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Biochemical and Biophysical Research Communications 303 (2003) 541–547
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Overexpression and characterization of hydantoin racemase from *Agrobacterium tumefaciens* C58

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Received 5 February 2003

Abstract

Hydantoin racemase enzyme together with a stereoselective hydantoinase and a stereospecific D-carbamoylase guarantee the total conversion from D,L-5-monosubstituted hydantoins with a low velocity of racemization to optically pure D-amino acids. In this work we have cloned and expressed the hydantoin racemase gene from two strains of *Agrobacterium tumefaciens*, C58 and LBA4404, in *Escherichia coli* BL21. The recombinant protein was purified in a one-step procedure by using immobilized cobalt affinity chromatography and showed an apparent molecular mass of 32,000 Da in SDS–gel electrophoresis. Size exclusion chromatography analysis determined a molecular mass of about 100,000 Da, suggesting that the native enzyme is a tetramer. The optimal conditions for hydantoin racemase activity were pH 7.5 and 55 °C with L-5-ethylhydantoin as substrate. Enzyme activity was slightly affected by the addition of Ni^{2+} and Co^{2+} and strongly inhibited by Cu^{2+} and Hg^{2+}. No effect on enzyme activity was detected with Mn^{2+}, EDTA, or DTT. Kinetic studies showed the preference of the enzyme for hydantoins with short rather than long aliphatic side chains or hydantoins with aromatic rings.

Keywords: Racemization; D-Amino acid; Hydantoin racemase; Purification

Optically pure D-amino acids are valuable intermediates for the preparation of semisynthetic antibiotics, pesticides, and other products of interest for the pharmaceutical, food, and agrochemical industries [1,2]. Enzymatic production of optically pure D-amino acid from D,L-5-monosubstituted hydantoins has been proved to be cheaper and less contaminating compared with chemoenzymatic production [3,4]. In this enzymatic reaction, called "hydantoinase process," first the chemically synthesized D,L-5-monosubstituted hydantoin ring is hydrolyzed by a stereoselective hydantoinase enzyme. Further hydrolysis of the resulting enantiospecific *N*-carbamoyl D-amino acid to the free D-amino acid is catalyzed by highly enantiospecific *N*-carbamoyl D-amino acid aminohydrolase (D-carbamoylase). At the same time as D-hydantoinase hydrolyses the enantiospecific D-5-monosubstituted hydantoin, the chemical and/or enzymatic racemization of L-5-monosubstituted hydantoin starts. Chemical racemization of the 5-monosubstituted hydantoins proceeds via keto-enol tautomerism under alkaline conditions [5]. The velocity is strongly dependent on the bulkiness and electronic factors of the substituent in 5-position [6] and is usually a very slow process [7]. Increased racemization rates are obtained at alkaline pH values and with rising temperatures [8]. Only high velocities of chemical racemization have been observed for D,L-phenyl and D,L-5-*p*-hydroxy-phenylhydantoin [6].

Enzymatic racemization of the D,L-5-monosubstituted hydantoins with very slow velocity of racemization has been shown in several microorganisms by total conversion and 100% production of optically pure D-amino acids [9–11]. These results suggested that a hydantoin racemase might be responsible for the fast racemization of the substrates. A recent work has presented more

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doi:10.1016/S0006-291X(03)00377-2

information about the genetic organization and genomic localization of the three genes involved in optically pure D-amino acids [12]. In that work a DNA fragment of 7.1 kb from *Agrobacterium* sp. IP I-671 was cloned and sequenced. Together with the D-hydantoinase and D-carbamoylase, a putative hydantoin racemase was described. Hydantoin racemase from *A. tumefaciens* C58 was cloned and overexpressed in our laboratory, and together with D-hydantoinase and D-carbamoylase from *Agrobacterium radiobacter* NRRL B11291 a reaction system for the production of D-amino acids from D,L-5-monosubstituted hydantoins with very slow rate of spontaneous racemization was developed [13]. However, from the biochemical and enzymatic point of view there is no information on hydantoin racemases involved in the production of D-amino acids. Only two hydantoin racemases have been studied from both the molecular and biochemical angle and they have been used in a system for the production of L-amino acids [7,14–16].

Here we report the biochemical, structural, and thermodynamic properties of hydantoin racemase from *A. tumefaciens* C58. These properties were compared with those of the *Arthrobacter aurescens* and *Pseudomonas* sp. hydantoin racemase enzymes.

Materials and methods

General protocols and reagents. Standard methods were used for the cloning and expression of the different genes [17,18]. Restriction enzymes, T4 DNA ligase, and the thermostable *Pwo* polymerase for PCR were purchased from Roche Diagnostic S.L. (Barcelona, Spain). The 5-monosubstituted hydantoins used in this work, D- and L-5-benzylhydantoin (D- and L-BH), D- and L-5-ethylhydantoin (D- and L-EH), D- and L-5-methylthioethylhydantoin (D- and L-MTEH), and D- and L-5-isobutylhydantoin (D- and L-MTEH) were synthesized according to the literature [19].

Microbes and culture conditions. Agrobacterium radiobacter CECT 4302, 4360, 477, and 4112T and *A. tumefaciens* CECT 472, 4067, 4119T, 4363, ATCC 33970 (named C58), and LBA4404 (from BD Biosciences Clontech, Madrid) were used as possible donors of the hydantoin racemase gene. These strains were cultivated at 30 °C for 20 h in Luria–Bertani (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2). *Escherichia coli* BL21 [20] was used to clone and express the hydantoin racemase gene.

Cloning and sequence analysis of hydantoin racemases. Agrobacterium genomic DNA was extracted as described previously [21] and the gene encoding hydantoin racemase was amplified by PCR. The primers used were designed based on GenBank sequence Accession Nos. NC003063 and NC003305 [22,23]. These were Rac5 (5′-A ATCT AGAGTGACAGGAAAGCTATTATGCGTGCGATGCAT-3′) and Rac3 (5′-AACTGCAGTTAATGATGATGATGATGATGGGCGC AGGCGACGAGGGCTGG-3′), the latter with six histidine residues before the stop codon. The *Xba*I and *Pst*I digested 720 bp fragment from hydantoin racemase was purified from agarose gel using QIAquick (Qiagen) and was then ligated into pBluescript II SK (+) plasmid (pBSK, Stratagene Cloning Systems) which was cut with the same enzymes to create plasmid pSER12.

After cloning, the fragment was sequenced into pBSK plasmid. The sequence was analyzed at least twice using standard T3 and T7 primers. Sequencing analysis was carried out using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosystems). The sequences were edited with Microsoft Word 97 and assembled using the CLUSTALW program [24]. The assembled sequences were aligned and compared with all of the amino acid sequence databases available from Internet using Basic Local Alignment Search Tool (BLAST) [25]. Manual comparisons of the sequences were performed with the CLUSTALW program. The percentage of homology between fragments was calculated with BLAST and SEAVIEW [26] programs.

Expression of hydantoin racemase. The construction in BL21 strain was grown in LB medium supplemented with 100 μg mL^{-1} ampicillin. A single colony was transferred into 10 mL LB medium with ampicillin at the concentration mentioned above in a 100-mL flask. This culture was incubated overnight at 37 °C with shaking. Five hundred milliliters of LB containing the appropriate concentration of ampicillin in a 2-L flask was inoculated with 5 mL of the overnight culture. After 2 h of incubation at 37 °C with vigorous shaking, the OD_{600} of the resulting culture was 0.3–0.5. For induction of the expression of the hydantoin racemase gene, isopropyl-β-D-thiogalactosidase (IPTG) was added to a final concentration of 0.2 mM and the cultivation was continued at 37 °C for 4 more hours. The cells were collected by centrifugation (Beckman JA2-21, 7000*g*, 4 °C, 10 min), washed twice, and resuspended in 50 mL wash buffer (100 mM NaCl, 0.02% NaN_3, 1 mM Tris, pH 7). The cell walls were disrupted in ice by sonication using UP 200 S Ultrasonic Processor (Dr. Hielscher GmbH, Germany) for 3 periods of 30 s, pulse mode 0.5, and sonic power 60%. The pellet was removed by centrifugation (Beckman JA2-21, 10,000*g*, 4 °C, 20 min) and discarded. The supernatant was applied to a column with TALON Metal affinity resin (Clontech Laboratories) and then washed three or four times with wash buffer. After complete washing, hydantoin racemase enzyme was eluted with elution buffer (50 mM imidazole, 100 mM NaCl, 0.02% NaN_3, and 2 mM Tris, pH 8). Before use the purified enzyme was dialyzed against 0.1 M potassium phosphate buffer pH 7.5 and stored at 4 °C.

Enzyme assay. The enzyme reaction was carried out with the purified hydantoin racemase (8 μM) together with 5 mM of optically pure 5-monosubstituted hydantoin dissolved in 100 mM phosphate buffer (pH 7.5) in 200 μL of reaction volume. The reaction mixture was incubated at 40 °C for 15 min and stopped by addition of twice the reaction volume of 1 M HCl. After centrifuging the stopped samples, the supernatant was analyzed by high performance liquid chromatography (HPLC). The HPLC system (Breeze HPLC System, Waters, Barcelona) equipped with a Chirobiotic T column (4.6 × 250 mm, ASTEC, USA) was used to separate the D- and L- forms of the 5-monosubstituted hydantoins. The mobile phase used in the analysis (60% water and 40% methanol) was pumped with a flow of 0.5 mL min^{-1}. The UV detector was fixed at 200 or 210 nm, depending on the substrate, and the 5-monosubstituted hydantoins were detected and quantified. The rates of all the enzymatic reactions in the study were obtained by subtracting the chemical racemization rates from the observed reaction rates. The specific activity of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 mM of D- or L-5-monosubstituted hydantoin at 40 °C min^{-1} mg^{-1} protein.

Molecular mass and protein characterization. Size exclusion chromatography-HPLC (SEC-HPLC) analysis was performed to calculate the molecular mass of the native enzyme using non-denatured protein molecular weight marker kit (Sigma–Aldrich Quimica S.A., Madrid). HPLC System (Breeze HPLC System, Waters, Barcelona) using a Superdex 200 HR 10/30 column (Amersham Biosciences, Barcelona) was equilibrated and eluted with 0.1 M potassium phosphate buffer, pH 7.5, at a flow rate of 0.5 ml min^{-1}. Molecular mass of the monomeric form was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by the Laemmli method [27] using a low molecular weight marker kit (Amersham Biosciences, Barcelona). The thermal stability of the hydantoin racemase enzyme was measured after 15 and 30 min of preincubation at temperatures from 30 to 80 °C in 0.1 M potassium phosphate buffer, pH 7.5. An enzyme assay was then

made at 40 °C for 15 min containing the L-5-ethylhydantoin (L-EH) substrate together with the hydantoin racemase enzyme. To analyze the effect of dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), $HgCl_2$, $NiCl_2$, $MnCl_2$, $CoCl_2$, and $CuSO_4$ on the enzyme, 2 mM of each metal, 0.1 M DTT, and EDTA were incubated with the hydantoin racemase (8 μM) in potassium phosphate buffer, pH 7.5 (final volume 200 μL) at room temperature for 45 min. The specific activities for the effect of metals were determined by standard enzyme assay.

Results

PCR cloning of the hydantoin racemase gene

Of all *Agrobacterium* strains studied in this work, only *A. tumefaciens* C58 (ATCC 33970) and *A. tumefaciens* LBA4404 gave a specific fragment for the hydantoin racemase gene. A DNA fragment of 720 bp corresponding to the hydantoin racemase gene was amplified in both *A. tumefaciens* genomic DNAs. Nucleotide sequence analysis showed 100% homology of these two fragments. From this moment on, therefore, we only worked with one gene *A. tumefaciens* C58 hydantoin racemase.

Expression and enzyme purification

To investigate the expression and the function of the enzyme, the *A. tumefaciens* C58 hydantoin racemase gene was cloned into pBSK plasmid. In order to avoid a fusion protein between the hydantoin racemase gene and the N-terminal end of the β-galactosidase gene present in the plasmid, TGA codon was included upstream from the RBS sequence and the beginning of the genes in Rac5 primer. The hydantoin racemase gene was expressed in *E. coli* BL21. The hydantoin racemase activity was determined in crude extracts by HPLC with a chiral column using 5 mM L-EH as substrate (see Materials and methods). A one-step purification procedure of the recombinant hydantoin racemase was employed by using immobilized cobalt affinity chromatography. The nucleotides corresponding to six histidine residues were included in the 3′-primer (Rac3) before the stop codon. Thus, the enzyme included a his-tag at the C-terminal end. The purified enzyme was checked by SDS–polyacrylamide gel (Fig. 1). The specific activity of the purified enzyme was calculated by standard enzyme assay. In elution buffer pH 8, the enzyme was stable at 4 °C for 8 weeks, and in the same buffer with 20% glycerol the purified enzyme could be stored at −20 °C over 6 months without noticeable loss of activity. The purified enzyme was active after 10 freeze–thawing cycles.

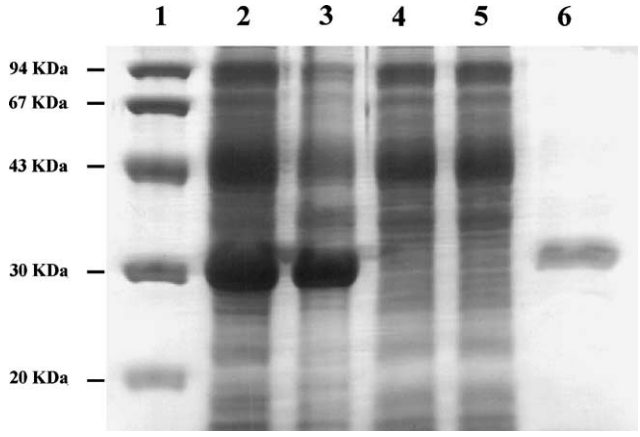

Fig. 1. SDS–PAGE analysis (15%) of each purification step of *A. tumefaciens* C58 hydantoin racemase from *E. coli* BL21 harboring the pSER12 plasmid after 4 h of induction. Lane 1, low molecular weight marker; lanes 2 and 3, supernatant and pellet of the resuspended crude extract after cell sonication; lane 4, eluate after adding the sonicated supernatant to the metal affinity column; lane 5, flow-through following wash buffer clean-up of metal affinity column; and lane 6, purified enzyme.

Molecular mass

The purified hydantoin racemase enzyme gave an apparent molecular mass of about 31,000 Da by SDS–polyacrylamide gel electrophoresis (Fig. 1). This value is a slightly higher than that deduced from the amino acid sequence (25,412 Da). The relative molecular mass of the native enzyme calculated by size exclusion chromatography, was estimated at 100,000 Da. These results suggest that the enzyme has a tetrameric structure, with four identical subunits of 25,000 Da in molecular mass.

Effects of pH and temperature on the activity

The hydantoin racemase enzyme showed maximum activity at pH 7.5 (Fig. 2). The optimum temperature for racemization of L-EH was 55–60 °C (Fig. 3A). However, in the temperature stability study and under the same

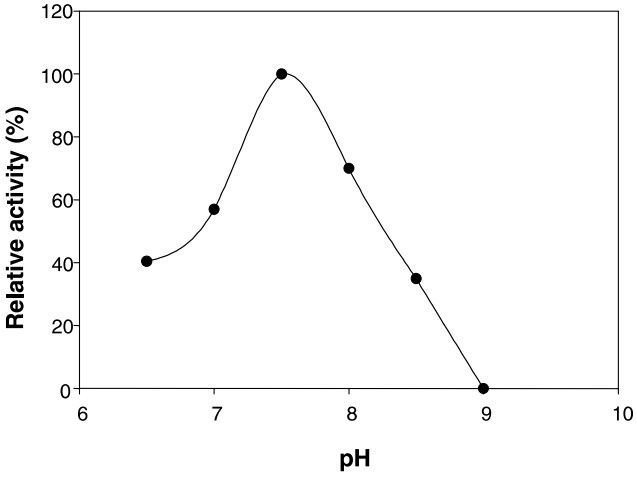

Fig. 2. Effect of pH on *A. tumefaciens* C58 hydantoin racemase activity. Hydantoin racemase activity was calculated at different pHs using enzyme assays at 40 °C for 15 min with L-5-ethylhydantoin (L-EH) substrate.

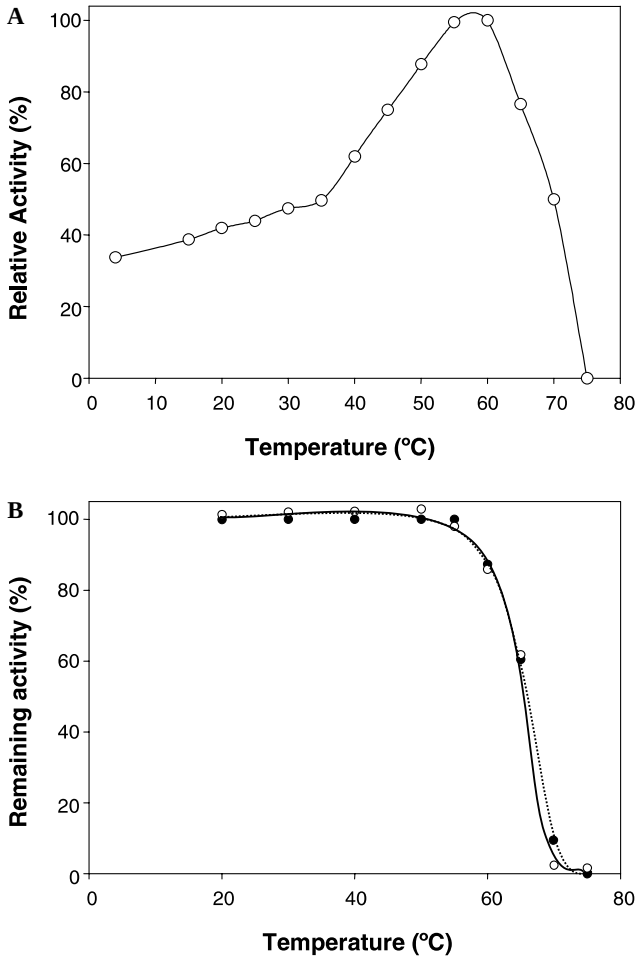

Fig. 3. Effect of temperature on *A. tumefaciens* C58 hydantoin racemase activity. (A) Hydantoin racemase activity measured at different temperatures using standard enzyme assay. (B) Hydantoin racemase activity after incubation at the indicated temperatures for 15 (black circles) or 30 min (white circles). The remaining hydantoin racemase activity was measured at 40 °C for 15 min using L-5-ethylhydantoin (L-EH) substrate.

assay conditions, when the enzyme was incubated over 55 °C for 15 and 30 min, the activity was gradually lost (Fig. 3B). Consequently, all standard assays were performed at 55 °C at pH 7.5.

Substrate enantioselectivity

The ability of the purified hydantoin racemase enzyme to racemize different 5-monosubstituted hydantoins with very low chemical racemization was examined. The D- and L-isomers of 5-monosubstituted hydantoins (BH, EH, IBH, and MTEH) were completely racemized (Fig. 4). A high rate of hydantoin racemase activity was detected for the three aliphatic D- and L-hydantoins (EH, IBH, and MTEH) compared to D- and L-BH, which was racemized slowly. These results indicate that this enzyme has less affinity to aromatic than to aliphatic hydantoins. When the racemization kinetics for the three aliphatic D- and L-hydantoins was analyzed, reaction velocity was found to depend on the substituent-chain-length of the hydantoin, where faster conversion times corresponded to shorter length. Conversion times for D- and L-EH were faster than those obtained with D- and L-MTEH and D- and L-IBH.

Kinetic characterization of hydantoin racemase enzyme

Kinetic parameters for the four D- and L-isomers of 5-monosubstituted hydantoins (BH, EH, IBH, and MTEH) were obtained from hyperbolic saturation curves by least-squares fit of the data to the Michaelis–Menten equation. Reactions were made with different concentrations of D- or L-5-monosubstituted hydantoins at 40 °C with a constant enzyme concentration of 2.9 $\mu g\,mL^{-1}$. Similar K_m values were obtained for D- and L-isomers of BH, EH, and MTEH, while a very significant decrease in K_m was noted for D- and L-isomers of BH (Table 1). In contrast, 10-fold increase in V_{max} was detected for D- and L-isomers of EH, compared to those obtained for D- and L-isomers of BH, IBH, and MTEH (Table 1).

Effect of metals and chemical agents

The activity of purified hydantoin racemase enzyme was assayed in the presence of different metal ions at 2 mM and 0.1 M of DTT and EDTA (Fig. 5). After 45 min of incubation with each reagent, activity was tested using the standard assay. Relative activity when compared to a non-treated reference sample decreased slightly in the presence of Ni^{2+} and Co^{2+}, whereas the presence of Mn^{2+} had no significant effect on enzyme activity. Incubation with Cu^{2+} and Hg^{2+} produced inactivation of the hydantoin racemase enzyme. The chelating agent EDTA and the reducing compound DTT had no significant effect on enzyme activity.

Discussion

Together with hydantoinase and D-carbamoylase from *A. radiobacter* NRRL B11291, the hydantoin racemase activity of *A. tumefaciens* C58 has recently been studied for the production of optically pure D-amino acids from D,L-5-monosubstituted hydantoins with a very slow rate of spontaneous racemization [13]. The amino acid sequence of this *A. tumefaciens* C58 hydantoin racemase enzyme has been studied to a level compared with that of other hydantoin racemases previously described, showing homology percentages of nearly 45% [13]. The apparent molecular mass of the subunit of the *A. tumefaciens* C58 purified enzyme is 31,000 Da, which is similar to that of previously purified recombinant

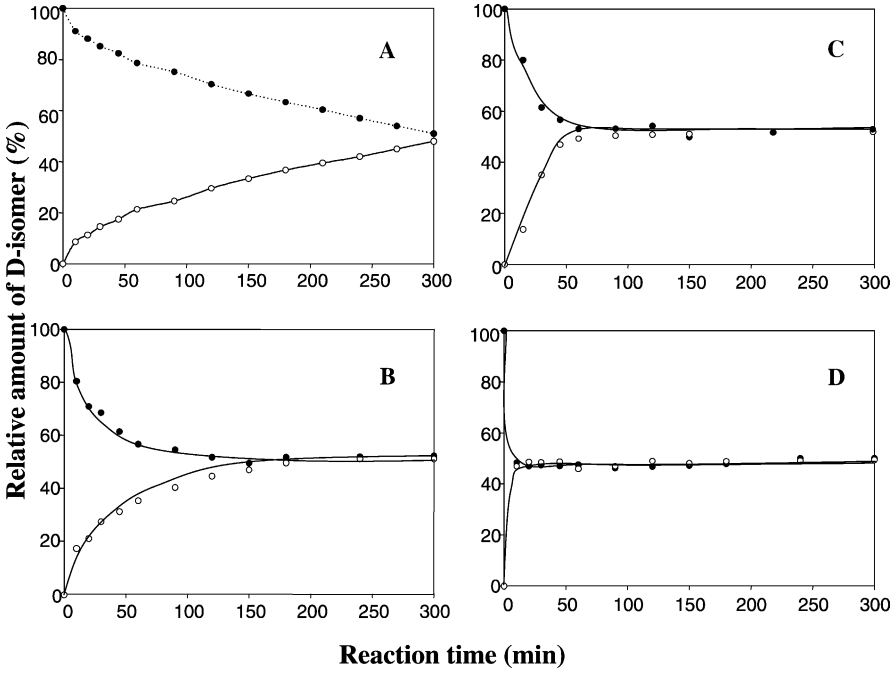

Fig. 4. Enzymatic racemization of the D-isomer (•) and L-isomer (○) of different 5-monosubstituted hydantoins by *E. coli* BL21/pSER12. The reaction was followed by chiral-HPLC at the points showed in the graphics. (A) 5-Benzylhydantoin; (B) 5-methylthioethylhydantoin; (C) 5-isobutylhydantoin; (D) 5-ethylhydantoin.

Table 1
Kinetic parameters of *A. tumefaciens* C58 hydantoin racemase enzyme for different 5-monosubstituted hydantoins

Substrate	K_m (mM)	V_{max} (U mg^{-1})
L-EH	4.45 ± 0.60	20.70 ± 2.80
D-EH	4.26 ± 0.70	19.17 ± 2.25
L-BH	5.56 ± 1.45	1.24 ± 0.25
D-BH	4.71 ± 0.62	2.75 ± 0.60
L-MTEH	5.41 ± 1.06	1.82 ± 0.40
D-MTEH	4.47 ± 0.96	2.37 ± 0.53
L-IBH	1.23 ± 0.20	2.39 ± 0.55
D-IBH	4.58 ± 0.51	4.84 ± 0.77

Note. Racemization of 1 mM substrate at 40 °C min^{-1} was defined as one unit enzyme.

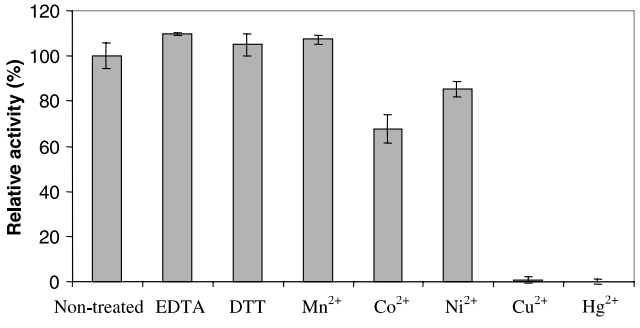

Fig. 5. Effect of the presence of different metal ions, DTT and EDTA, on *A. tumefaciens* C58 hydantoin racemase activity. Relative enzyme activity was expressed in percentage after comparison with a non-treated reference sample.

hydantoin racemase enzymes purified until now from *Pseudomonas* sp. (32,100 Da) and *A. aurescens* (31,000 Da) [7,15]. All three apparent molecular masses are larger than those calculated from the amino acid sequence: 25,412 Da for *A. tumefaciens* C58 hydantoin racemase enzyme, 27,090 Da for *Pseudomonas* sp. hydantoin racemase enzyme, and 25,085 Da for *A. aurescens* DSM 3747 hydantoin racemase enzyme. These differences can be explained by a "molten globule" state, where the enzymes in SDS–PAGE conditions are compact denatured structures but with near native secondary structure, significant loss of tertiary structure, and increased exposure of hydrophobic surface area [28,29]. In the SDS–PAGE, these partially denatured hydantoin racemases moved slower than if they were totally denatured. The relative molecular mass of the *A. tumefaciens* C58 hydantoin racemase native enzyme (100,000 Da) was approximately half than that reported for *Pseudomonas* sp. hydantoin racemase enzyme (190,000 Da) and for *A. aurescens* DSM 3747 hydantoin racemase enzyme (175,000 Da). Thus, a tetrameric structure is deduced for *A. tumefaciens* C58 hydantoin racemase enzyme, whereas the *Pseudomonas* sp. hydantoin racemase enzyme has been described as hexameric [15] and *A. aurescens* DSM 3747 hydantoin racemase enzyme as hexameric, heptameric, or octameric [7]. However, a previous work concerning the isolation and characterization of hydantoin racemase enzyme from the wild organism *A. aurescens* DSM 3747 gave a relative molecular mass of about 84,000 Da, with four

subunits of 21,000 Da each [30]. These results are in accordance with those obtained in this work for *A. tumefaciens* C58 hydantoin racemase enzyme with a molecular mass of almost 100,000 Da and tetrameric structure.

The *A. tumefaciens* C58 hydantoin racemase enzyme showed higher thermal stability (55 °C) than *Pseudomonas* sp. and *A. aurescens* hydantoin racemases (both 45 °C). Likewise, *A. tumefaciens* C58 hydantoin racemase enzyme showed maximum activity at a higher temperature range (55–60 °C) than *A. aurescens* hydantoin racemase (55 °C) and *Pseudomonas* sp. hydantoin racemase (45 °C). However, the optimal pH for *A. tumefaciens* C58 hydantoin racemase activity was 7.5, lower than *A. aurescens* and *Pseudomonas* sp. hydantoin racemases (8.5 and 9.5, respectively). This low alkaline pH avoids chemical racemization, which only occurrs at alkaline pHs [5]. Consequently, the racemization of the D- or L-5-monosubstituted hydantoins is an enzymatic process.

The natural substrates for hydantoin racemases are as yet unknown, *Pseudomonas* sp. hydantoin racemase enzyme has shown better racemization for hydantoins with aliphatic substituents, whereas *A. aurescens* hydantoin racemase enzyme racemized faster than aromatic ones [7,15]. As a result, these enzymes, involved in L-amino acids formation together with hydantoinase and L-carbamoylase, should yield higher amounts of aliphatic L-amino acids using the "hydantoinase process" from *Pseudomonas* sp. On the other hand, hydantoin utilizing enzymes of *A. aurescens* should be more suitable for the production of aromatic L-amino acids. *A. tumefaciens* C58 hydantoin racemase enzyme is the first hydantoin racemase described to date for D-amino acid production, and its activity was higher with aliphatic substituents than with aromatic ones at the 5-position. For hydantoins with aliphatic substituents a reciprocal dependence was detected between *A. tumefaciens* C58 hydantoin racemase kinetic activity and the substituent-chain-length. The highest racemization velocity corresponded to the shortest length in the substituent-chain. Therefore, the "hydantoin process" using *Agrobacterium* enzymes should be more effective for the production of aliphatic D-amino acids, although almost no differences have been obtained in the conversion time of aromatic and aliphatic hydantoins to the corresponding D-amino acids in a whole cell system [13]. Differences have been shown in the kinetic parameters of the enzymatic racemization for *Pseudomonas* sp. hydantoin racemase enzyme when the reaction started from the D- or the L-5-monosubstituted hydantoin. The K_m values of *Pseudomonas* sp. hydantoin racemase enzyme for D- and L-MTEH were 29.8 and 73.7 mM, respectively. Likewise, the V_{max} values for the D- to L-isomer and the L- to D-isomer were 35.2 and 79.0 $\mu mol\,min^{-1}\,mg^{-1}$ protein, respectively. However, the K_m and V_{max} values of *A. tumefaciens* C58 hydantoin racemase enzyme for the same D- and L-5-monosubstituted hydantoin were almost identical (Table 1). The similarity of these parameters for the enzymatic racemization of the D- and L-isomer shows that *A. tumefaciens* C58 hydantoin racemase enzyme has the same affinity for both potential substrates.

In this work, we have described the structural and enzymatic properties of the first described hydantoin racemase involved in the production of optically pure D-amino acids. This enzyme has shown similar kinetic and thermodynamic properties to those previously described for the production of L-amino acids. However, structural characteristics of the *A. tumefaciens* C58 hydantoin racemase native enzyme have proved to be different to those previously studied.

Acknowledgments

This work was supported by the project SAF-2001 2067 from DGICYT and FIT-010000-2001-177 from Programa Nacional de Biotecnología, Ministerio de Educación y Cultura, Spain. The authors thank Andy Taylor for the critical discussion of the manuscript and Pedro Madrid-Romero for technical assistance.

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