Process Biochemistry 122 (2022) 120–128

Contents lists available at ScienceDirect

Process Biochemistry

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

Immobilization-stabilization of the dimeric *D*-amino acid oxidase from porcine kidney

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ARTICLE INFO

Keywords:
Enzyme immobilization/stabilization
Enzyme subunit dissociation
Glutaraldehyde versatility

ABSTRACT

This paper shows the immobilization of the dimeric D-amino acid oxidase (DAAO) following different methodologies. Immobilization on glyoxyl-agarose yielded activity recoveries under 5%, and it was discarded. The anion exchange on aminated supports permits the rapid enzyme immobilization, but its activity decreased and the final biocatalysts is less stable than the free enzyme, suggesting that the near presence of a cationic surface is negative for enzyme performance. Treating this biocatalyst with glutaraldehyde led to the full enzyme inactivation. However, immobilization at pH 7 on glutaraldehyde preactivated agarose beads permitted very high stabilizations (enzyme dissociation is prevented) with expressed activity near 95%. The immobilization at pH 9 produced a slightly higher stabilization, but the expressed activity was 70%. The treatment of the biocatalyst with polyethylenimine produced the slowdown of the initial enzyme inactivation. The use of vinyl sulfone agarose permitted the immobilization of the enzyme, but even immobilizing the enzyme at pH 5 the expressed activity was 50%. The blocking step with aspartic permitted to maintain the activity and stability of the unblocked biocatalyst, while the aminated compounds led to enzyme destabilization/inactivation. Considering all factors, immobilization on glutaraldehyde support at pH 7 seems to be the recommended immobilization protocol for DAAO.

1. Introduction

Enzymes are potential biocatalysts of multiple reactions of interest in many different industrial areas [1–6]. However, they have many limitations imposed by their biological origin, which have delayed their implementation [7]. That way, enzymes are soluble molecules, with moderate stability under physiological conditions, and very active, selective and specific for their physiological substrate and reaction. However, in industry, one needs a biocatalyst with these features for non-physiological substrates and to be able to function under non-physiological conditions. In many instances they will not be used to catalyze their physiological reaction (e.g., use of hydrolases as catalysts in kinetically controlled synthesis [8] or as catalysts of promiscuous reactions [9–13]). Fortunately, the researcher has many different tools to overcome these limitations, whose power has given a huge leap in the last years. That way, all enzyme biodiversity may be available to the researcher thanks to metagenomics [14–18], and the enzyme targeted properties may be improved via site-directed mutagenesis [19,20], or imitating an accelerated natural evolution, via directed evolution [21–23]. Enzyme physicochemical modification enables a final polishing of the enzyme features [24–28].

In this context, enzyme immobilization was initially developed to solve the problem of enzyme solubility [29,30]. This first objective of enzyme immobilization is nowadays surpassed, as a proper immobilization permits to greatly improve other enzyme features, such as stability, activity, selectivity or specificity [31–35]. It may also reduce inhibitions, increase the resistance versus deleterious chemicals, and increase the operation window of the enzyme [36–38]. Moreover, a properly designed immobilization protocol may be coupled to enzyme purification, saving costs and time [39]. The potential of the joint application of different techniques to design a proper enzyme may be exemplified when designing enzymes bearing several active centers [40], one of the outstanding examples being plurizymes [41–43]. In the case of immobilization, it can be combined with any other strategy to

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https://doi.org/10.1016/j.procbio.2022.10.002
Received 1 September 2022; Received in revised form 26 September 2022; Accepted 2 October 2022
Available online 4 October 2022

improve the enzyme features, such as metagenomics, use of thermophilic enzymes, directed mutagenesis or chemical modification [35,44–51]. Whatever the case, to benefit from all the advantages that immobilization can provide, the immobilization protocols must be adequately designed and controlled [52].

D-amino acid oxidases (DAAOs) are dimeric flavine adenine dinucleotide (FAD)-containing flavoenzymes that oxidize *D*-amino acids. They catalyze the oxidative deamination of *D*-amino acids yielding the corresponding imino acid, which is spontaneously hydrolyzed to yield the corresponding α-keto acid and ammonia [53–58]. The re-oxidation of FAD produces hydrogen peroxide, being this an advantage when the enzyme is utilized in biosensors but it is a problem when the enzyme is utilized as biocatalyst [59]. DAAOs have found application in diverse sectors, such as analytical chemistry (biosensors) [60–63] or in the production of keto-acids [64–66] and glutaryl 7- amino cephalosporanic acid [67–71]. The hydrogen peroxide oxidation of the reaction product in the cephalosporin C modification by DAAO is positive, as this yield 7-amino cephalosporanic, which is a good substrate for glutaryl acylase, while keto-adipic derivative is not a good substrate for the enzyme. New routes for synthetic cephalosporins production have been proposed to avoid this matter [72]. There are some publications even preparing fusion enzymes for this cephalosporin production (coupling DAAO+-glutaryl acylase), even with the problems that this fusion protein may raise [40]. Among the available DAAOs, the one from pig kidney presents special interest [73–75].

The immobilization of this enzyme may be key to facilitate their implementation. As a dimeric protein, it is interesting to immobilize both enzyme subunits on the support, to avoid the release of some subunits to the supernatant and to prevent the contamination of the reaction product [34]. This is due to two reasons. First, it has been reported that the first step of the multimeric enzyme inactivation under certain conditions (e.g., certain pH values) may be subunits dissociation, making the enzyme to become less and less stable when it is more and more diluted. Second, even if subunit dissociation is not related to enzyme stability, the release of one enzyme subunit to the medium will produce the contamination of the product by the enzyme, one of the points that one wants to avoid using an immobilized enzyme [34].

Our objective is the stabilization of this enzyme via immobilization. To reach this goal, we have utilized some of the activated supports described in the literature as suitable for this goal [36]. First, we have tried glyoxyl-agarose beads. Glyoxyl groups produce reversible imino bonds with the amino groups of Lys and the terminal amino of the protein, and enzymes are not fixed to the support by just one point [76,77]. That makes immobilization of the enzyme at alkaline pH value (to have not ionized the amino groups of the lateral chain of Lys) compulsory, except when the enzyme bears two low pK amino groups in the same face, (e.g. two amino terminal), when the enzyme may be immobilized even at neutral pH values. In this circumstance, immobilization may be coupled to purification [78,79]. Second, the enzyme has been immobilized on glutaraldehyde supports. They are very versatile supports, as they are heterofunctional ones that permit the first immobilization via ion exchange or a covalent reaction, that way altering the orientation of the enzyme on the support [37,80–84]. It is also possible to immobilize the enzyme on pre-activated supports or to treat the previously ionically adsorbed enzyme with glutaraldehyde, being the latter the protocol that usually produces the highest stabilizations [37,80–84]. Finally, we have tried vinyl sulfone (VS) supports. VS can react with non-ionized amino groups (primary or secondary), but also with imidazole (from His), thiol (from Cys) or phenol (from Tyr), and it has been described as a potent tool to stabilize the enzymes via multipoint covalent attachment [85,86]. Using this support, immobilization conditions will mark the orientation of the enzyme on the support surface, long term incubation will facilitate the enzyme-support multi--interaction (determining the intensity of the multipoint covalent attachment) and the final blocking step will prevent undesired enzyme-support covalent reactions, [85,86] while also permitting a final tuning of the enzyme stability and activity by altering the immobilized enzyme-support interactions [87–89]. These interactions, together with producing conformational changes and altering the enzyme features, can alter even the enzyme inactivation pathway [90]. Finally, this blocking step also permits to coimmobilize enzymes on the support via different events, even enabling the reuse of the most stable enzymes, always that they are covalently immobilized and the least stable enzymes only physically adsorbed [91,92]. This is especially relevant for this enzyme than can be coupled to diverse enzymes, as stated above. During the studies, enzyme activity and stability have been the studied variables. Agarose has been utilized in this first approach, as it is an inert support and the only reactive groups will be those introduced by the user [93], facilitating the understanding of the results [52]. The use of agarose 4BCL may permit the future DAAO coimmobilization with large enzymes, such as catalases [64–66],

2. Materials and methods

2.1. Materials

4BCL agarose beads standard was purchased from ABT (Burgos, Spain). 2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS®) was purchased from Roche (Mannheim, Germany). Glutaraldehyde solution grade I (GLU), 25% (v/v) in water, ethylenediamine (EDA), *D*-(+)-glucose, cysteamine, *L*-aspartic acid (Asp), ethanolamine (ETA), *L*-Cysteine (Cys), dextran sulphate (DS) from *Leuconostoc* spp (Mw, 9000–20,000 Da), branched polyethylenimine (PEI) (Mw 25,000 Da) and *D*-amino acid oxidase from porcine kidney (DAAO) (7.2 U/mg of powder) were acquired from Sigma Aldrich (Madrid, Spain). Alfa Aesar (Heysham, UK) provided *D*-alanine. Glycine, flavin adenine dinucleotide (FAD) disodium salt, divinyl sulfone (DVS) and horseradish peroxidase (268 U/mg of protein) were acquired from Fisher scientific Spain (Madrid, Spain). Low molecular weight (LMW)- sodium dodecyl sulfate (SDS) markers (14.4–97.0 kDa) for electrophoresis reference were purchased from GE Healthcare Life Sciences (Madrid, Spain). Protein concentration was determined using Bradford's method using bovine serum albumin as standard [94]. All other reagents were of analytical grade. Monoaminoethyl-N-aminoethyl (MANAE) agarose was produced as previously described [95].

2.2. Methods

2.2.1. Determination of DAAO activity

The activity of DAAO was determined via ABTS®/peroxidase assay using a spectrophotometer thermoregulated at 25 ºC with magnetic stirring. The ABTS® oxidation catalysed by the peroxidase was caused by the hydrogen peroxide released in the DAAO oxidation reaction was monitored by the change in absorbance at 414 nm ($\varepsilon_{414} = 36{,}000\ M^{-1}\ cm^{-1}$ under these conditions)[96]. It was performed using 2.2 mL of 100 mM sodium carbonate at pH 8.3 containing 200 μL of *D*-alanine (using as an standard, although the enzyme can recognize a battery of different d-amino accids) [73–75] at 200 mM, 100 μL of ABTS® at 10 mg/mL prepared in 100 mM sodium phosphate at pH 7.0 and 50 μL of horseradish peroxidase at 0.1 mg/mL prepared in 100 mM sodium phosphate at pH 6.0. The reaction started when 10–100 μL of the enzyme sample were added. One unit (U) of activity was defined as the amount of enzyme that oxidizes 1 μmol of substrate per minute under the specified conditions. It was checked that using double and half concentration of peroxidase, the determined DAAO activity was identical.

2.2.2. Enzyme immobilization

DAAO immobilization was characterized by the immobilization course, immobilization yield and expressed activity as recommended by Boudrant et al. [97]. All enzyme immobilizations were performed following the activity of supernatant, suspension and a reference

suspension of the enzyme under identical conditions where inert agarose was used (the enzyme was not immobilized on this support) [97].

2.2.3. Immobilization of DAAO on glyoxyl agarose beads

The immobilizations were performed at pH 10.0, using 50 mM sodium carbonate and employing two different temperatures; 4ºC and 25ºC [77]. DAAO was immobilized using 2 mg of enzyme per g of support, in a proportion 1/10 g of support per mL of enzyme solution. In some instances, 0.11 mM FAD was added.

2.2.4. Immobilization of DAAO via anionic exchange on MANAE-support

DAAO (final concentration 2 mg enzyme/g of support) was dissolved in 5 mM sodium acetate at pH 5.0, 5 mM sodium phosphate at pH 7.0 or 5 mM sodium carbonate at pH 9.0 at 25 ºC. Then, 1 g of MANAE support was added to 10 mL of enzyme solution. The solutions were kept in orbital stirring for 2 h. The enzymatic activity of the suspension, supernatant and reference was followed during the whole process [97]. After the immobilization, the biocatalyst was washed several times with distilled water and stored at 4–6 ºC.

In some instances, the immobilized DAAO was suspended in a solution containing 1% (v/v) of glutaraldehyde (GLU) in 50 mM of sodium phosphate at pH 7.0 and 25 ºC for 1 h. This treatment guarantees the modification of all primary amino groups in the support and in the enzyme mainly with just one molecule of glutaraldehyde [98]. The modified biocatalysts were washed several times with distilled water using a sintered filter to eliminate the excess of glutaraldehyde and stored at 4 − 6 °C.

2.2.5. Immobilization of DAAO on glutaraldehyde-MANAE- agarose beads

First, MANAE- agarose [95] was incubated for 16 h at 25 ºC and pH 7 in 10% (v/v) glutaraldehyde to introduce two glutaraldehyde molecules per amino residue [98,99].

Immobilization on pre-activated glutaraldehyde-MANAE-agarose was performed using 5 g of support per 50 mL of enzyme solution (0.2 mg/mL) prepared in 5 mM sodium acetate buffer pH 5.0, 5 mM sodium phosphate buffer at pH 7.0 or 5 mM of sodium carbonate buffer at pH 9.0 for 1 h. The enzymatic activity of the suspension and supernatant was followed during the whole process using the ABTS® assay described above. Then, the biocatalyst was washed with 50 mM sodium phosphate at pH 8 and incubated for 16 h, then they were washed with distilled water, vacuum dried and stored at 4–6 ºC.

2.2.6. Immobilization of DAAO on vinyl sulfone-agarose (VS-agarose) support

The 4% BCL agarose beads were activated with DVS according to the methodology previously described by Santos et al. [85] with some modifications. A mass of 1 g of 4% BCL agarose beads was added to 20 mL of 350 mM DVS in 333 mM sodium carbonate at pH 11.5. The suspension was kept in orbital stirring for 2 h and then thoroughly washed with distilled water and stored at 4–6 °C.

DAAO (0.2 mg/mL) was immobilized on VS-agarose support using a ratio of 1/10 g of support/mL of enzyme solution prepared in 5 mM sodium acetate buffer pH 5.0, 5 mM sodium phosphate buffer at pH 7.0 or 5 mM of sodium carbonate buffer at pH 9.0. During the process the activities of the suspension, supernatant and a reference suspension were followed using the ABTS® assay described above. As a reaction end point, the remaining VS groups on the support were blocked with 1 M of different agents at 25ºC and pH 8.0 during 24 h; Asp, Gly, EDA, ETA, *D*-glucose, *D*-glucosamine, Cys or cysteamine. Then, the biocatalysts were washed with distilled water, vacuum dried and stored at 4–6 ºC.

2.2.7. Physical modification of immobilized enzymes with some ionic polymers

A mass of 1 g of biocatalysts was suspended in 10 mL of a 10% (w/v) PEI solution (Mw 25,000) at pH 7.0 at 4 °C during 18 h, or 10% (w/v) dextran sulphate solution (Mw 9000–20,000) dissolved in 50 mM Tris-HCl at pH 7.0 and 25 °C during 24 h [100]. Afterward, the modified biocatalysts were washed several times with abundant distilled water to eliminate any excess polymer and put in storage at 4 − 6 °C.

2.2.8. Thermal inactivation of the different biocatalyst

The immobilized enzyme samples were suspended in 10 mM Tris-HCL at pH 7.0. Samples were periodically withdrawn, and the residual activity (the percentage of activity at that time divided by the initial one) was quantified using the ABTS® assay. The inactivation temperatures were selected to ensure reliable inactivation courses.

2.2.9. SDS-PAGE of different enzyme preparations

SDS-PAGE analyses were carried out following Laemmli's protocol [101]. The protein samples were diluted in 4% SDS (w/v) and 10% mercaptoethanol (v/v) to have a protein concentration of 0.3 − 0.5 mg of protein/mL solution in the samples. This suspension was boiled for 8 min. The support was discarded after centrifuging the suspension at 10, 000 rpm for 2 min. After taking 15 μL aliquots of the supernatants of each sample, they were utilized to carry out the SDS-PAGE analysis and 5 μL of low-molecular-weight marker proteins (LMW-SDS Marker 14.4 −97 kDa), which was run at 100 V. Finally, gels were stained utilizing Coomassie brilliant blue stain.

3. Results and discussion

3.1. Immobilization of DAAO on glyoxyl agarose beads

The immobilization of the enzyme was tried at pH 10, but a rapid enzyme inactivation could be observed at both 4 and 25ºC at this pH value, the addition of free FAD does not mitigate this inactivation (Fig. 1). When the immobilization was intended, the sodium borohydride reduced biocatalyst exhibited a negligible activity (under 5%). Then, considering that this is a multimeric enzyme, the immobilization was tried at pH 7 and 8 (results not shown). However, enzyme immobilization was not found. Fig. 2 shows that this enzyme has the two terminal amino groups in opposite planes, making enzyme immobilization by both terminal amino groups, and thus, at these neutral pH values, unfeasible [67]. That way, this immobilization protocol must be discarded for this enzyme, although it was useful for other DAAOs [102].

3.2. Immobilization of DAAO on MANAE-agarose beads

Fig. 3 shows the immobilization course of DAAO on MANAE agarose beads at pH 5, 7 and 9. Immobilization proceeded rapidly at all pHs values. Curiously, the activity decreased after immobilization, more when performed at pH 5 or 7 than when performed at pH 9. We selected

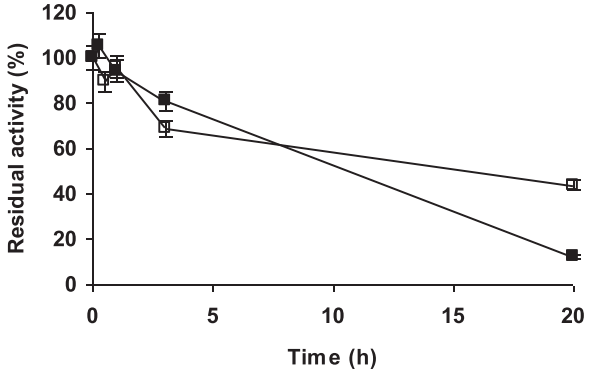

Fig. 1. Immobilization courses of DAAO on glyoxyl-agarose beads. Immobilization was performed at 50 mM sodium carbonate pH 10.0 using 2 mg DAAO/g of support and 0.11 mM of FAD. **(a)**: 25 ºC, **(b)**: 4 ºC. Other specifications are described in Materials and methods section. Solid squares: reference at 25 ºC; Empty squares: reference at 4 ºC.

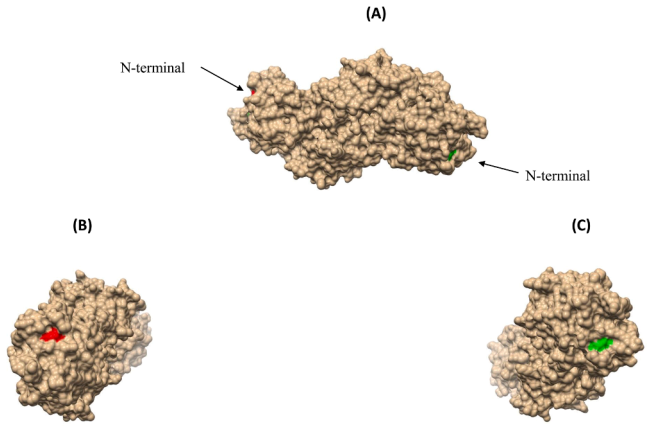

Fig. 2. Structure of D-amino acid oxidase from porcine kidney (DAAO). The figure shows a surface diagram of DAAO showing some amino acids: **(a)**: Structure of DAAO; **(b):** Red: N-terminal residue in the helix A; **(c)**: Green: N-terminal residue in the helix B.

the enzyme immobilized at pH 9 to analyze the enzyme stability (Fig. 4). The resulting biocatalyst was less stable than the free enzyme, suggesting that this could not be a good immobilization method for the enzyme.

To improve enzyme stability, the immobilized enzyme was treated with 1% glutaraldehyde, trying to get a multipoint covalent immobilization. Unfortunately, the enzyme was fully inactivated after this treatment.

That way, it looks that the enzyme interaction with the amino groups in the support produced a strong enzyme destabilization. Therefore, these immobilization protocols were also discarded for this enzyme.

3.3. *Immobilization of DAAO on glutaraldehyde-MANAE-agarose beads*

Next, we utilized the pre-activated glutaraldehyde support. The presence of glutaraldehyde dimers over the amino groups could perhaps reduce the negative enzyme-aminated surface interactions. The immobilization courses of the enzyme at pH 5, 7 and 9 on the glutaraldehyde preactivated support may be found in Fig. 5. Immobilization was rapid in all cases, and initially an increment on the enzyme activity could be obserbed, perhaps due to some positive conformational changes of the enzyme structure indued by the immobilization. These increases in activity were lost after some incubation hours. After the overnight incubation at pH 8, the enzyme immobilized at pH 7 gave 95% of expressed activity, while at pH 5 and pH 9 the expressed activity was around 70% (Table 1). This suggested that, as it is described in other instances in the literature, the immobilization pH may affect the enzyme-support interactions by altering the enzyme orientation [37,38,82,84,103].

Fig. 6 shows that the stabilities of the 3 biocatalysts were much higher than those of the free enzyme, the enzyme immobilized at pH 9 was slightly more stable than the enzyme immobilized at pH 7, but this did not justify the decrease in activity observed for this biocatalyst preparation. That way, the enzyme immobilized at pH 7 on the pre-activated glutaraldehyde support was selected as optimal immobilization protocol.

In order to investigate if the reason for this stabilization is the prevention of the free enzyme subunit dissociation (described to be relevant for the enzyme inactivation of this enzyme under these conditions [104], a SDS-PAGE of the biocatalyst was performed. Fig. 7 shows that the immobilized biocatalysts did not release any enzyme subunit to the supernatant after this treatment, confirming that both enzyme subunits were covalently attached to the support.

Next, we investigated the effects of the enzyme coating with poly ionic polymers on enzyme stability. This treatment can further stabilize some enzymes in certain cases [28]; moreover, this may be a strategy to coimmobilize DAAO and other enzymes [105–111], (for example catalase or glutaryl acylase, as discussed in the introduction section), enabling the reuse of DAAO (that will be covalently immobilized) if it turned out to be the most stable enzyme after the inactivation of the other coimmobilized enzymes. The treatments produced a decrease by around 2/3 of the activity of the enzyme activity using poly-cationic (PEI) or poly anionic polymers (DS) (Table 2), perhaps due to the decrease of oxygen in the enzyme environment cause by the ionic polymers [112]. The enzyme stability using DS decreased (Fig. 8), but still the immobilized enzyme stability was above that of the free enzyme. The PEI coating slowed down the enzyme inactivation in the first moment, while after three hours of inactivation, the residual activity decreased below that of the residual activity of the unmodified enzyme (Fig. 8). In any case, it might be an adequate strategy to treat immobilized DAAO to coimmobilize enzymes [105–111]. This effect seems to

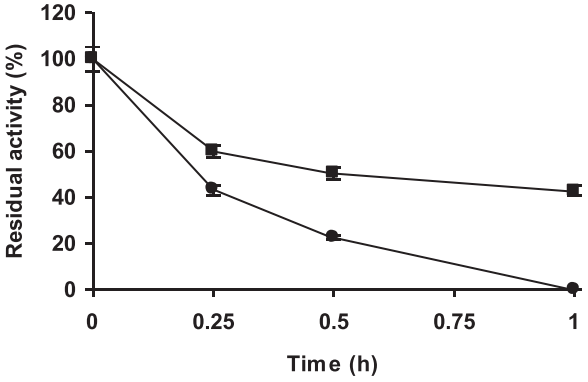

Fig. 4. Thermal inactivation of DAAO immobilized on MANAE agarose support compared to the free enzyme. The inactivation was performed in 10 mM of Tris-HCL at pH 7.0 and 40ºC. Other specifications can be found in the Materials and methods section. Solid squares: Free enzyme (0,2 mg/mL) and solid circles: Biocatalyst immobilized at pH 9.0.

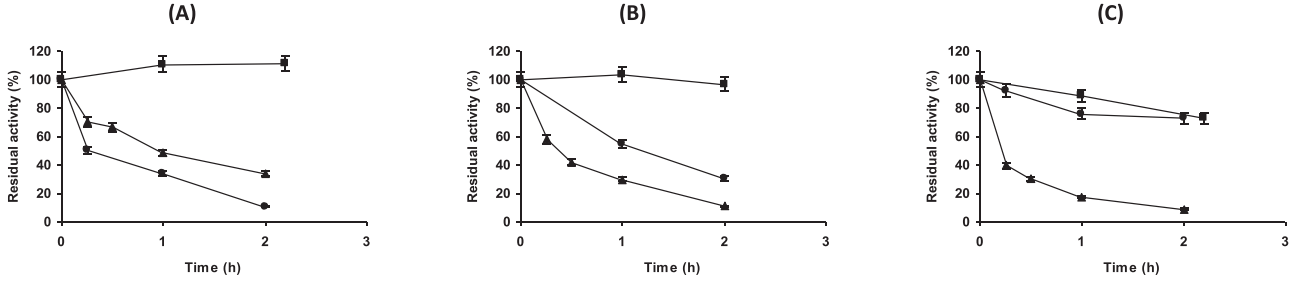

Fig. 3. Immobilization courses of DAAO (0.2 mg/mL) on MANAE agarose support at 25 ºC. The immobilization was performed using: **(a)** 5 mM sodium acetate buffer at pH 5.0, **(b)** 5 mM of sodium phosphate buffer at pH 7.0 and **(c)** 5 mM of sodium carbonate buffer at pH 9.0. Other specifications can be found in the Materials and methods section. Solid squares: reference; Solid circles: suspension and solid triangles: supernatant.

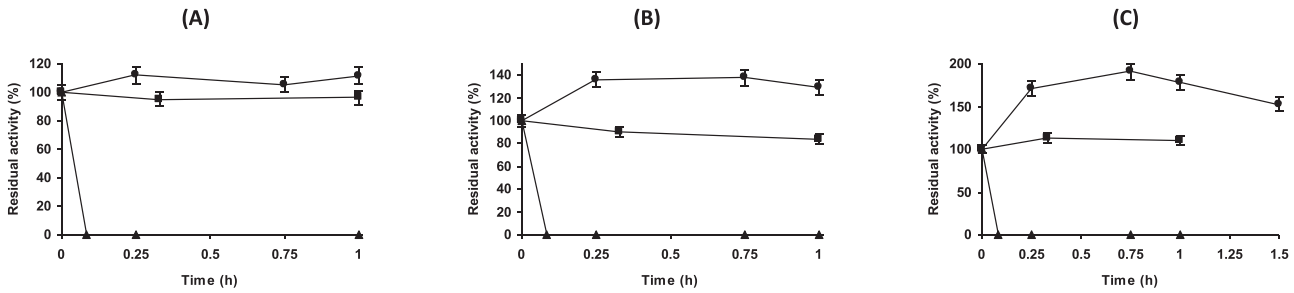

Fig. 5. Immobilization courses of DAAO (0.2 mg/mL) on glutaraldehyde agarose support at 25 ºC. The immobilization was performed at: **(a)** 5 mM sodium acetate buffer at pH 5.0, **(b)** 5 mM of sodium phosphate buffer at pH 7.0 and **(c)** 5 mM of sodium carbonate buffer at pH 9.0. Other specifications can be found in the Materials and methods section. Solid squares: reference; Solid circles: suspension and solid triangles: supernatant.

Table 1
Effect of the immobilization pH in the hydrolysis of *D*-Alanine at pH 7.0 and 25 °C. The data are given as relative activity, considering the activity of the free enzyme (0,2 mg/mL) as 100%. Experiments were conducted as described in methods.

Biocatalyst	Relative activity (%)
Ag-GLU-DAAO (pH 5.0)	67.61 ± 3.38
Ag-GLU-DAAO (pH 7.0)	94.50 ± 4.72
Ag-GLU-DAAO (pH 9.0)	70.09 ± 3.50

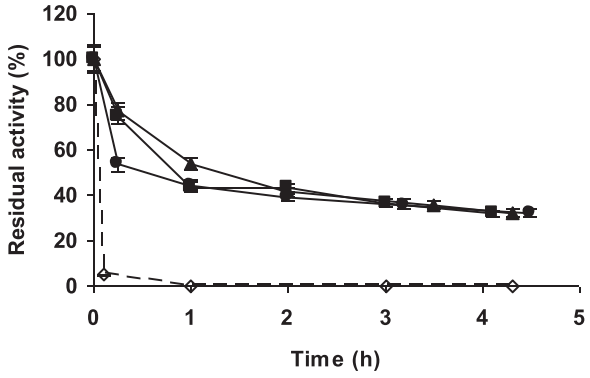

Fig. 6. Thermal inactivation of DAAO immobilized on glutaraldehyde agarose support and free enzyme (0.2 mg/mL). The inactivation was performed in 10 mM of Tris-HCL at pH 7.0 and 48ºC. Other specifications can be found in the Materials and methods section. Solid line and solid squares: Immobilized at pH 5.0; Solid line and solid circles: immobilized at pH 7.0; Solid line and solid triangles: immobilized at pH 9.0 and dotted line and empty rhombus: Free enzyme.

contradict the effect of cationic surfaces on enzyme stability, but it should be considered that while the support surface is rigid the PEI molecule is flexible.

3.4. Immobilization of DAAO on VS-agarose

The immobilization courses of DAAO on VS-agarose beads are shown in Fig. 9. At pH 5, the immobilization was total after 3 h, maintaining the enzyme activity almost intact. At pH 7, the immobilization rate was similar, but the costs in terms of activity were much higher, perhaps by a more intense multipoint covalent attachment at this pH value. The situation was more drastic at pH 9. If the enzyme was incubated to have more enzyme-support attachments, almost all activity was gone.

We compared the stabilities of the unblocked biocatalyst prepared at pH 5 and 7. Due to some problems in the blocking step (see below), this was performed using the unblocked biocatalysts. Fig. 10 shows that the enzyme immobilized at pH 5 was more stable than the enzyme

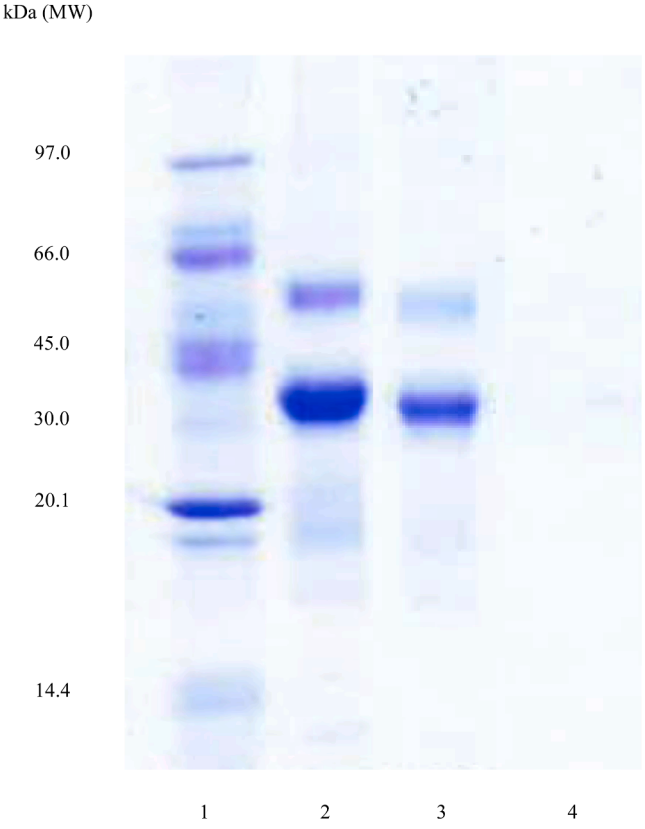

Fig. 7. SDS-PAGE gels of the analysis of free enzyme, MANAE-agarose biocatalyst and glutaraldehyde agarose biocatalyst. Lane 1: Molecular weight markers; Lane 2: Free enzyme (1 mg/mL); Lane 3: MANAE-agarose biocatalyst; Lane 4: DAAO (0,2 mg/mL) immobilized on glutaraldehyde agarose support. Other specifications can be found in the Materials and methods section.

Table 2
Effect of the polyethyleneimine (PEI) and dextran sulphate (DS) treatment of the DAAO biocatalyst in the hydrolysis of *D*-Alanine at pH 7.0 and 25 °C. The data are given as relative activity, considering the activity of the free enzyme (0,2 mg/mL) (0.657 U/mg) as 100%. Experiments were conducted as described in methods.

Biocatalyst	Relative activity (%)
Ag-GLU-DAAO-PEI	32.18 ± 1.60
Ag-GLU-DAAO (pH 7.0)	94.50 ± 4.72
Ag-GLU-DAAO-DS	28.36 ± 1.41

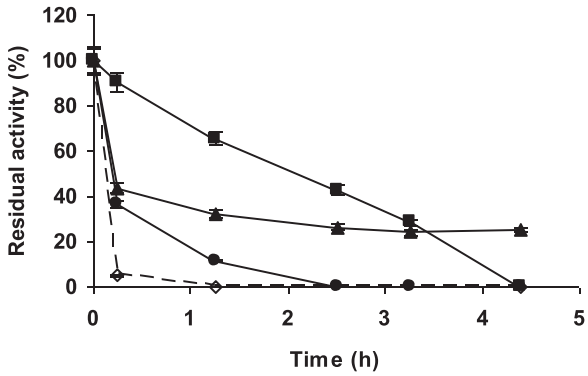

Fig. 8. Thermal inactivation of DAAO immobilized on glutaraldehyde agarose support at pH 7.0 and free enzyme (0.2 mg/mL). The inactivation was performed in 10 mM of Tris-HCL at pH 7.0 and 48 ºC. Other specifications can be found in the Materials and methods section. Solid line and solid squares: Biocatalyst modified with PEI; Solid line and solid circles: Biocatalyst modified with DS; Solid line and solid triangles: Biocatalyst immobilized at pH 7.0 and dotted line and empty rhombus: Free enzyme.

immobilized at pH 7, although at this pH the enzyme-support reactivity was higher and, therefore, the possibilities of getting a higher multipoint covalent attachment should be increased. Table 3.

That way, the enzyme immobilized at pH 5 was used to analyze the effect of the blocking agent on the enzyme activity and stability. Some of the biocatalysts were fully inactive (blocked using EDA, ethanolamine or Cys). The incubation of the optimal glutaraldehyde preparation on this media did not produce any effect on enzyme activity, suggesting that the enzyme-support surface interactions are responsible for this enzyme inactivation. Many other blocking agents significantly decreased the expressed activity [86] of the biocatalyst. Only the blocking with Asp maintained the activity of the unblocked biocatalyst. Fig. 10 shows the inactivation courses of the different biocatalysts. While using other enzymes the blocking step is highly recommended [36,75], in this instance only the blocking with Asp permitted to maintain the enzyme stability at values similar to those of the unblocked biocatalyst. This biocatalyst was much more stable than the free enzyme. The SDS-PAGE of these biocatalysts confirms that both enzyme subunits had been attached to the enzyme (results not shown).

3.5. Comparison of optimal GLU and VS biocatalysts

Fig. 11 shows the inactivation of both immobilized biocatalysts for a direct comparison. The stability of the glutaraldehyde biocatalysts was lower than that of the VS biocatalysts. However, the difference was not as large as to compensate the 50% versus 95% expressed activity for both biocatalysts. That way, it seems that although both may be valid, DAAO immobilized at pH 7 on preactivated glutaraldehyde support could be the recommended protocol for this enzyme considering activity/stability.

4. Conclusions

The enzyme DAAO from porcine kidney is quite a difficult enzyme to immobilize. The enzyme becomes inactivated/destabilized by interaction with ionic surfaces, and it is not very stable at alkaline pH value, and an intense multipoint covalent immobilization also led to enzyme inactivation. Among the many immobilization protocols assayed in this paper, the best results have been achieved utilizing preactivated

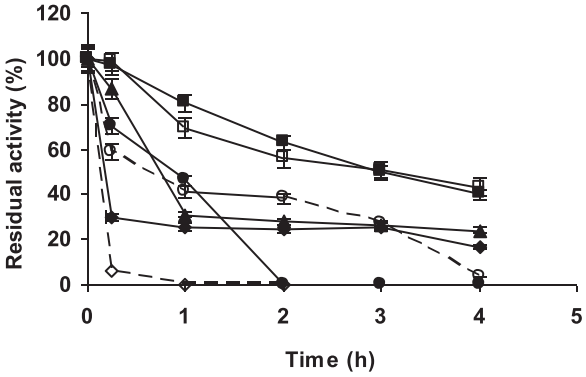

Fig. 10. Thermal inactivation of DAAO immobilized on VS- agarose support at pH 5.0 and modified with different agents block. The inactivation was performed in 10 mM of Tris-HCL at pH 7.0 and 48 ºC. Other specifications can be found in the Materials and methods section. Solid line and solid squares: Biocatalyst blocked with 1 M Aspartic acid; Solid line and solid circles: Biocatalyst blocked with 1 M Glycine; Solid line and solid triangles: Biocatalyst blocked with 1 M *D*-Glucose; Solid line and solid rhombus: Biocatalyst blocked with 1 M *D*-Glucosamine; Dotted line and empty circles and: Biocatalyst blocked with 1 M cisteamine; Dotted line and empty squares: Biocatalyst immobilized at pH 5.0 and dotted line and empty rhombus: Free enzyme.

Table 3
Effect of the different agents block at 1 M and pH 8.0 on the DAAO biocatalyst in the hydrolysis of *D*-Alanine at pH 7.0 and 25 °C. The data are given as relative activity, considering the activity of the free enzyme (0,2 mg/mL) (0.5976 U/mg) as 100%. Experiments were conducted as described in methods.

Biocatalyst	Relative activity (%)
AG-VS-DAAO (1 M Aspartico)	53.51 ± 2.67
AG-VS-DAAO (1 M Glycine)	34.29 ± 1.71
AG-VS-DAAO (1 M Glucosamine)	21.81 ± 1.09
AG-VS-DAAO (1 M Glucosa)	30.68 ± 1.53
AG-VS-DAAO (1 M Cysteamine)	19.74 ± 0.98
AG-VS-DAAO (1 M Cysteine)	0
AG-VS-DAAO (1 M Ethanolamine)	0
AG-VS-DAAO (1 M Ethylendiamine)	0
AG-VS-DAAO	51.96 ± 2.59

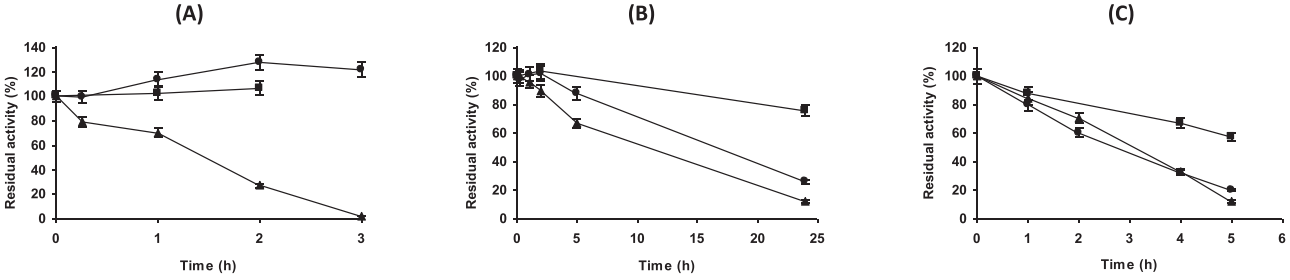

Fig. 9. Immobilization courses of DAAO (0.2 mg/mL) on VS-agarose support at 25 ºC. The immobilization was performed at: **(a)** 5 mM sodium acetate buffer at pH 5.0; **(b)** 5 mM of sodium phosphate buffer at pH 7.0 and **(c)** 5 mM of sodium carbonate buffer at pH 9.0. Other specifications can be found in the Materials and methods section. Solid squares: reference; Solid circles: suspension and solid triangles: supernatant.

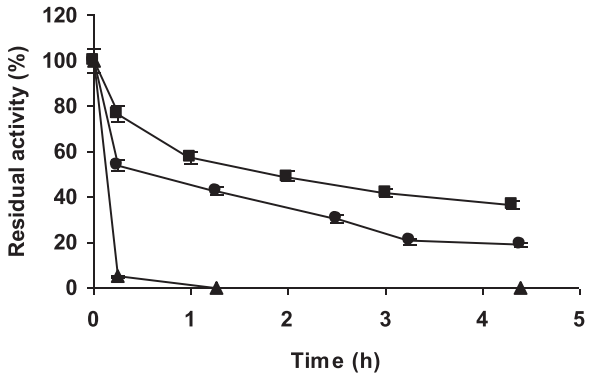

Fig. 11. Thermal inactivation of DAAO immobilized on VS- agarose support at pH 7.0 and immobilized on glutaraldehyde agarose support. The inactivation was performed in 10 mM of Tris-HCL at pH 7.0 and 48 ºC. Other specifications can be found in the Materials and methods section. Solid line and solid triangles: Free enzyme (0,2 mg/mL); Solid line and solid circles: glutaraldehyde biocatalyst and solid line and solid squares: VS- biocatalyst.

glutaraldehyde and performing the immobilization at pH 7 (considering both activity and stability). This immobilized enzyme presented all enzyme subunits attached to the support, making enzyme subunit dissociation impossible. The stabilization achieved using this protocol is slightly lower than the stabilization found using optimal VS biocatalyst.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

DC thanks to Ministerio de Ciencia e Innovación-Spanish Government by a FPI (PRE2018–084809). The help and suggestions from Dr. Ángel Berenguer (Departamento de Química Inorgánica, Universidad de Alicante) are gratefully recognized.

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