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Resolution of DL-hydantoins by D-hydantoinase from *Vigna angularis*: Production of highly enantioenriched *N*-carbamoyl-D-phenylglycine at 100% conversion

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Summary. D-Hydantoinase from *Vigna angularis* hydrolyzed *rac*-5-monosubstituted-hydantoins with polar and aromatic side chains and dihydrothymine but *rac*-5,5-disubstituted-hydantoins were not substrates of this enzyme. 5-Phenylhydantoin was the best substrate. By using this substrate, *N*-carbamoyl-D-phenylglycine was obtained in quantitative yield and over 98% ee.

Keywords: Amino acids – D-Hydantoinase – *N*-Carbamoyl-D-phenylglycine – Substrate specificity – *Vigna angularis*

Abbreviations: ACN: acetonitrile, HPLC: high performance liquid chromatography, TFA: trifluoroacetic acid, ODS: octadecylsilane, FTIR: Fourrier Transform Infrared.

Introduction

Enantiopure or highly enantioenriched D-amino acids are considered as important chiral building blocks for a variety of biologically active compounds such as pesticides, semisynthetic β-lactam antibiotics, peptides and to pholipeptin, a specific phosphatidylinositol phospholipase C inhibitor (Gokhale et al., 1996; Lee and Lin, 1996; Kim and Kim, 1994; Garcia and Azerad, 1997; Ui et al., 1997). Particulary, D-amino acids, which can be obtained by ring opening of aromatic-5-substituted-hydantoins, catalyzed by D-hydantoinases, especially D-phenylglycine and D-*p*-hydroxyphenylglycine (Gokhale et al., 1996; Lee and Lin, 1996; Kim and Kim, 1995), are recognized as excellent chiral building blocks for the production of semisynthetic penicillins and cephalosporins. In addition, several D-phenylglycine derivatives have been described as potent and selective antagonists at ionotropic glutamate receptors (Garcia and Azerad, 1997).

The enzyme D-hydantoinase (dihydropyrimidine amidohydrolase EC. 3.5.2.2), which is widespread in nature (Morin, 1993), catalyzes enantiospecifically the hydrolytic ring opening of *rac*-5-substituted hydantoins to the corresponding *N*-carbamoyl-D-amino acid. This latter compound 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) isolated from *Agrobacterium sp* (Kim and Kim, 1994).

In addition to bacterial D-hydantoinases (Gokhale et al., 1996; Lee and Lin, 1996; Kim and Kim, 1994; Garcia and Azerad, 1997; Runser and Meyer, 1993), a D-hydantoinase extracted from legumes (*Phaseolus vulgaris*) has been used to prepare enantiomerically pure *N*-carbamoyl-D-methionine (Morin, 1993). In this report (Morin, 1993) described the distribution of the D-hydantoinase activity among eighteen legume species, the effect of temperature and pH on its activity and stability, and the determination of its enzymatic activity towards dihydrouracil, hydantoin and three *rac*-5-monosubstituted hydantoins harboring aliphatic side chains.

Therefore, considering the facts that: a) the hydantoinase extracted from *Vigna angularis* is commercially available at a rather inexpensive price, b) this enzyme has been rarely used, probably because of the lack of information concerning its fundamental properties such as substrate specificity, c) as far as we know there is so far only one report, in the literature, concerning the use of hydantoinases from plant origin to produce enantiopure or highly enantioenriched D-amino acids (Morin, 1993), d) aromatic-5-substituted-hydantoins have not yet been used as substrates for hydrolysis, by using the hydantoinase from *Vigna angularis*, we decided to investigate the hydrolytic activity of this enzyme towards *rac*-5-monosubstituted-hydantoins with polar and aromatic side chains, with *rac*-5,5-disubstituted-hydantoins and dihydrothymine in order to produce highly enantioenriched D-amino acids.

Results and discussion

The hydrolysis of hydantoin and different 5-mono-, 5,5-disubstituted-*rac*-hydantoins and dihydrothymine were studied by D-hydantoinase from *Vigna angularis* and the results concerning its activity towards these substrates are displayed in Table 1.

Table 1. Activities of D-hydantoinase from *Vigna angularis* towards different feedstocks. The specific activity is expressed as International Units of enzyme (U) per mg of protein

Substrate	U/mg	Substrate	U/mg
hydantoin	0.92	*rac-p*-hydroxy-5-phenylhydantoin	0.25
rac-hydantoin-5-acetic acid	0	*rac*-dihydrothymine	0.58
rac-hydantoin-5-acetic acid methyl ester	0.80	*rac*-5-methyl-5-phenylhydantoin	0
ras-5-phenylhydantoin	0.88	*rac*-5-methyl-*p*-metoxy-5-phenylhydantoin	0

As expected towards the unsubstituted substrate the enzyme showed the best activity. *rac*-5-phenylhydantoin was transformed by the enzyme with an specific activity corresponding to 95% of that obtained with hydantoin. However, substitution of the *p*-hydrogen atom of the aromatic ring by an hydroxyl group resulted in a significant decrease in the specific activity of the enzyme. It is worth to point out that very similar results were reported in the literature for several microbial D-hydantoinases (Garcia and Azerad, 1997). On the other hand, *rac*-5-methyl-5-phenylhydantoin, *rac*-5-methyl-*p*-methoxy-5-phenylhydantoin and *rac*-hydantoin-5-acetic acid, were not recognized as substrates by this enzyme. The observation that *rac*-hydantoin-5-acetic acid is not a substrate for this enzyme can be attributed to the strong negative charge present in the side chain of this compound at pH 9.0, the pH of the reaction medium. This interpretation seems to be plausible, since the corresponding methyl ester derivative (obtained by methylation of *rac*-hydantoin-5-acetic acid with CH_2N_2) was shown to be a good substrate of the enzyme. As depicted in Table 1, in the presence of methyl ester of *rac*-hydantoin-5-acetic acid, the enzyme elicit an specific activity that corresponds to 87% of the specific activity obtained in the presence of hydantoin. Finally, the enzyme was able to catalyze the hydrolytic ring opening of dihydrothymine, although its specific activity was 63% compared to that determined in the presence of hydantoin.

Considering the fact that, in the present work, *rac*-5-phenylhydantoin was found to be a very good substrate of D-hydantoinase from *Vigna angularis*, and that the reaction product, D-phenylglycine, is itself used in antibiotic synthesis as well as it is valuable chiral building block, we undertook the hydrolysis of *rac*-5-phenylhydantoin (100 mg scale) for the production of *N*-carbamoyl-D-phenylglycine. Under the experimental conditions, described under Materials and methods, the reaction attained 100% of conversion, after 2.5 hours of incubation at 30°C, since the unreacted L-hydantoin epimerized to the desired D-isomer in the reaction medium.

The target product was isolated from the reaction mixture with a yield of 82%. Its absolute configuration was confirmed by polarimetry, $[\alpha]_D^{25} = -142.3$ (c = 1, MeOH) which corresponds to an optical purity higher than 98% according to the value found with a microbial purified D-hydantoinase (Keil et al., 1995).

The analysis of *N*-carbamoyl-D-phenylglycine by chiral HPLC as described under Materials and Methods shows that the product obtained by the action of D-hydantoinase from *Vigna angularis* displayed only one peak with retention time of 1.90 min. When this product was submitted to racemization with NaOH 1 M for 1 h, two peaks of the same area were obtained with retention times of 1.75 and 1.90 min, respectively. The absence of the peak with the lower retention time together with the value of $[\alpha]_{25}^{D}$ determined are indicate of the production of *N*-carbamoyl-D-phenylglycine with an enantiomeric excess greater than 98%.

D-Hydantoinase from *Vigna angularis* can be considered as an important biotechnological tool for preparation of enantiomerically pure D-amino acids and their derivatives and presents some important advantages over the micro-

bial enzymes already used for this purpose. This crude commercially available enzyme is able to produce *N*-carbamoyl-D-amino acids with the same enantiospecificity than those obtained with purified bacterial enzymes (Keil et al., 1995). The experimental requirements needed for its action are simpler than those required by bacterial enzymes, it does not require for instance of anaerobic conditions such as D-hydantoinases from thermophylic microrganisms (Garcia and Azerad, 1997), nor cofactors such as Mn^{2+} (Keil et al., 1995). Moreover, this enzyme hydrolyzes dihydropyrimidines more efficiently (Table 1) than the enzyme from *Agrobacterium sp* (Morin, 1993; Kim and Kim, 1994).

Materials and methods

D-Hydantoinase from *Vigna angularis*, hydantoin, *rac*-hydantoin-5-acetic acid, *N*-carbamoyl-glycine and dihydrothymine were purchased from Sigma. Methanol (chromatographic grade) and the Diazald® kit (for CH_2N_2 generation) were products of Aldrich. Acetonitrile (ACN) (chromatographic grade), trifluoroacetic acid (TFA) (chromatographic grade), $CuSO_4$ and all the 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. Polarimetric analysis were conducted in a Jasco DIP-370 Polarimeter, infrared spectra in a Perkin-Elmer 467 FTIR Spectrometer and ^1H NMN spectra in a NMR Bruker 200 spectrometer. HPLC was carried out by using ISCO 2350 (pumps), Pharmacia LKB/VWM 2141 (UV-Detector) and Pharmacia LKB/REC 102 (Register).

Determination of protein concentration

Protein concentration was determined according to Lowry. Bovine serum albumin (Fraction V) was used as standard.

Synthesis of rac-5-substituted hydantoins

5-Phenylhydantoin, *p*-hydroxy-5-phenylhydantoin, 5-methyl-5-phenylhydantoin and 5-methyl-*p*-methoxy-5-phenylhydantoin were synthesized according to the method described by Henze and Speer (1942), by using the corresponding aldehyde or ketone and $(NH_4)_2CO_3/NH_4CN$. *rac*-hydantoin-5-acetic acid methyl ester was prepared by methylation of the free acid with CH_2N_2/Et_2O (Blank et al., 1955).

Assay of D-hydantoinase activity

Hydantoinase activity was determined in reaction mixtures containing in 0.3 mL: 0.1 M 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 the addition of 0.6 mL of 12% (w/v) TCA, the precipitated proteins were eliminated by centrifugation. The residual concentration of each substrate was determined in aliquots (50 to 200 μL) of the supernatant fluid by HPLC using a reverse phase C_{18} Spherisorb ODS-2 column (4.6 × 250 mm, ISCO). The mobile phase used was ACN/H_2O 10% (v/v) with 0.1% TFA (v/v) at a flow rate of 1.0 mL/min. The column eluent was detected at 230 nm. For each hydantoin tested and dihydrothymine the respective stand-

ard curve was constructed in order to determine its concentration by integration of the corresponding peak. The initial velocity of the reaction was calculated in terms of mM of substrate consumed per min.

Production of N-carbamoyl-D-phenylglycine

N-carbamoyl-D-phenylglycine was produced by using a jacketed batch reactor (35 mL). The reaction medium (20 mL) contained 0.1 M H_3BO_3/KCl buffer, pH 9.0, 20 mM *rac*-5-phenylhydantoin and 156 International units (U) (assayed with 120 mM of hydantoin) of D-hydantoinase from *Vigna angularis* corresponding to 174 mg of protein, which was added to start the reaction. The reaction mixture was maintained under continuous magnetic stirring and the temperature was kept at 30°C by circulating water through the jacket of the reactor with a thermocirculating bath. The extent of reaction was followed by removing at different times aliquots (50 to 200 μL) of the reaction mixture which were treated with TCA and analyzed by HPLC as described before. After completion, proteins were eliminated by precipitation with TCA and centrifugation. The supernatant fluid was concentrated *in vacuo* to about 2 mL and *N*-carbamoyl-D-phenylglycine was precipitated by acidification with concentrated HCl. The precipitate was recovered by centrifugation, washed with bidistilled water and dried *in vacuo*. FTIR analysis showed the following bands of axial deformation: 1,690 cm^{-1} (C = O bond of free COOH); 1,550 cm^{-1} (NH of primary amide of carbamoyl group); 3,300 cm^{-1} (free OH of carboxylic acid). The following band of angular deformation was also observed: 1,300 cm^{-1} (O—H bond of free COOH) in addition to the bands of axial and angular deformations characteristic of the aromatic ring. ^{1}H NMR (CD_3OD) spectrum showed a aromatic multiplet at δ 7.4–7.2 (5H) and a singlet δ 5.3 (1H). Chiral HPLC performed at 25°C with a Nucleosil Chiral-1 column (4.6 × 250 mm) (Macherey-Nagel, Germany) and using $CuSO_4$ 2 mM as the mobile phase at a flow rate of 1.0 mL/min and with the column eluent being detected at 235 nm.

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References

Blank I, Mager J, Bergman ED (1955) Studies of organic fluorine compounds. IV Synthesis of esters of fluoro-oxaloacetic and of fluoropyruvic acid. J Chem Soc 2190–2193

Garcia MJ, Azerad R (1997) Production of ring-substituted D-phenylglycines by microbial or enzymatic hydrolysis/deracemisation of the corresponding DL-hydantoins. Tetrahedron: Asymmetry 8: 85–92

Gokhale DV, Bastawde K, Patil SG, Kalkote UK, Joshi R, Joshi RA, Raviadratan T, Galkwad BG, Jodand VV, Nene S (1996) Chemoenzymatic synthesis of D-(-)-phenylglycine using hydantoinase of *Pseudomonas desmolyticum* resting cells. Enzyme Microb Technol 18: 353–357

Henze HR, Speer RJ (1942) Identification of carbonyl compounds through conversion into hydantoins. J Am Chem Soc 64: 522–523

Keil O, Schneider MP, Rasor JP (1995) New hydantoinases from thermophylic microorganisms – Synthesis of enantiomerically pure D-amino acids. Tetrahedron: Asymmetry 6: 1257–1260

Kim GJ, Kim HS (1994) Adsorptive removal of inhibitory by-product in the enzymatic production of optically active D-*p*-hydroxyphenylglycine from 5-substituted hydantoin. Biotechnol Lett 16: 17–22

Lee CK, Lin KC (1996) *N*-carbamoyl-D-*p*-hydroxyphenylglycine production using immobilized D-hydantoinase from recombinant *E coli*. Enzyme Microb Technol 19: 623–627

Morin A (1993) Use of D-hydantoinase extracted from legumes to produce *N*-carbamyl-D-amino acids. Enzyme Microb Technol 15: 208–214

Runser S, Ohleyer E (1990) Properties of the hydantoinase from *Agrobacterium sp.* IP I-671. Biotechnol Lett 12: 259–264

Ui H, Miyake T, Iinuma H, Imoto M, Naganawa H, Hattori S, Hamada M, Takeuchi T, Umezawa S, Umezawa K (1997) Pholipeptin, a novel cyclic lipoundecapeptide from *Pseudomonas fluorescens*. J Org Chem 62: 103–108

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