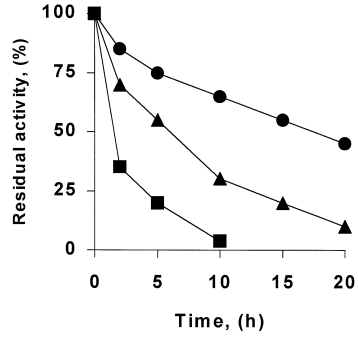

Fig. 6. Effect of hiding the sensitive group of DAAO from *Trigonopsis* to the action of hydrogen peroxide. The different derivatives were immobilized in poorly activated supports (15 μmol/ml of support) using glutaraldehyde-agarose (squares) or glyoxyl-agarose (triangles). The enzyme immobilized on glyoxyl-agarose was further modified with poly-aldehyde-dextran (circles) Inactivations were carried out at pH 7 and 25°C in the presence of 10 mM hydrogen peroxide.

enzyme might be reduced and the enzyme stability was increased. However, when immobilized via the amino terminal on glutaraldehyde supports [14], the sensitive group seemed to be fully exposed to the action of hydrogen peroxide.

3.4. Stabilization of DAAO against hydrogen peroxide by generation of barriers via chemical modification of the enzyme with poly-aldehyde dextrans

The chemical modification of DAAO from *T. variabilis* immobilized on glyoxyl-agarose with aldehyde dextrans promoted a slight decrease in the enzyme activity (by 20%), associated with a further increase in the enzyme stability by a 3-fold factor (Fig. 6). This suggested that these big macromolecules were able to protect the sensitive residue and, thus, increase the enzyme stability.

4. Discussion

The results presented in this paper show that it is possible to increase the enzyme stability against the action of hydrogen peroxide by using physico-chemical tools such as protein immobilization and chemical modification. We have been able to prepare DAAO derivatives 10–20-fold more stable than non-stabilized DAAO, preserving in both cases very high levels of enzyme activity.

When the enzyme inactivation requires movements of the enzyme structure (i.e, if the sensitive group is protected by the core of the protein or if the modified group is very related to the maintenance of the enzyme structure), enzyme rigidification via multipoint covalent attachment is enough to increase the enzyme stability (Figs. 1 and 2). That seems to be the case of the inactivation of DAAO from *R. gracilis* by hydrogen peroxide.

However, in other cases, the hydrogen peroxide may directly attack a group that is fully exposed to the medium and that is very related to the catalytic function. In this case, the rigidification may have no effect on the enzyme stability, being necessary to 'hide' the sensitive group(s) from the attack of the hydrogen peroxide. This may be achieved by placing the group very near the support surface or using modification with polymers to seclude the reagent from the enzyme environment. That seems to be the case of the enzyme from *T. variabilis*. Even in this complex case, we have been able to increase the enzyme stability by a 12-fold factor preserving 70% of the DAAO activity. One of the advantages of these immobilization and post-immobilization techniques is the possibility of using the additive stabilizing effects of the different strategies (Fig. 6). Of course, we can expect that the effects of both strategies may be additive. Optimization of these processes will be subject of forthcoming papers (Guisan et al., unpublished results).

Acknowledgements

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