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Letter

Abstract

Polyaniline (PANI) is a water-insoluble polymer that has been used as support for enzyme immobilization due to its desirable characteristics, such as ease of preparation, high synthesis yield, high stability to temperature and pH, and resistance to microbial attack. In this work an investigation was carried out to determine the best conditions to immobilize D-hydantoinase (E.C. 3.5.2.2) in this support. As result, a simple and fast methodology for D-hydantoinase immobilization in PANI is described. 100% of proteins were immobilized on the support in concentrations up to 2 mg solid/ml. Higher concentrations led to a lower protein percentage immobilized. After five reaction cycles about a half of D-hydantoinase initial activity was conserved.

Keywords: D-Hydantoinase; Immobilization process; Polyaniline; Adsorption; Amino acids

Immobilization of D-hydantoinase in polyaniline

1. Introduction

D-Hydantoinase (dihydropyrimidine amidohydrolase E.C. 3.5.2.2), extracted from adzuki bean (*Vigna angularis*), catalyses enantiospecifically the ring opening of *rac*-5-substituted hydantoin to the corresponding *N*-carbamoyl-D-amino acid [1]. This product can be easily converted into the respective D-amino acid either by diazotation or by a second enzymatic step using *N*-carbamoyl amino acid amidohydrolase (E.C. 3.5.1.6) [2]. Enantiomerically pure D-amino acids are considered important chiral building blocks for a variety of biologically active compounds such pesticides, semisynthetic β-lactam antibiotics, peptides and enzyme inhibitors [2–5].

Biocatalyst stability is a major concern in almost all bioprocesses, because it may affect the overall cost of the process. The immobilization of enzymes in a heterogeneous support is a cost-effective approach, and contributes to the repeated use of enzyme and to efficient separation of the reaction product [6].

Different papers regarding D-hydantoinase immobilization by adsorption in different supports, like DEAE anion exchange resins, silica gel, celite and zeolite Y have been published [7]. This enzyme was also immobilized covalently in different supports, for example in Eupergit C, Eupergit C 250 L, EAH Sepharose [8,9], polystyrene anion exchange resins [10] and amino propyl functionalized glass beads [1a–d].

Polyaniline (PANI) is a water-insoluble polymer that has been used as support for enzyme immobilization [11–15] due to its desirable chemical characteristics, such as high stability to temperature and pH, and resistance to microbial attack [11,16]. Some works explain this immobilization as a covalent linkage between enzyme and the support utilizing glutaraldehyde as a bifunctional reagent [11,14], while other explain this immobilization by physical adsorption [12,13]. In this sense, this work is focused on the development of a methodology to immobilize D-hydantoinase on PANI and to establish a short comparison about the nature of the linkage between enzyme and support.

2. Materials and methods

2.1. Materials

Hydantoin (analytical grade) was purchased from Sigma –Aldrich. Boric acid, ammonium sulfate, and other chemicals (analytical grade) were purchase from VETEC S.A. All materials were used as received.

2.2. D-Hydantoinase extraction

D-Hydantoinase was extracted from adzuki bean (*V. angularis*) obtained from a local market. Extraction of the crude enzyme was performed according to the procedure described by Fan and Lee [17]. Briefly, an amount of 60 g of beans was soaked for 3 h in 100 mL of 100 mM, pH 9.0 borate buffer at 8 °C. The soften beans were homogenized with 1000 mL borate buffer using a blender. The homogenate was centrifuged for 60 min at 8000 rpm at 4 °C to remove insoluble material. Then, ammonium sulfate was added to the clarified supernatant (extract) to reach up to 40% saturation and the suspension was centrifuged for 60 min at 8000 rpm at 4 °C. The precipitate was removed (first precipitate). Ammonium sulfate was then added to the clarified supernatant (first supernatant) to reach up to 60% saturation, and the suspension was centrifuged for 60 min at 8000 rpm at 4 °C. The precipitate was removed (second precipitate) and the supernatant was discarded (second super-

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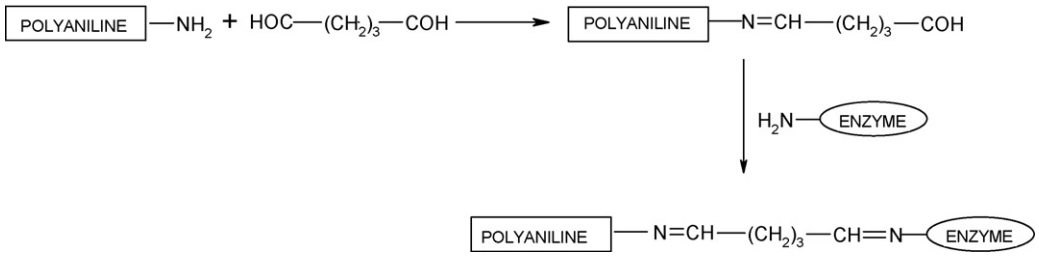

Fig. 1. Scheme of polyaniline activation with glutaraldehyde and enzyme immobilization with activated PANI.

natant). The precipitate was dissolved in a minimal volume of borate buffer and lyophilized in an Edwards Lyophilizator during 20 h.

2.3. Preparation of support

HCl-doped PANI was synthesized by chemical oxidation of a 0.44 mol L^{-1} aniline solution with a 0.61 mol L^{-1} ammonium persulfate solution, as described by Pron et al. [18]. After chemical synthesis, polyaniline was filtered and solids were washed with phosphate buffer 0.1 mol L^{-1}, pH 7.0, to neutralize the resin. The resin was filtered again and dried under high vacuum.

2.4. Treatment of the support

20 mg of dried PANI were as treated with 0.6 mL of a glutaraldehyde solution 2.5% (v/v) prepared in 0.1 mol L^{-1} phosphate buffer, pH 7.0. This system was shaken by 2 h at 30 °C and 150 rpm. After that, the resin was washed with 1 mL phosphate buffer 0.1 mol L^{-1}, pH 7.0, and subsequently with 1.0 mL of borate buffer 0.1 mol L^{-1}, pH 9.0.

2.5. Enzyme immobilization

Immobilization of D-hydantoinase was carried out by adding 1.0 mL of an enzyme solution prepared in phosphate buffer 0.1 mol L^{-1} (pH 5.0, 6.0, 7.0 and 8.0) and borate buffer 0.1 mol L^{-1} (pH 8.0, 9.0, 10.0 and 12.0) to 20 mg of dried PANI (treated and not treated). The mixtures were gently stirred (150 rpm) for 2 h, at 10 °C, and the solids were washed with the same buffer (2× 1 mL). The immobilization procedure was performed with different amounts of D-hydantoinase (0.14, 0.28, 0.56 and 1.4 mg protein) in the enzyme solution.

2.6. Enzyme immobilization for recycle usage

Immobilization of D-hydantoinase was carried out by adding 1.0 mL of an enzyme solution 0.28 mg protein/mL prepared in phosphate buffer 0.1 mol L^{-1}, pH 9.0, to 20 mg of dried PANI (treated and not treated). The mixtures were gently stirred (150 rpm) for 2 h and 20 h, at 10 °C. After this procedure the immobilized enzyme was submitted to five reaction cycles of hydantoin hydrolysis.

2.7. Determination of D-hydantoinase activity

The enzymatic hydantoinase activity was determined by a colorimetric method, based on the change of absorbance at 450 nm after addition of *p*-dimethylaminobenzaldehyde [19]. Hydantoinase assay medium contained 100 mM H_3BO_3 buffer, pH 9.0, 100 mM hydantoin as substrate, and an adequate concentration of enzyme to determinate the initial velocity after 30 min of incubation at 40 °C. One unit of enzyme (U) was defined as the quantity of enzyme enough to produce 1 μmol of the product (*N*-carbamoylglycine) in 1 min at 40 °C and specific pH for this reaction. The reaction progress was followed in a UV–vis Agilent 8453 spectrophotometer. Control experiments (blank runs) were always carried out using the same procedure but in the absence of D-hydantoinase.

2.8. Protein determination

The total protein concentration was determined by the Bradford method [20], using bovine serum albumin as standard.

3. Results and discussion

3.1. Characterization of PANI

The polymer synthesized presented an intermediary oxidation state, as discussed by Fernandes et al. [11]. This polymer can show differences in the FTIR spectra, especially in the relative intensities of the benzoid and quinoid related bands, characterizing variations in the oxidation states [21]. A weak NH_2 band

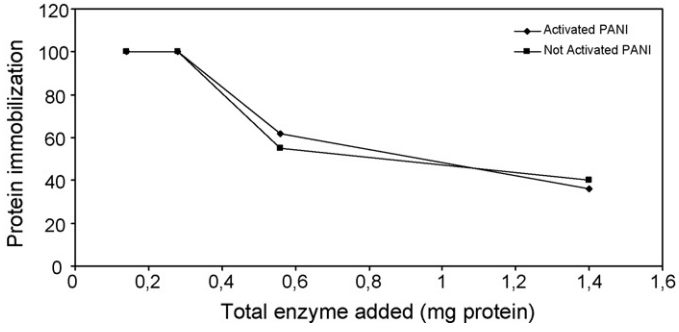

Fig. 2. Effects of enzyme charge on the immobilization of D-hydantoinase in PANI. Protein immobilized in relation to the total enzyme added (total mass of protein). The results presented for each experimental condition are the mean value of three replications.

Table 1
D-Hydantoinase activity recuperation after the immobilization process in PANI

Enzyme (protein available to immobilization)	Enzyme units available to immobilization	Activated PANI		Not activated PANI	
		Enzyme units immobilized	Activity recuperation	Enzyme units immobilized	Activity recuperation
0.14	0.104	0.086	82.7	0.088	84.6
0.28	0.270	0.235	86.9	0.230	85.2
0.56	0.603	0.306	50.8	0.290	48.1
1.4	1.429	0.404	28.2	0.420	29.4

Activity recuperation = (number of units immobilized/number of units available to immobilizing process) × 100. All experimental conditions were replicated three times.

appears at 3433 cm^{-1}. The low intensity of this band is justified by the fact that polyaniline presents only one NH_2 group in each macromolecule unit.

3.2. Enzyme binding efficiency

Literature points out that covalent binding is the best way to immobilize enzymes [8,9,22–24]. However, the expensive costs of supports used in these processes has limited the general application of these protocols.

In the present work, a short comparison about the nature of the linkage between enzyme and support was carried out and the choice of PANI as support is justified by its very low cost and very simple synthesis.

PANI could binds covalently to enzyme by action of the bifunctional reagent glutaraldehyde as hypothesized in Fig. 1. Initially, PANI could be activated with glutaraldehyde. After that, the activated PANI could react with the enzyme (via H_2N-Lys groups) resulting in a covalent link between support and enzyme. To study the characteristic of this particular binding, two sets of experiments were carried out, one using PANI treated with glutaraldehyde and other with PANI not activated.

Table 1 shows the recovery of the enzyme activity. It can be noted from this table that the activity recovery is about 80% (number of units immobilized/number of units available to immobilization process) when the ratio protein/PANI is up to 0.28 mg protein/20 mg. Both PANIs (treated and not treated) showed the same behavior.

Fig. 2 presents the effect of the enzyme added to the support. It can be noted that 100% of the protein content was immobilized on the support up to 0.28 mg protein. Above this quantity, as the maximum charge of the support was reached, a lower percentage of protein was immobilized. For instance, when 1.4 mg protein was used, only about 36% of the protein content was immobilized on support and, again, the same behavior was observed for both PANIs (treated and not treated).

Based on these results, it should be stressed that the best ratio between enzyme and support was found to be is 0.28 mg of protein to 20 mg of support. At this a study to search the best immobilization pH (Fig. 3) was performed and a plateau of maximum activity (pH 7–9) found and the same behavior was observed for both PANIs (treated and not treated).

Covalent immobilization should conduct to a lower protein binding and lost of enzyme activity when compared

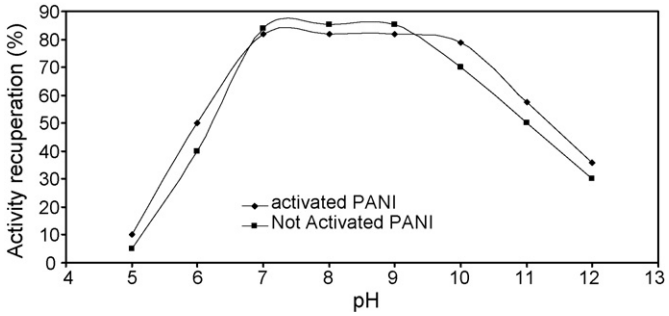

Fig. 3. Activity recuperation (%) × pH of immobilization.

to the corresponding adsorption process. For example, a covalent immobilization of a commercial extracellular invertase on nylon-6 presented only 15 mg protein per 1 g of support [22]. The covalent immobilization of hydantoinase from *Arthrobacter aurescens* on Eupergit C and EAH Sepharose 4B presented 48 and 95% of protein binding, with 49 and 15% of activity recovery, respectively [8,9]. Our results presented better yields on protein immobilization and enzyme activity recovery than the results obtained with covalent linkage of the enzyme to the support.

3.3. Activity after various cycles

In order to check the activity of the immobilized enzyme, the system was submitted to five consecutive reaction cycles. In this set of experiments, it was used 0.28 mg protein/20 mg PANI at pH 9 (the best immobilization results) during 2 and 20 h of immobilization time. After each reaction of hydantoin cleavage, the product was separated, the support was washed by 1.0 mL borate buffer pH 9.0, and a new reaction was initiated by addition of new hydantoin solution. Fig. 4 shows the results of activity recovery after five reaction cycles. After 2 h of immobilization time, it can be noted that the system lost about a half of its initial activity after the second cycle, keeping this activity on the subsequent cycles. When increasing the immobilization time to 20 h, the activity decrease from 82 to 55% on the third cycle and remains almost constant from the third cycle to the fifth cycle. Both PANIs (treated and not treated) showed the same behavior once again.

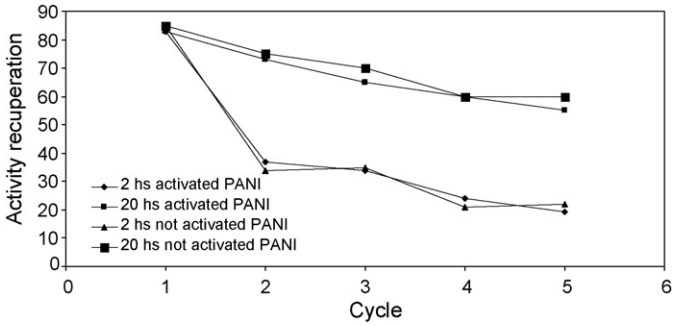

Fig. 4. Effects of number of reaction cycles on the activity recuperation of D-hydantoinase immobilized in PANI.

4. Conclusion

In this work it has been made possible to immobilize and reuse D-hydantoinase using PANI as support. It has been demonstrated that this immobilization is a pure adsorption process since there is no effect in treating the support with glutaraldehyde the supposed activation reagent.

Nevertheless, the adsorption was proved to be stable since the system was successfully reused. PANI retained around 80% of the enzyme activity available to immobilization, preserving 55% its initial activity after five reaction cycles.

The advantages of this new system includes the simple polymeric support synthesis, high immobilization yield, simplicity of handling, recycle usage and low costs.

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