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Stability of immobilized *D*-hydantoinase from *Vigna angularis* and repeated cycles of highly enantioenriched production of *N*-carbamoyl-*D*-phenylglycines

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Summary. *D*-hydantoinase from *Vigna angularis* was immobilized by covalent linkage to aminopropyl glass beads. Thermal stability, resistance to storage at different pH values and temperatures of this biocatalyst were studied. This enzyme preparation was used as a catalyst to prepare enantioenriched *N*-carbamoyl-*D*-phenylglycine, *N*-carbamyl-*D*-*p*-fluorophenylglycine and *N*-carbamoyl-*D*-*p*-trifluoromethylphenylglycine, using a stirred batch reactor. Reactions were conducted during eight repeated reaction cycles, without loss of enzymatic activity or variation of the enantiomeric excess of the respective product (>98%).

Keywords: *D*-hydantoinase – *Vigna angularis* – Biocatalysis – *N*-carbamoyl-*D*-amino acids – Immobilized enzyme

Introduction

Enantiomerically pure or highly enantioenriched *D*-amino acids and their derivatives are considered as valuable chiral building blocks for the synthesis of a variety of biologically active compounds, such as semi-synthetic β-lactam antibiotics, pesticides, peptides and enzymatic inhibitors (Gokhale et al., 1996; Garcia and Azerad, 1997; Lee and Lin, 1996; Ui et al., 1997).

D-Amino acids can be efficiently synthesized by biocatalytic conversion of hydantoins substituted in the 5 position of the hydantoinic ring by action of a *D*-hydantoinase (dihydropyrimidine amidohydrolase EC 3.5.2.2), an enzyme that is widespread in nature (Morin, 1986; Bernheim, 1946; Kim et al., 1997). This enzyme is able to catalyze the enantiospecific hydrolysis and ring opening of *rac*-5-mono-substituted hydantoins to the corresponding *N*-carbamoyl-*D*-amino acid. These compounds can be easily converted into the respective *D*-amino acids either by diazotation or by means of a second hydrolytic enzymatic step by using *N*-carbamoyl amino acid amidohydrolase (EC 3.5.1.6) (Gohkale et al., 1996; Kim and Kim, 1995).

D-Hydantoinase from *Vigna angularis* was first reported by Morin (1993) as a good catalyst for the synthesis of *D*-amino acids and it has been used in our laboratory in the form of a soluble enzyme preparation for the production of *N*-carbamoyl-*D*-phenylglycine (CPG) with 100% conversion and enantiomeric excess (EE) higher than 98% (Arcuri et al., 2000). Later this enzyme was immobilized by covalent linkage to aminopropyl glass beads (Arcuri et al., 2002) and used to produce *N*-carbamoyl-*D*-*p*-fluorophenylglycine (CFPG) and *N*-carbamoyl-*D*-*p*-trifluoromethylphenylglycine (CTFMPG) (Arcuri et al., 2003a) with over 98% EE and 100% conversion.

The experimental conditions needed for optimal enzymatic action of the enzyme from *Vigna angularis* are simpler than those required by bacterial D-hydantoinases (Arcuri et al., 2000; Fan and Lee, 2001). It does not require, for instance, anaerobic conditions such as D-hydantoinase for thermophylic microorganisms (Garcia and Azerad, 1997), nor divalent cations, such as Fe^{2+}, as cofactors (*Pseudomonas putida*, Morin et al., 1995), Mn^{2+} (thermophylic microorganisms, Keil et al., 1995) or Zn^{2+} (*Agrobacterium radiobacter*, Huang et al., 2003).

Since *D*-hydantoinase from *Vigna angularis* is commercially available at a rather inexpensive price, it shows quite a large spectrum of substrates, and its immobiliza-

tion is a straightforward and efficient procedure (Arcuri et al., 2002). We decided to analyze the stability of this enzyme preparation and to utilize it to prepare CPG, CFPG and CTFMPG, using repeated reaction cycles.

The results of the present article show that immobilized D-hydantoinase from *Vigna angularis* can be used as a valuable biocatalyst to synthesize D-amino acids with both high EE and conversion, having, in addition, good operational, thermal and storage stability.

Materials and methods

D-Hydantoinase from *Vigna angularis*, hydantoin, *p*-dimethylaminobenzaldehyde, aminopropyl glass beads and glutaraldehyde 2.5% (v/v) solution were purchased from Sigma. $CuSO_4$ and all other chemicals used were of analytical grade and obtained from Merck S.A. Indústrias Químicas, Brazil. Nucleosil Chiral-1 column (4.6 × 250 mm) was purchased from Macherey-Nagel, Germany. HPLC analyses were performed by using an ISCO 2340 chromatograph (pumps), a Pharmacia LKB/VWM 2141 UV-detector, and a Pharmacia LKB/REC 103 recording integrator. Polarimetric analyses were conducted on a Jasco DIP-370 Polarimeter.

Synthesis of rac-5-substituted hydantoins

5-Phenylhydantoin, 5-*p*-fluorophenylhydantoin and 5-*p*-trifluoromethylphenylhydantoin were synthesized according to the method described by Henze and Speer (1942), using the corresponding aldehydes and $(NH_4)_2CO_3/NH_4CN$.

Immobilization procedure

D-Hydantoinase from *Vigna angularis* was immobilized on aminopropyl glass beads activated with glutaraldehyde, 2.5% (v/v), according to the procedure described by Arcuri et al. (2002).

Assay of D-hydantoinase activity

Enzyme activity was determined in reaction mixtures containing 100 mM H_3BO_3/KCl buffer, pH 9.0, 20 mM of substrate and an adequate enzyme concentration to determine the initial velocity in 15 min of incubation at 30°C. The reaction was stopped by centrifugation at 12,000 × g for 1 min by using a microcentrifuge. The concentration of products and the residual substrate concentration were determined in aliquots (10–30 μL) of the supernatant fluid by chiral HPLC using the column already described and 1 mM $CuSO_4$ as the mobile phase at a flow rate of 1.0 mL/min and with the column eluent being detected at 235 nm. For kinetic experiments the reaction rate was followed by determination of the product concentration with *p*-dimethylaminobenzaldehyde, essentially as described by Morin (1993). One International Unit (IU) was defined as the amount of enzyme catalyzing the formation of 1 μmole of product per min at pH 9.0 and 30°C.

Production of N-carbamoyl-D-phenylglycines with repeated batch reactions

Production of CPG, CFPG and CTFMPG was performed in a jacketed batch reactor (35 mL) containing 100 mM H_3BO_3/KCl buffer, pH 9.0, 12.5 mM of substrate and 100 mg of immobilized D-hydantoinase (4.84 IU). The reaction mixture was kept at 30°C by circulating water through the jacket of the reactor with a thermocirculating bath. During one reaction cycle (100 min) 50 μL aliquots of the respective reaction mixture were removed at different time intervals, centrifuged to recover the enzyme, and 10 μL of the supernatant fluid was used to follow the extent of reactions by chiral HPLC, determining both product and residual substrate concentrations as described before. At the end of each cycle, the reaction mixture was centrifuged to separate the catalyst, and the respective reaction product was precipitated by acidification of the supernatant with concentrated HCl. The product was recovered by filtration, washed with glass distilled water and vacuum dried. The recovered immobilized enzyme was washed three times with the buffer solution and was employed immediately for the next batch reaction. After eight consecutive reactions cycles, the respective product recovered in each cycle was pooled and analyzed by chiral HPLC and polarimetry to determine the EE.

Theoretical calculations

Molecular orbital calculations were performed with the Titan program (Wave Function Inc., Irvine, CA; Schrödinger, Inc., Portland, OR). Optimized structures and harmonic vibrational frequencies were determined by the AM1 semi empirical method. The structures were confirmed to be true local minima by means of the vibrational frequency calculations that showed all the frequencies to be real.

Determination of protein concentration

Protein concentration was determined according to Hartree (1972) using bovine serum albumin (Fraction V) as standard.

Results and discussion

D-Hydantoinase from *Vigna angularis* has been immobilized by two research groups. Fan and Lee (2001) reported the immobilization of a partially purified preparation of this enzyme by entrapment in calcium alginate beads. The kinetic parameters for the hydrolysis of 5-phenylhydantoin were determined, as well as, the activity of the enzyme as a function of the pH and temperature of the reaction medium, and finally, its behavior during five repeated batch reactions for the production of CPG. Arcuri et al. (2002) reported the immobilization of a crude extract of D-hydantoinase from *Vigna angularis* by covalent linkage to aminopropyl glass beads by using glutaraldehyde as spacer agent. The kinetic parameters for the hydrolysis of hydantoin were estimated, its substrate specificity was determined and its utilization for the preparation, in laboratory scale (≈200 mg), of highly enantioenriched CPG was reported.

Since one of the objectives of the immobilization of an enzyme is to increase its stability when compared to that of the free enzyme, the thermal stability of immobilized D-hydantoinase was tested by pre-incubating the enzyme at 70°C for different times. The results obtained are illustrated in Fig. 1 and clearly show that while the soluble enzyme loses its enzymatic activity very rapidly (60% in

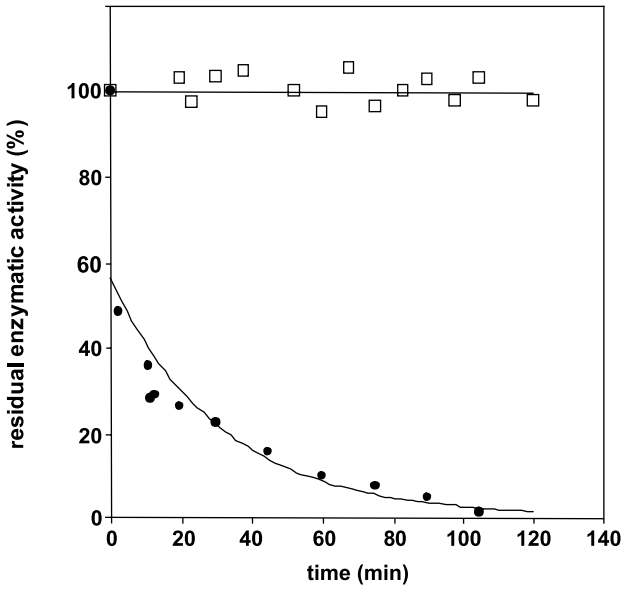

Fig. 1. Thermal inactivation of *D*-hydantoinase from *Vigna angularis*. One sample of the immobilized enzyme containing 0.12 IU (□) and another one of the free enzyme (●), containing the same enzymatic activity were incubated at 70°C in 100 mM H_3BO_3/KCl buffer, pH 9.0. At the times indicated in the figure aliquots were removed, chilled on an ice bath and the enzymatic activity remaining at each time of both samples was assayed with 200 mM hydantoin as substrate, under initial rate conditions at 30°C as described under Materials and methods by using *p*-dimethylaminobenzaldehyde

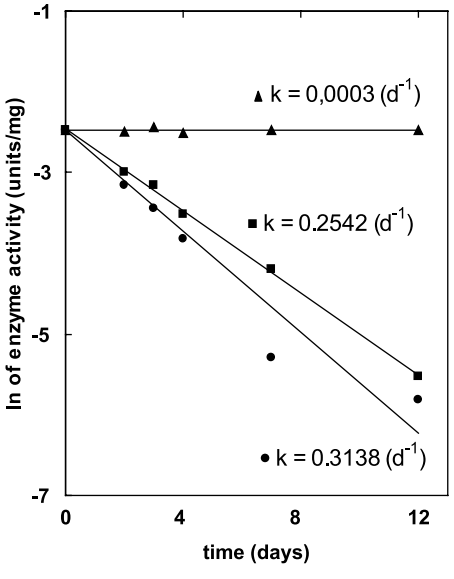

Fig. 2. Effect of pH upon the stability to storage of immobilized *D*-hydantoinase. Immobilized *D*-hydantoinase was stored at −5°C in 100 mM H_3BO_3/KCl buffer, pH 8.0 (●), pH 9.0 (▲) and pH 10.0 (■). At the times indicated in the figure aliquots of 10–50 μL of each sample were assayed for enzymatic activity at pH 9.0, as described in legend of Fig. 1. The natural logarithm of the residual activity was plotted against the time of storage at the different pH values. The value of the pseudo-first order constant of inactivation (k) was estimated by least-squares linear regression

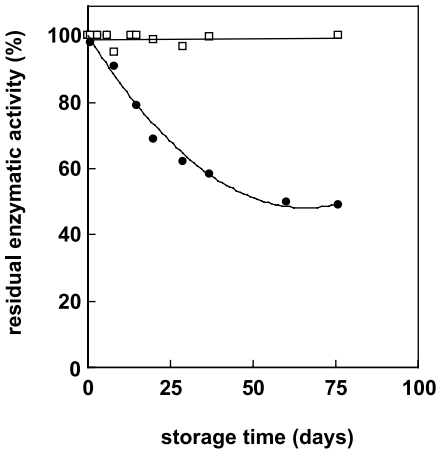

Fig. 3. Effect of the storage temperature upon de stability of *D*-hydantoinase from *Vigna angularis*. One sample of the immobilized enzyme containing 0.12 IU (□) and another one of the free enzyme (●), containing the same enzymatic activity were stored at −5°C in 100 mM H_3BO_3/KCl buffer, pH 9.0. At times indicated in the figure, aliquots of 10–50 μL were taken to assay the enzymatic activity at 30°C as described under Materials and Methods and the legend of Fig. 1

10 min), *D*-hydantoinase covalently linked to aminopropyl glass beads retains full activity during all the period of time tested at this temperature (120 min).

The effect of pH upon the stability of *Vigna angularis* *D*-hydantoinase was also analyzed (Fig. 2). While the immobilized enzyme was completely stable at pH 9.0, the pH used to assay its activity, the enzyme significantly inactivated at pH 10 and at pH 8.0 (Fig. 2). The values estimated for the pseudo-first order constant of inactivation (k) at these pH values are also displayed in Fig. 2. Considering these values of k, the half-time period ($t_{1/2}$) of the immobilized enzyme is 2,310 days at pH 9.0, 2.73 days at pH 8.0, while at pH 10, $t_{1/2}$ is 2.2 days, i.e., the stability of the immobilized enzyme at pH 9.0 is about one thousand times greater than at the other pH values tested. This is an important result since both the soluble and immobilized enzymes show maximal activity at pH 9.0 (results not shown).

Since resistance to storage is another important factor to be considered in order to characterize an enzyme for biotechnology applications, this issue was also analyzed in this work. The enzyme was stored at three different temperatures, 25, 5 and −5°C, respectively. Preliminary studies performed with immobilized *D*-hydantoinase showed that this enzyme preparation was not fully stable either at 25°C or at 5°C (results not shown). However when the enzyme was stored at −5°C, the immobilized preparation showed remarkable stability (Fig. 3). The

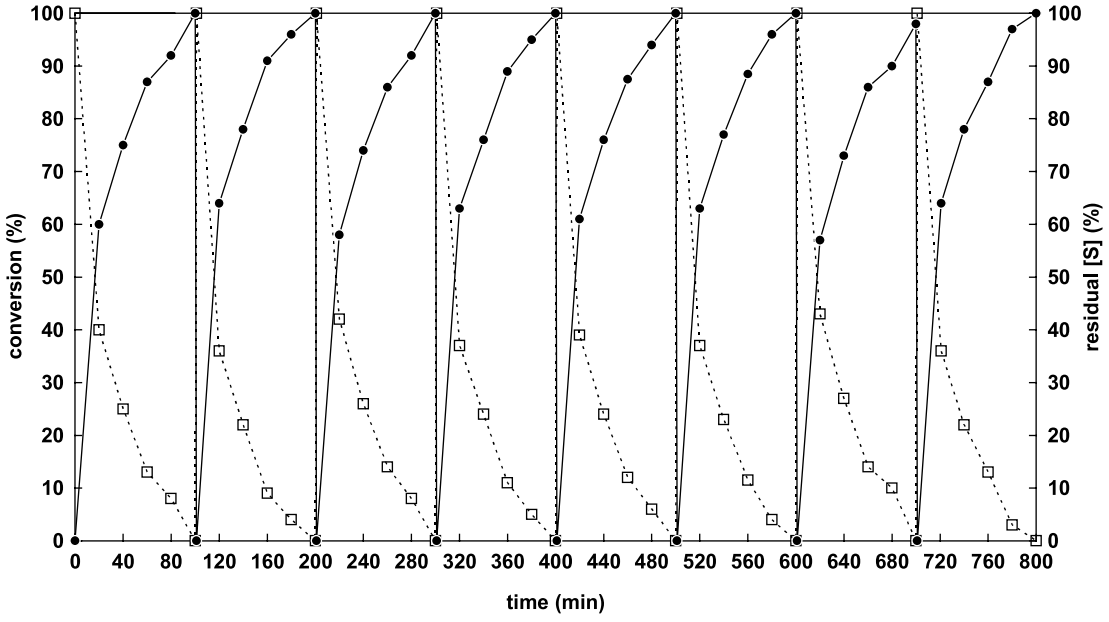

Fig. 4. Repeated use of immobilized D-hydantoinase for converting 12.5 mM of *rac*-5-phenylhydantoin (S) (□) to *N*-carbamoyl-*D*-phenylglycine (CPG) (●). Reactions conditions and analytical procedures are described under Materials and methods

enzymatic activity was measured during 80 days of storage, remaining unchanged for *D*-hydantoinase linked to the support while the free enzyme lost approximately 50% of its original activity during this same period of time.

All experiments depicted in Figs. 1 to 3 suggest that *D*-hydantoinase from *Vigna angularis* covalently linked to aminopropyl glass beads shows desirable stability properties to be used as the catalyst in preparative-scale production of *D*-amino acids since the enantiospecificity of the free enzyme is maintained in the immobilized preparation (Arcuri et al., 2000; Arcuri et al., 2002). In order to establish the operational stability of the immobilized enzyme, this preparation was used in repeated batch reactions. Figure 4 shows that the immobilized enzyme is able to convert 100% of *rac*-5-phenylhydantoin (**1**) (12.5 mM) into *N*-carbamoyl-*D*-phenylglycine (CPG) in one reaction cycle of 100 min. The fact that this picture was maintained essentially the same during eight consecutive reaction cycles (Fig. 4) shows that immobilized *D*-hydantoinase elicited good operational stability.

The same kind of experiment was repeated under identical reaction conditions with *rac*-5-*p*-fluorophenylhydantoin (**2**) and *rac*-5-*p*-trifluoromethylphenylhydantoin (**3**). With the former one, the results obtained by using cycles of 100 min were very similar (results not shown) to those reported in Fig. 4. However, when **3** was used as substrate, the reaction rate was slower and in one cycle of 100 min the conversion achieved was 87% (results not shown). This latter result can be explained by the difference of molecular volumes of these substrates that were calculated by using the AM1 semi empirical method included in the Titan program. The molecular volumes of **1** and **2** are very similar 194.68 $Å^3$ and 201.59 $Å^3$, respectively) and thus these hydantoins fit well into the hydrophobic pocked present at the active site of this enzyme (Arcuri et al., 2003b). On the other hand the greater molecular volume of **3** (235.27 $Å^3$) probably diminished the catalytic efficiency of this *D*-hydantoinase although its enantiospecificity remained high (Table 1). The optimized structures of **1**, **2** and **3** are shown in Fig. 5.

In conclusion, in the present work we presented a very simple, reproducible and reliable method to produce important *D*-amino acid derivatives with high enantio-

Table 1. Characteristics of carbamoyl-*D*-phenylglycines produced with repeated reaction cycles catalyzed by immobilized *D*-hydantoinase from *Vigna angularis*

Product	Chemical yield (%)	EE (%)	$[\alpha]_D^{25}$ (c = 1, MeOH)
CPG	90	>98	−142.3
CFPG	87	>98	−134.7
CTFMPG	75	>98	−73.9

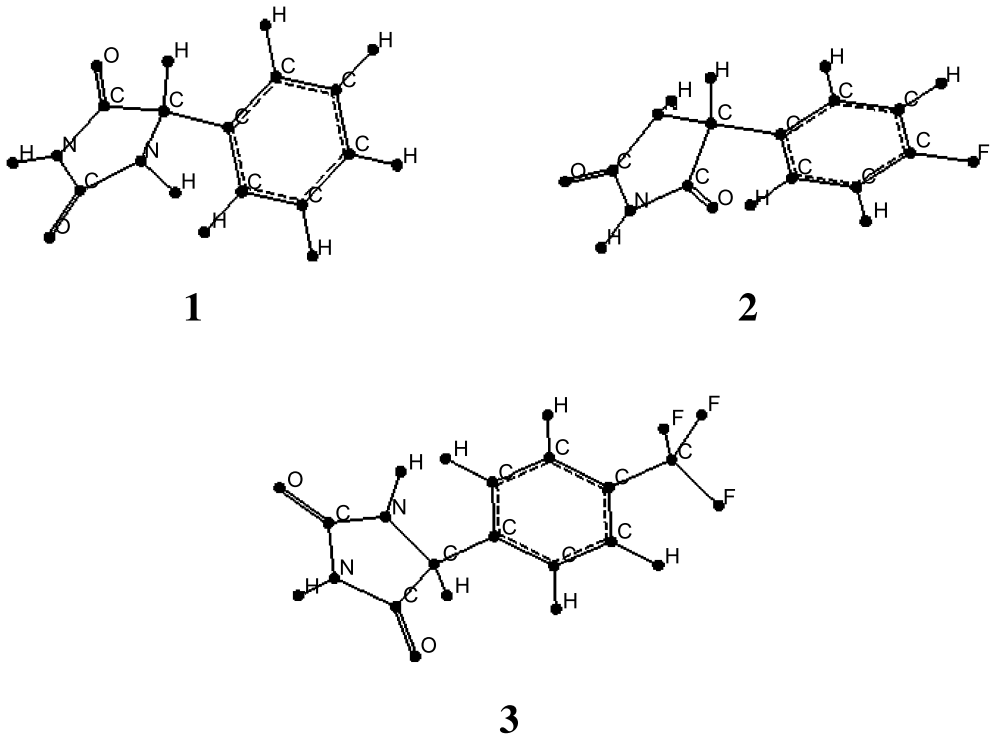

Fig. 5. Optimized structures of **1**, **2** and **3**

meric excess using immobilized *D*-hydantoinase from *Vigna angularis* as the biocatalyst. Since it was shown that this enzyme preparation has good stability properties, this procedure is suitable to be scaled up.

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