Kinetic study and production of *N*-carbamoyl-D-phenylglycine by immobilized D-hydantoinase from *Vigna angularis*

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D-hydantoinase from *Vigna angularis* was covalently linked to aminopropyl glass beads. Comparative kinetic studies between immobilized and free D-hydantoinase showed that the immobilization procedure did not modify the catalytic properties nor the substrate specificity of the enzyme but increased its stability. In addition, *N*-carbamoyl-D-phenylglycine was produced in good yield with enantiomeric excess higher than 98%.

KEY WORDS: biocatalysis; D-hydantoinase; D-amino acids; *Vigna angularis*; *N*-carbamoyl-D-phenylglycine.

Enantiomerically pure D-amino acids are considered to be important chiral building blocks for a variety of biologically active compounds such as pesticides, semi-synthetic β-lactam antibiotics, peptides and enzyme inhibitors [1–6]. The enzyme D-hydantoinase (dihydropyrimidine amidohydrolase EC 3.5.2.2) which is widespread in nature [7], catalyzes enantiospecifically the hydrolytic ring opening of *rac*-5-substituted hydantoins to the corresponding *N*-carbamoyl-D-amino acid (scheme 1) that 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 (EC 3.5.1.6) [4].

Since the enzyme extracted from *Vigna angularis* (one of the enzymes tested by Morin [7]) is commercially available, it has been used in our laboratory for the production of D-amino acids and its derivatives. Therefore, *N*-carbamoyl-D-phenylglycine was obtained in quantitative yield and over 98% enantiomeric excess (ee) [8]. In addition, several mono- and di-substituted hydantoins were tested as substrates of this enzyme. This study revealed that 5-mono-substituted hydantoins with polar and aromatic side chains and dihydrothymine are recognized as substrates but 5,5-di-substituted hydantoins are not [8].

Quite recently, the partial purification of this enzyme, its encapsulation in calcium alginate beads and its use for the production of the same D-amino acid [9] have been described, so we decided to disclose our results concerning the immobilization of a crude *V. angularis* D-hydantoinase preparation.

The crude extract of D-hydantoinase from *V. angularis* was covalently linked to aminopropyl glass beads by using glutaraldehyde as spacer agent. The kinetic properties of this enzyme preparation were studied and *N*-carbamoyl-D-phenylglycine was obtained in 100 mg scale.

The global immobilization yield (η_G), a relationship between the total enzymatic activity and the amount of enzymatic activity bound to the support was 50%. On the other hand, the retention of the enzymatic activity (R) was 98%, where $R = (IU_{initial} - IU_{theoretic}) \times 100$ and $IU_{theoretic} = IU_{total} - IU_{not\ linked}$ (IU, international units of the enzyme, are defined as μmol of product per minute, under experimental conditions). These immobilization parameters suggest that this procedure can be considered highly efficient.

A stability study was performed with this enzyme preparation at different pH and temperatures. The best results were obtained by stocking the immobilized enzyme at pH 10.0 and 278 K. Under these experimental conditions no loss of enzymatic activity was detected up to 14 days of storage. However, optimum conditions for assays of enzymatic activity were pH 9.0 and 303 K, the same conditions as found for the free enzyme [8].

Kinetic studies of the free and immobilized D-hydantoinase were performed at pH 9.0, borate buffer 100 mM and 303 K by using different concentrations of hydantoin (10–100 mM). The kinetic parameters estimated for both enzyme preparations are summarized in table 1. The estimates of K_m and V_m were not significantly different ($p < 0.05$ according to Student's t test adapted for non-linear regression [10]).

The enzymatic activity was assayed as described in the appendix. The results obtained in the present work showed that the immobilization procedure had no influence upon the catalytic properties of D-hydantoinase. Therefore, contrary to the results described by Fan and Lee [9], the immobilization procedure described caused no inactivation of the enzyme. In addition, no mass

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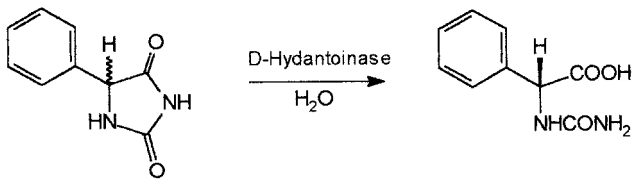

Scheme 1. Hydrolytic ring opening of *rac*-5-phenylhydantoin catalyzed by D-hydantoinase.

transfer resistance phenomenon was observed since the estimated values of V_m for the free and immobilized enzyme are not significantly different (table 1). This result was expected since the immobilization procedure described in the present work does not involve encapsulation of the enzyme in particles as in the procedure described by Fan and Lee [9].

Different *rac*-hydantoins mono-substituted at position 5 of the hydantoinic ring (*rac*-hydantoin-5-acetic acid, *rac*-hydantoin-5-acetic methyl ester, *rac*-5-phenyl hydantoin, *rac-p*-hydroxy-5-phenyl hydantoin) and dihydrothymine were tested as potential substrates of immobilized D-hydantoinase from *V. angularis*. The immobilized enzyme shows the same substrate specificity, catalyzing the hydrolytic ring opening of these 5-monosubstituted hydantoins at the same rate as the free enzyme [8]. This result suggests that the present method of D-hydantoinase immobilization does not interfere with the enzyme substrate specificity.

The specific activity of immobilized D-hydantoinase for *rac*-5-phenyl hydantoin and *rac-p*-hydroxy-5-phenyl hydantoin were 0.88 and 0.25 IU/mg respectively, which means that the activity found for *rac-p*-hydroxy-5-phenyl hydantoin is 30% of the activity found for *rac*-5-phenyl hydantoin. On the other hand, values reported by Fan and Lee [9] for these two substrates showed that the latter was hydrolyzed with a specific activity corresponding to 17% of the specific activity obtained with *rac*-5-phenyl hydantoin. This difference in the utilization of *rac-p*-hydroxy-5-phenyl hydantoin as substrate of immobilized D-hydantoinase from the same source can be attributed to a better availability of the active site of the enzyme covalently linked to glass, as compared with that of the enzyme encapsulated in calcium alginate beads [9].

Table 1
Comparison of estimates of kinetic parameters of free and immobilized D-hydantoinase from *V. angularis*

Parameter value	Free enzyme	Immobilized enzyme
$V_m \pm SE$ [a] (μmoles/min mg)	1.56 ± 0.04	1.68 ± 0.10 [b]
$K_m \pm SE$ [a] (mM)	30.40 ± 2.10	35.88 ± 4.31

[a] Asymptotic standard error.
[b] Corrected value according to η_G obtained.
Kinetic studies were performed at pH 9.0. 100 mM borate buffer and 30 °C, using different concentrations of hydantoin (10–100 mM). Other experimental conditions are described in the appendix.

The immobilized D-hydantoinase was assayed in batch process at pH 9.0, 100 mM borate buffer and 303 K. After an initial period of 60 min during which 27% of the enzymatic activity was lost, the activity of the immobilized enzyme remained constant during eight consecutive reaction cycles of 100 min, thus showing good operational stability. The loss of activity mentioned above is probably due to non-covalently-bound enzyme molecules released from the beads.

The production of *N*-carbamoyl-D-phenylglycine using immobilized D-hydantoinase and *rac*-5-phenyl hydantoin as substrate, in a laboratory scale (100 mg), was followed by chiral HPLC using a Nucleosil Chiral-1 column with 1 mM $CuSO_4$ as mobile phase. After 24 h of reaction the target product was obtained with more than 90% of conversion (100% of conversion was achieved with 30 h of reaction) and with ee higher than 98%, the same ee as obtained with the free enzyme. This result is indicative of a total retention of the enzyme enantiospecificity upon immobilization.

The analysis of *N*-carbamoyl-D-phenylglycine by chiral HPLC showed that the product obtained by the action of D-hydantoinase from *V. angularis* displayed only one peak with a retention time of 1.9 min. When a pure sample of *N*-carbamoyl-D-phenylglycine was co-injected with the product obtained in the enzymatic process, only one peak was observed with higher area and at the same retention time (1.9 min).

The product of the enzymatic reaction was also submitted to chemical racemization with 1 M NaOH for 1 h at 353 K and analyzed by chiral HPLC. Two peaks of approximately the same area were obtained with retention times of 1.75 and 1.90 mm respectively. The absence of the peak with the lower retention time together with the observation that the target product co-eluted with the internal standard are indicative of the production of *N*-carbamoyl-D-phenylglycine as the specific product of the enzymatic reaction.

The immobilization procedure described in the present work is simpler and less time-consuming than that reported by Fan and Lee [9]. This latter procedure involves, initially, the reaction of the enzyme with polyglutaraldehyde particles followed by entrapping the immobilized enzyme particles in calcium alginate beads and hardening with polyethyleneimine. Our procedure just requires reaction of D-hydantoinase with aminopropyl glass beads in the presence of glutaraldehyde as cross-linking agent. The particles containing the enzyme covalently bound are heavy and thus they can be easily isolated from the reaction medium, either by gravity (5 min) or by centrifugation at 15 500*g* for 1 min (to stop reaction).

This crude commercially available enzyme is able to produce *N*-carbamoyl-D-phenylglycine with high enantiomeric excess, showing that no previous purification is necessary in order to use this enzyme as a free or immobilized catalyst to produce highly enantioenriched *N*-carbamoyl-D-amino acids.

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Appendix

Experimental

Synthesis of rac-5-hydantoins: *rac*-hydantoin-5-acetic acid, *rac*-hydantoin-5-acetic methyl ester, *rac*-5-phenyl hydantoin, *rac-p*-hydroxy-5-phenyl hydantoin and dihydrothymine were synthesized according to the method described by Henze and Speer [11] by using the corresponding aldehyde or ketone and $(NH_4)_2CCO_3$/NH_4CN. Hydantoin-5-acetic methyl ester was prepared by methylation of the free acid with CH_2N_2/Et_2O according to Blank *et al*. [12].

Immobilization procedure: 100 mg of aminopropyl glass beads were activated with glutaraldehyde 2.5% v/v, during 30 min at 303 K and washed with deionized water. A crude extract of D-hydantoinase from *V. angularis* (10 mg, 0.484 IU) in borate buffer, 100 mM, pH 9.0 was incubated for 16 h at 278 K with the activated support. The immobilized enzyme was exhaustively washed with deionized water and borate buffer in order to remove non-covalently linked protein. This preparation was used in kinetic assays and production of *N*-carbamoyl-D-phenylglycine.

Assay of D-hydantoinase activity: hydantoinase activity was determined in reaction mixtures containing 100 mM H_3BO_3/KCl buffer, pH 9.0, 20 mM of substrate and an adequate concentration of enzyme to determine the initial velocity after 15 min of incubation at 30 °C. The reaction was stopped by centrifugation and the concentrations of the products were determined in aliquots (10 μl) of the supernatant fluid by chiral HPLC using a Nucleosil Chiral-l column (4.6 × 250 mm) (Macherey-Nagel, Germany) and using 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 the kinetic reaction experiments the reaction rate was followed by determination of the product concentration with *p*-dimethylaminobenzaldehyde essentially as described by Morin [7].

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