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Use of Physicochemical Tools to Determine the Choice of Optimal Enzyme: Stabilization of D-Amino Acid Oxidase

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An evaluation of the stability of several forms (including soluble and two immobilized preparations) of D-amino acid oxidases from *Trigonopsis variabilis* (TvDAAO) and *Rhodotorula gracilis* (RgDAAO) is presented here. Initially, both soluble enzymes become inactivated via subunit dissociation, and the most thermostable enzyme seemed to be TvDAAO, which was 3−4 times more stable than RgDAAO at a protein concentration of 30 μg/mL. Immobilization on poorly activated supports was unable to stabilize the enzyme, while highly activated supports improved the enzyme stability. Better results were obtained when using highly activated glyoxyl agarose supports than when glutaraldehyde was used. Thus, multisubunit immobilization on highly activated glyoxyl agarose dramatically improved the stability of RgDAAO (by ca. 15 000-fold) while only marginally improving the stability of TvDAAO (by 15−20-fold), at a protein concentration of 6.7 μg/mL. Therefore, the optimal immobilized RgDAAO was much more stable than the optimal immobilized TvDAAO at this enzyme concentration. The lower stabilization effect on TvDAAO was associated with the inactivation of this enzyme by FAD dissociation that was not prevented by immobilization. Finally, nonstabilized RgDAAO was marginally more stable in the presence of H_2O_2 than TvDAAO, but after stabilization by multisubunit immobilization, its stability became 10 times higher than that of TvDAAO. Therefore, the most stable DAAO preparation and the optimal choice for an industrial application seems to be RgDAAO immobilized on glyoxyl agarose.

Introduction

Nature offers enzymes with very different properties in terms of activity, stability, and selectivity. The selection of new industrial enzymes by exploring the biodiversity is a powerful tool in biocatalysis that allows researchers to choose the enzyme with the best properties in terms of activity, selectivity, stability, etc. Besides these properties, it may also be useful to know the main causes of inactivation to allow development of strategies to prevent them. Among such causes are the following: distortion of the tertiary structure, a general mechanism of enzyme inactivation (*1*, *2*); dissociation of the enzyme subunits, the first step of inactivation of most multimeric enzymes (*3*, *4*); chemical inactivation (*5*); and dissociation of the cofactors and prosthetic groups (*6*, *7*) (Scheme 1).

Considering that the industrial use of enzymes commonly requires their immobilization, it may be very convenient to explore these physicochemical techniques to solve some of problems mentioned above. For example: stabilization of the tertiary structure of enzymes may be achieved by multipoint covalent attachment (*8*, *9*); multisubunit immobilization may prevent enzyme inactivation by subunit dissociation (*10*); and the orientation and rigidification of the enzyme on the support surface may reduce or even prevent inactivation of the enzyme by chemical modification (*11*).

DAAOs (D-amino acid oxidases, EC 1.4.3.3) constitute a practical example of enzymes that suffer the four inactivation causes previously mentioned, since they are multimeric enzymes that have FAD as a cofactor (*12*, *13*) and produce hydrogen peroxide as a side product (which promotes their inactivation by chemical modification) (*14*).

These enzymes are very interesting from an applied point of view (*12*). They may be used to detect D-amino acids (*15*, *16*), eliminate traces of D-amino acids from L-amino acid preparations (*17*), produce keto-acids (e.g., coupled to catalases) (*14*, *18*, *19*), etc. However, perhaps their most relevant application is in the first step of the enzymatic hydrolysis of cephalosporin C to 7-aminocephalosporanic acid (7-ACA) (*12*, *20*).

In this work, we compare the properties of the two most widely used DAAOs (those from *Trigonopsis variabilis* and *Rhodotorula gracilis*). Both enzymes present very similar kinetic constants in many of the applications previously described (*21*), but their low stability is the current obstacle in their industrial implementation. Here we report the stability of different DAAO preparations during the optimization of an industrial biocatalyst.

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Materials and Methods

Materials. Cephalosporin C and DAAO from both *T. variabilis* (TvDAAO) and *R. gracilis* (RgDAAO) were kindly donated by Antibioticos S.A. (Leon, Spain) and used without further purification or additional treatments (*22*, *23*). Cross-linked 10% agarose, glyoxyl, and monoaminoethyl-*N*-aminoethyl (MANAE) derivatives were kind gifts from Hispanagar S.A. (Burgos, Spain) and prepared as previously described (*24*−*26*). Peroxidase and *o*-phenylenediamine dihydrochloride were obtained from Sigma (Sigma Chemical Co., St. Louis, MO). All other chemical reagents used were of analytical grade.

Enzymatic Activity Assays. DAAO activity was analyzed spectrophotometrically using cephalosporin C as a substrate by measuring the increment in the absorbance at 420 nm promoted by coupling the oxidative deamination of the substrate with the reaction between the hydrogen peroxide and *o*-phenylenediamine catalyzed by peroxidase. The reaction mixture consisted of 1.5 mL of 6.5 mM cephalosporin C in 100 mM potassium phosphate pH 7.5, 0.5 mL of 1.85 mM *o*-phenylenediamine in distilled water, and 0.1 mL of a 2 mg/mL peroxidase solution in 100 mM potassium phosphate at pH 7.5. The reagents were kept at 4 °C and preincubated for 5 min at 25 °C before adding a maximum of 0.1 DAAO units.

The amount of enzyme required to oxidize 1 μmol of cephalosporin C per minute in the previously described conditions was defined as 1 DAAO unit.

Immobilization of DAAO on Glyoxyl-agarose. A 2 mg/mL protein solution (20 mL) in 0.1 M sodium bicarbonate and 20% (v/v) glycerin at pH 10 and 20 °C was prepared, and then 10 mL (packed bed volume) of highly activated glyoxyl agarose (220 μmol/ml of support) was added. After 24 h and 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 was added, and after 30 min, the gel was washed with 0.1 M sodium phosphate pH 7 and distilled water. The preparation of derivatives on poorly activated glyoxyl supports was carried out in the conditions mentioned above, but at 4 °C and using a higher protein concentration. In this case, the immobilizates were reduced after 1 h. Reduction and washing were performed as described before, but at 4 °C.

Immobilization of DAAO on Glutaraldehyde-agarose. Glutaraldehyde activation (220 μmol reactive groups/mL) of the MANAE support was performed as previously described (*25*). A suspension (90 mL) containing 40 mg of protein in 0.1 M sodium phosphate at pH 7 and 20 °C was prepared. Then, 10 mL (packed bed volume) of agarose-glutaraldehyde was added and the suspension was stirred very gently. As the end point to the enzyme−support reaction, after 24 h, the pH was increased to 8.5 and the derivatives were reduced by adding 200 mg of solid sodium borohydride (*24*, *25*). After 30 min, the enzyme derivative was washed with 0.1 M phosphate and distilled water.

Thermal Inactivation Experiments. Enzyme preparations were incubated at the desired pH, temperature, and enzyme concentration. Samples were withdrawn periodically, and their activity was analyzed as described above. Stability was defined as the half-life of the preparation and stabilization as the ratio between the half-lives of the compared enzyme preparations.

Stability Assays in the Presence of Hydrogen Peroxide. Enzyme derivatives (1 mg/mL of enzyme to decrease the impact of dissociation processes on enzyme inactivation) were incubated in 200 mM phosphate containing 5 mM hydrogen peroxide at pH 7.5 and 25 °C. Periodically, samples of these inactivation suspensions were withdrawn, washed with 5 volumes of 25 mM sodium phosphate at pH 7 and 4 °C, resuspended in 25 mM sodium phosphate buffer, and assayed as previously described.

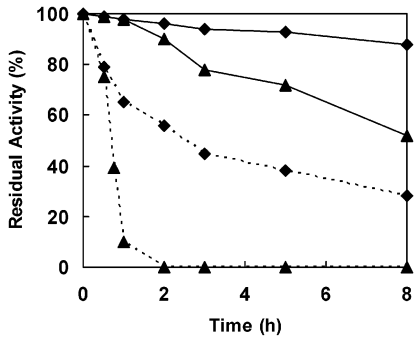

Figure 1. Effect of the enzyme concentration on the thermal inactivation of soluble RgDAAO and TvDAAO. The inactivation was carried out at 40 °C in 25 mM sodium phosphate buffer, pH 7.5. Solid lines: protein concentration corresponding to 0.15 mg/mL. Dotted lines: protein concentration of 50 μg/mL. Diamonds: TvDAAO. Triangles: RgDAAO.

SDS−PAGE Analysis of the Immobilized Enzymes. Immobilized DAAO preparation (100 mg) containing 0.2 mg of protein (or a control sample consisting of an equivalent amount of soluble protein) was boiled with 100 μL of 100 mM Tris-HCl buffer containing 2% (w/v) SDS and 5% (v/v) mercaptoethanol. This treatment is not able to break the secondary amine bonds formed between the protein and the support (*26*), but it is able to release any enzyme subunit that has not been covalently attached (*10*). The supernatant was analyzed by SDS-PAGE on 12% polyacrylamide gels (*28*) using a Hoefer Electrophoresis Apparatus (Amersham Biosciences). Gels were stained using Coomasie Brilliant Blue and analyzed by laser densitometry.

Results and Discussion

Inactivation of Soluble Enzymes. Figure 1 shows that the soluble TvDAAO is much more stable than RgDAAO (by approximately 8-fold) at a protein concentration of 0.15 mg/mL. Their stabilities were significantly reduced when diluted 5-fold, the dilution effect being more significant for TvDAAO than for RgDAAO. Nevertheless, TvDAAO remained more stable than RgDAAO (by 3-fold). This negative effect of dilution on enzyme stability suggests that some dissociation mechanism is occurring (e.g., FAD or subunit dissociation, see Scheme 1) (*3*).

At this point, the enzyme with the best stability seemed to be TvDAAO.

Immobilization of Enzymes on Different Supports. The immobilization of both DAAOs on highly activated glyoxyl agarose and glutaraldehyde agarose occurred rapidly, with no detectable activity in the supernatant after 30 min (results not shown). However, the immobilized enzymes were allowed to react with the support for 24 h before reduction. This long reaction time was used in order to increase the number of enzyme−support bonds (*27*, *29*), aiming to increase the enzyme stability. Table 1 shows that enzyme activity recovery was very high in all cases (over 80%).

Both DAAOs were also immobilized on poorly activated supports at low temperatures and short reaction times.

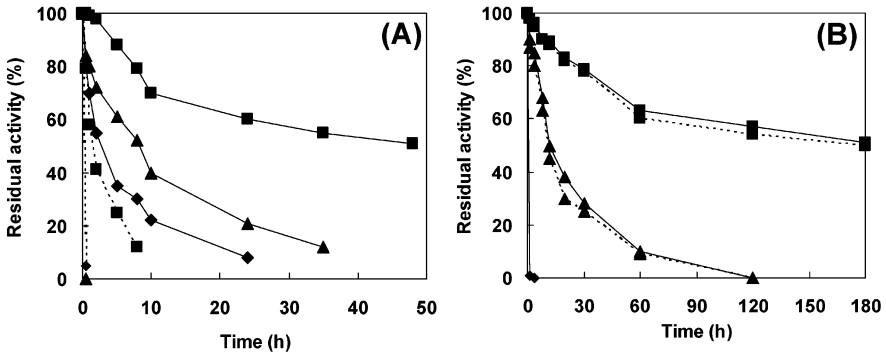

Figure 2. Thermal inactivation of different TvDAAO and RgDAAO derivatives at different enzyme concentrations. (A) TvDAAO derivatives. (B) RgDAAO derivatives. Solid lines: protein concentration corresponding to 0.2 mg/mL. Dotted lines: protein concentration of 6.7 μg/mL. Squares: highly activated glyoxyl derivatives. Triangles: highly activated glutaraldehyde. Diamonds: poorly activated glyoxyl derivatives. The inactivations were carried out at 40 °C, pH 7.5.

Scheme 1. General Mechanisms of Enzyme Inactivation

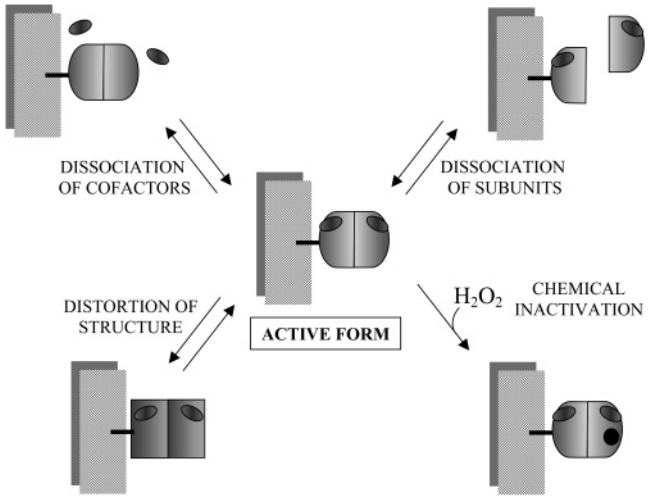

Table 1. Results Achieved in the Immobilization of RgDAAO and TvDAAO onto Different Supports[a]

enzyme source	support	immobilization yield (%)	recovered activity (%)
RgDAAO	glyoxyl (220 μmol/mL)	100	85
	glyoxyl (5 μmol/mL)	<5	~100
	glutaraldehyde (220 μmol/mL)	100	90
TvDAAO	glyoxyl (220 μmol/mL)	100	95
	glyoxyl (5 μmol/mL)	<5	~100
	glutaraldehyde (220 μmol/mL)	100	95

[a] Immobilizations were carried out as described in Materials and Methods (enzyme concentration = 2 mg/mL)

In this case, immobilization yields were very low and the intrinsic activity of immobilized enzyme remained unaltered.

Thermal Stability of DAAO Preparations. Derivatives of both DAAOs prepared on poorly activated supports exhibited a stability similar that of soluble enzymes at similar protein concentrations (data not shown). Moreover, a dilution effect on enzyme stability was found, similar to that of the soluble enzyme (results not shown)

However, as shown in Figure 2, the immobilization on highly activated glutaraldehyde supports improved the enzyme stability by 2-fold for TvDAAO and 60-fold for RgDAAO (compared with the enzyme immobilized on poorly activated supports) when using 0.2 mg/mL protein solutions. Derivatives on highly activated glyoxyl supports became even more stabilized (10-fold for TvDAAO and approximately 40-fold for RgDAOO) than the glutaraldehyde preparations at 0.2 mg/mL. Dilution of these enzyme preparations by 30-fold promoted a decrease in the stability of all immobilized TvDAAO preparations, although not as significant as in the case of the soluble enzyme. On the other hand, this dilution had no relevant effect on the stability of immobilized RgDAAO preparations. This optimized immobilized RgDAAO preparation was 15 000 times more stable than either the soluble enzyme or RgDAAO immobilized on poorly activated glyoxyl supports at a protein concentration of 6.7 μg/mL.

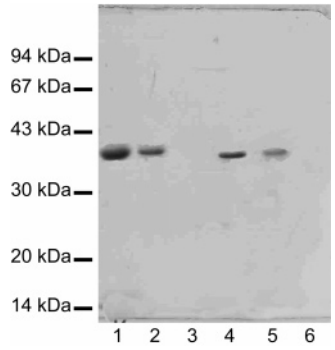

Figure 3. SDS-PAGE analysis of protein subunits released by DAAO derivatives. Samples were obtained by boiling the immobilized preparations in the presence of SDS as described in Materials and Methods. Lane 1, soluble TvDAAO; lane 2, TvDAAO immobilized onto poorly activated glyoxyl; lane 3, TvDAAO immobilized on highly activated glyoxyl; lane 4, soluble RgDAAO; lane 5, RgDAAO immobilized onto poorly activated glyoxyl; lane 6, RgDAAO immobilized on highly activated glyoxyl.

To investigate the reason for this dilution effect on the stability of immobilized DAAOs, the supernatant obtained from boiling the enzyme preparations in the presence of SDS and mercaptoethanol was analyzed by SDS-PAGE. Figure 3 shows the protein subunits released from glyoxyl supports. Using highly activated glyoxyl supports, neither TvDAAO nor RgDAAO released any subunits after the treatment, suggesting that both subunits had become covalently attached to the support through at least a single point. However, both DAAOs immobilized on poorly activated supports released protein subunits to the supernatant, giving a band with an intensity of approximately 40−50% of the intensity that corresponds to the total amount of immobilized enzyme. This result shows that in this derivative, most proteins were immobilized by only one subunit.

Thus, RgDAAO immobilized on highly activated supports presented a fully stabilized quaternary structure, explaining the negligible effect of dilution on the enzyme stability. On the other hand, TvDAAO immobilized on

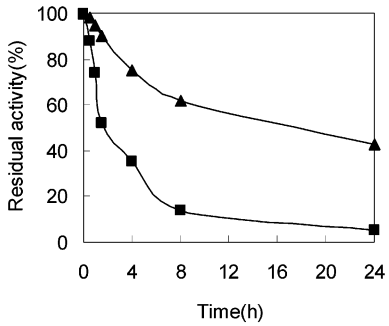

Figure 4. Effect of the presence of FAD on the thermal inactivation of TvDAAO immobilized on highly activated glyoxyl. Inactivations were carried out at 42 °C and pH 9.0. Suspensions contained 67 μg of protein/mL. Triangles: inactivation carried out in the presence of 50 mM FAD. Squares: inactivation carried out in the absence of exogenous FAD.

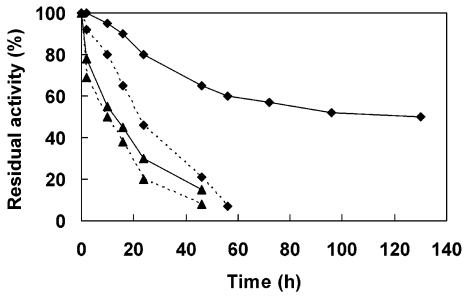

Figure 5. Effect of the presence of 5 mM H_2O_2 on the thermal inactivation of TvDAAO and RgDAAO derivatives. Experimental conditions were pH 7.5 and 25 °C. Suspensions contained 1 mg of protein/mL. Triangles: TvDAAO derivative. Diamonds: RgDAAO derivative. Solid line: derivatives prepared with highly activated glyoxyl agarose. Dotted line: derivatives prepared with poorly activated glyoxyl agarose.

these supports also has a stabilized quaternary structure, but its stability still depends on dilution. This suggests that a dissociation mechanism different from subunit dissociation was taking place. After checking the effect of FAD on TvDAAO stability (Figure 4), we found that this compound conferred a high degree of stabilization on this preparation, while no effect was found in the case of RgDAAO (results not shown). Thus, reversible loss of FAD seemed to be the key point in the inactivation of TvDAAO immobilized on highly activated glyoxyl agarose.

Stability of the Immobilized Preparations against Hydrogen Peroxide. Both enzymes proved to be very sensitive to the presence of hydrogen peroxide (Figure 5). Nevertheless, RgDAAO immobilized via one subunit resulted in a 2-fold greater stability than TvDAAO. This difference increased when using preparations immobilized on highly activated glyoxyl agarose, which presented a significant stabilizing effect for RgDAAO (more than 5-fold) while having a marginal effect on the stability of TvDAAO. Thus, immobilized RgDAAO became almost 10 times more stable than immobilized TvDAAO.

Conclusions

The results presented in this paper show that not only the stability but also the mechanism of enzyme inactivation should be considered when selecting an enzyme as an industrial biocatalyst. The use of physicochemical tools that are required to prepare these biocatalysts (e.g., immobilization), if properly designed, can decrease the impact of some of these mechanisms and even completely avoid them. Nevertheless, in some other instances, the immobilization tools may be inefficient. Thus, the evaluation of the final enzyme preparation may be more convenient than the evaluation of the soluble enzyme.

In the example used in this paper, TvDAAO initially seemed to be the best option in terms of stability. However, after multipoint and multisubunit covalent immobilization, RgDAAO became the most stable of the two enzymes. This can be explained because, in the case of RgDAAO, FAD seemed to be strongly bonded to the enzyme ($K_d = 2.0 \times 10^{-8}$ M) (*30*), while in the case of TvDAAO, FAD seems to be reversibly released from the active enzyme since the enzyme is stabilized by the addition of FAD (K_d between 10^{-6} and 10^{-7} M) (*31*). Stabilization of proteins that become inactivated by cofactor dissociation may not be easily achieved by immobilization techniques, but there are other tools that may be helpful. For example, a recent elegant publication (*32*) reports the use of site-directed mutagenesis and chemical modification to covalently fix the cofactor to the active center of human DAAO.

To take advantage of these positive effects of immobilization, it was necessary to use adequate immobilization systems and conditions. Thus, both the activation degree of the support (high activation degrees are preferred) and the suitability of the groups introduced in the support (optimal results were obtained using glyoxyl agarose) were found to be critical points to be considered in preparing stable DAAO derivatives. A detailed description of the features that make glyoxyl agarose adequate for enzyme immobilization may be found in the literature (*33*).

The high thermostability of the optimal RgDAAO preparation, together with its high resistance to the action of hydrogen peroxide, made this preparation very suitable for use in some of the industrial processes discussed in the Introduction.

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