Complete Conversion of D,L-5-Monosubstituted Hydantoins with a Low Velocity of Chemical Racemization into D-Amino Acids Using Whole Cells of Recombinant *Escherichia coli*

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A reaction system was developed for the production of D-amino acids from D,L-5-monosubstituted hydantoins with a very slow rate of spontaneous racemization. For this purpose the D-hydantoinase and D-carbamoylase from *Agrobacterium radiobacter* NRRL B11291 were cloned in separate plasmids and expressed in *Escherichia coli*. The third enzyme, hydantoin racemase, was cloned from *Agrobacterium tumefaciens* C58. The hydantoin racemase amino acid sequence was significantly similar to those previously described. A reaction system consisting of recombinant *Escherichia coli* whole cell biocatalysts containing separately expressed D-hydantoinase, D-carbamoylase, and hydantoin recemase showed high substrate specificity and was effective toward both aliphatic and aromatic D,L-5-monosubstituted hydantoins. After optimizing reaction conditions (pH 8 and 50 °C), 100% conversion of D,L-5-(2-methylthioethyl)-hydantoin (15 mM) into D-methionine was obtained in 30 min.

Introduction

A wide spectrum of D,L-5-monosubstituted hydantoins are used as substrate for the production of optically pure amino acids. D-Amino acids are involved in the synthesis of antibiotics, pesticides, and other products of interest for the pharmaceutical, food, and agrochemical industries. 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 (Kim and Kim, 1995; Park et al., 2000). In this enzymatic reaction, called the hydantoinase process, first the chemically synthesized D,L-5-monosubstituted hydantoin ring is hydrolyzed by the D-hydantoinase enzyme. Further hydrolysis of the resulting enantioselective *N*-carbamoyl-D-amino acid to the free D-amino acid is catalyzed by highly stereoselective *N*-carbamoyl-D-amino acid aminohydrolase (D-carbamoylase).

At the same time as D-hydantoinase hydrolyses the enantioselective D-5-monosubstituted hydantoin, the chemical and/or enzymatic racemization of L-5-monosubstituted hydantoin starts. The velocity of the chemical racemization of D,L-5-monosubstituted hydantoins depends on the electronic nature of the residue at the 5′-position and is usually very slow (Wiese et al., 2000). One of the highest velocities of chemical racemization is presented by D,L-5-*p*-hydroxy-phenylhydantoin (D,L-pHPH) (Pietzsch and Syldatk, 2002). This D,L-5-monosubstituted hydantoin is the substrate of D-5-*p*-hydroxyphenylglycine (D-pHPG), a highly commercial D-amino acid used as a precursor of semisynthetic β-lactam antibiotics. However, there are other commercially interesting D-amino acids from D,L-5-monosubstituted hydantoins with slow chemical racemization, which can be racemated faster via enzymatic reaction. This reaction is catalyzed in the presence of hydantoin racemase, and together with the two above-mentioned enzymes it is responsible for the total conversion of the substrate into the product. 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 (Watabe et al., 1992a,b; Wiese et al., 2000; Wilms et al., 2001).

A recent work has presented more information about the genetic organization and genomic localization of the three genes involved in optically pure D-amino acids (Hils et al., 2001). In that work a cluster of 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. At about the same time, two different groups have reported the genome sequence of *Agrobacterium tumefaciens* C58 (Goodner et al., 2001; Wood et al., 2001). This genome comprises a circular chromosome, a linear chromosome, and two plasmids: the tumor-inducing plasmid pTIC58 and a second one named pATC58. These authors assigned a putative function to 3475 of a total 5419 predicted protein-coding genes. In the linear chromosome, together with 1880 other protein-coding genes, a putative hydantoin racemase was found.

In the present work, we have developed a three-step enzymatic reaction using recombinant whole cell biocatalysts after separate expression of a D-hydantoinase and D-carbamoylase from *Agrobacterium radiobacter* NRRL B11291 (Olivieri et al., 1981) and putative hydantoin racemace from *A. tumefaciens* C58. This whole cell system was capable of producing optically pure D-amino

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acids from D,L-5-monosubstituted hydantoins with low or very low chemical racemization. Biochemical parameters of the whole cell system were optimized in order to decrease the reaction time and increase D-amino acid production.

Materials and Methods

General Protocols and Reagents. Standard methods were used for the cloning and expression of the different genes (Sambrook et al., 1989; Ausubel et al., 1990). *E. coli* BL21 (Studier and Moffart, 1986) was used to clone and express D-hydantoinase, D-carbamoylase, and hydantoin racemase genes. All the D,L-5-monosubstituted hydantoins used in this work, D,L-5-benzyl-hydantoin (D,L-BH), D,L-5-ethyl-hydantoin (D,L-EH), D,L-5-(3′-indolyl-methylene)-hydantoin (D,L-IMH), D,L-5-isopropyl-hydantoin (D,L-IPH), D,L-5-(2-methylthioethyl)-hydantoin (D,L-MTEH), and D,L-5-*p*-hydroxy-phenylhydantoin (D,L-pHPH), were purchased from TCI Co. (Tokyo Chemical Industry, Japan). The resulting *N*-carbamoyl-D-amino acids were synthesized according to previous works (Stark and Smyth, 1963). The optically pure D-amino acids were purchased from Sigma Aldrich Quimica S.A. (Madrid, Spain). Restriction enzymes, T4 DNA ligase, and the thermostable *Pwo* polymerase for polymerase chain reaction (PCR) were purchased from Roche Diagnostic S.L. (Barcelona, Spain).

Cloning of D-Hydantoinase and D-Carbamoylase in *E. coli*. The genes encoding D-hydantoinase and D-carbamoylase were amplified by PCR from the isolated genomic DNA of *A. radiobacter* NRRL B11291. The primers were designed on the basis of GenBank sequence accession number X91070 (Grifantini et al., 1998). D-Hydantoinase gene was amplified with primers Hid5 (5′-AAGAATTCGTGACAGGAAAGCTTTATGGA-TATCATCATC-3′) and Hid3 (5′-AAGGTACCTTATT-GCTTGT-ATTTGCGGCG-3′). The *Eco*RI and *Kpn*I digested 1423-bp fragment was purified from agarose gel using QIAquick (Qiagen), and was then ligated into pBluescript II SK plasmid (Stratagene Cloning Systems) to create plasmid pSER11. D-Carbamoylase gene was amplified using Car5 (5′-AAGGATCCGTGACAGG-AAAGCTTTATGACACG-TCAGATGATACTTGC-3) and Car3 (5′-AACTGCAGTTAGAATTCCGCGATCAG-ACCG-3′). The digested 914-bp fragment was purified and ligated between the *Bam*HI and *Pst*I sites of pBluescript II SK plasmid (Stratagene Cloning Systems) to give plasmid pJMC38.

Cloning of Hydantoin Racemase in *E. coli*. A fragment corresponding to a putative hydantoin racemase from *A. tumefaciens* C58 was amplified by PCR. The primers used were designed on the basis of GenBank sequence accession numbers NC003063 and NC003305. These were Rac5 (5′-AAGAATTCGTGACAGGAAAGCT-ATTATGCGTGCGATGCAT-3′) and Rac3 (5′-AAGGTAC-CTTAGGCGCAGGCGA-3′). The hydantoin racemase fragment (720 bp) was digested with *Eco*RI and *Kpn*I. It was cloned into pBluescript II SK plasmid (Stratagene Cloning Systems). The plasmid with the hydantoin racemase fragment was named pSER17.

Expression of Enzymes in *E. coli*. The different constructions in BL21 strain were grown in Luria-Bertani liquid medium supplemented with 100 μg mL^{-1} of ampicillin. A single colony from each plate was transferred into 10 mL of LB medium with ampicillin at the concentration mentioned above in a 100-mL flask. This culture was incubated overnight at 37 °C with shaking. Then, 100 mL of LB containing the appropriate concentration of ampicillin in a 500-mL flask was inoculated with 1 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 D-hydantoinase, D-carbamoylase, and hydantoin racemase genes, isopropyl-β-D-thiogalactosidase (IPTG) was added to a final concentration of 0.2 mM and the cultivation was continued at 37 °C for an additional 4 h. For D-hydantoinase culture $MnCl_2$ at a final concentration of 2 mM was added at the same time as IPTG. The cells were collected by centrifugation at 7000*g* at 4 °C for 10 min. The pellets were then stored at −20 °C until the activity was measured.

Enzyme Reaction and Activity Assay. As the D-hydantoinase and D-carbamoylase react at different rates, different volumes of cells containing each gene were added to the reaction tube for the whole system. These volumes were selected in order to achieve similar activity of both proteins. For the hydantoin racemase gene the volume was the same as the D-hydantoinase. Previously, all the pellets were resuspended with 100 mM phosphate buffer (pH 8) to an OD_{600} of 10. The enzyme mixture was added to prewarmed D,L-MTEH solution (at a final concentration of 15 mM) and incubated at 50 °C. Aliquots were taken during the reaction and stopped by addition of twice the reaction volume of 1 M HCl. After centrifuging the stopped samples, the supernatant was analyzed by HPLC. The HPLC system (Breeze HPLC System, Waters, Barcelona) equipped with a Symmetry C_{18} column (4.6 × 150 mm, Waters, Barcelona) was used to determine concentrations of D,L-5-monosubstituted hydantoins, *N*-carbamoyl D-amino acids, and D-amino acids. The mobile phase used in the analysis (20 mM phosphoric acid pH 3 (85%) and methanol (15%)) was pumped with a flow of 0.75 mL min^{-1}. The UV detector was fixed at 210 nm, and D,L-MTEH, *N*-carbamoyl D-methionine, and D-methionine were detected and quantified.

Results

Sequence Analysis of Hydantoin Racemase from *Agrobacterium tumefaciens*. The hydantoin racemase gene from *A. tumefaciens* (720 bp) showed a deduced molecular weight of 25412 Da. When compared to GenBank database hydantoin racemase amino acid sequences from different sources, the *A. tumefaciens* hydantoin racemase was found to be homologous. Amino acid sequence identity between the studied hydantoin racemase and the *Agrobacterium* sp. IP I-67 putative hydantoin racemase (Hils et al., 2001) was 46.67%. When compared with hydantoin racemase of *Pseudomonas* sp. NS671 (Watabe et al., 1992a) it showed the same identity percentage (46.67%). Lower identity percentages were obtained when compared with *Arthrobacter aurescens* DM3747 (Wiese et al., 2000), 41.53%. However, the lowest identity percentage was obtained when comparing the new sequence with *Schizosaccharomyces pombe* putative hydantoin racemase (GenBank accession no. Z67998), 30.29%. Alignment of the amino acid sequences showed greatly conserved regions, especially in the N-terminal ends (Figure 1). Comparisons with amino acid sequences of other racemaces or putative racemaces in the databases available showed very low or negligible similarity with the hydantoin racemase studied.

Expression of Hydantoin Racemase in *E. coli*. To investigate the expression and the function of the enzyme, the gene was cloned into pBluescript II SK plasmid (Stratagene Cloning Systems). To avoid a fusion protein between the hydantoin racemase gene and the N-terminal end of the β-galactosidase gene present in the

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AtHyuA      1 -MHIRLINPNSTASMTAQALDSALRVKQKDIHVSAAN-PVDTPVSIEGQADEAMAVPGLL
AspHyuA     1 -MKIKVINPNTTWTMTDKIAGAARAAAAPGTEIVAVS-PDMGPVSIEGYYDEVEAAVGVV
PsHyuE      1 -MKIKVINPNTTLAMTKGIEHAAKSAARSDTQIVAVS-PKMGPASIESYYDEYLSIPGVI
AaHyuA      1 -MRILVINPNSSSALTESVADAAQQVVATGTIISAIN-PSRGPAVIEGSFDEALATFHLI
SpC1F7      1 MIRILVINPNSTVQMTESVKSVLDDCTPPNVQLEYLTCPPEGPKAIECVSDGVRSAAVLM

AtHyuA     59 AEIRKGEGHG-VDAYVIACFDD-PGLHAAREVARGPVIGICQAAVQVAMTISRRFSIITT
AspHyuA    59 DEVRKGELEG-CDGYVIACFGD-PGLNAAREVARGPVIGIAEAAMHAASLIGGSFSIISM
PsHyuE     59 EEIKKGEEEG-VDAFVIACWGD-PGLHAAREVTDKPVVGIAESSVYLASMLAARFSVVTV
AaHyuA     59 EEVERAERENPPDAYVIACFGD-PGLDAVKELTDRPVVGVAEAAIHMSSFVAATFSIVSI
SpC1F7     61 KYFEDHPPQV--DAFLVSCYSDHPLVTTLRETYRKPCTGIMQASILTALSLGRKVSVVTT

AtHyuA    117 LPRSIPIIEDLVEDYGAQRYCRK-VRAIDLPVLGLEEDP-EVAEALLRREIEAAKREDAA
AspHyuA   117 LGRSRGVLEHLVHSYGMAHKCRS-VRMTDLPVLEFEEEG-SDARRIVVEECRRAIEQDHA
PsHyuE    117 LPRIKTMLEDLVDSYGMQKRVLN-IRTTPMGVLDFERDP-EAGIEMLRQEGKRAVEEDNA
AaHyuA    118 LPRVRKHLHELVRQAGATNRLAS-IKLPNLGVMAFHEDE-HAALETLKQAAKEAVQEDGA
SpC1F7    119 TKRYEPLLTDGIHAMGISDSVFAGIASTGLAPLELDSKPRAEVDALLARTALRAVNEMGA

AtHyuA    175 EAIILGCAGMSSLCDRLRDATG--VPVIDGVTAAIKLAEALVGAGYTTSKVNAYDYPRVK
AspHyuA   175 DSVLLGCGGMSDLMAYVSQEIG--APAIDGVSAGVKLVEALVGMGLGTSKRAGLRLSDRE
PsHyuE    175 EAILLGCAGMAEFADSLEKELG--VPVIDGVVAGVKFAETIVDLGKKTSKLKTYKYPEKK
AaHyuA    176 ESIVLGCAGMVGPARQLSDELG--VPVIDPVEAACRVAESLVALGYQTSKANSYQKPTEK
SpC1F7    179 DVICLGCAGMIHMAHVLEKAVGPNIPIIDGTKAGVELLASLVRMNLFTSKQGVYQAVGSD

AtHyuA    233 GPALVACA---------
AspHyuA   233 SLHCSFSSFAPSRPR--
PsHyuE    233 EYVGALENFGRNQTTTK
AaHyuA    234 QYL--------------
SpC1F7    239 ------------------
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Figure 1. Multiple alignment of the amino acid sequences of hydantoin racemases. Hydantoin racemase from *Agrobacterium tumefaciens* (AtHyuA); putative hydantoin racemase from *Agrobacterium* sp. IP-671 (AspHyuA), GenBank accession no. AF335479; hydantoin racemase from *Pseudomonas* sp. NS761 (PsHyuE), GenBank accession no. M84731; hydantoin racemase from *Arthrobacter aurescens* DSM 3747 (AaHyuA), GenBank accession no. AF146701; putative hydantoin racemase from *Schizosaccharomyces pombe*, GenBank accession no. Z67998 (SpC1F7).

plasmid, TGA codon was included upstream from the RBS sequence and the beginning of the hydantoin racemase gene in Rac5 primer. The plasmid together with the insert was named pSER17. The kinetics of the gene induction in BL21 pSER17 was studied in order to find the minimum time necessary to obtain the maximum expression level. This proved to be 4 h, after which nearly 15% of the whole protein cell had been expressed. SDS−PAGE analysis of the crude extract revealed a protein band of 31 KD in the soluble fraction (Figure 2).

Expression of D-Hydantoinase and D-Carbamoylase in *E. coli*. D-Hydantoinase and D-carbamoylase genes were cloned from *A. radiobacter* NRRL B11291 in separate plasmids. D-Hydantoinase gene was amplified by PCR using Hid5 and Hid3 primers and ligated into pBluescript II SK plasmid (Stratagene Cloning Systems). TGA codon was included upstream from the RBS sequence and the beginning of the D-hydantoinase gene in the Hid5 primer to avoid a fusion protein between D-hydantoinase gene and the N-terminal end of the β-galactosidase gene present in pBluescript II SK plasmid (Stratagene Cloning Systems). The same strategy was followed to design the Car5 primer, which amplified D-carbamoylase from *A. radiobacter* NRRL B11291. The plasmid with the D-hydantoinase gene was named pSER11 and the plasmid containing the D-carbamoylase gene was named pJMC38.

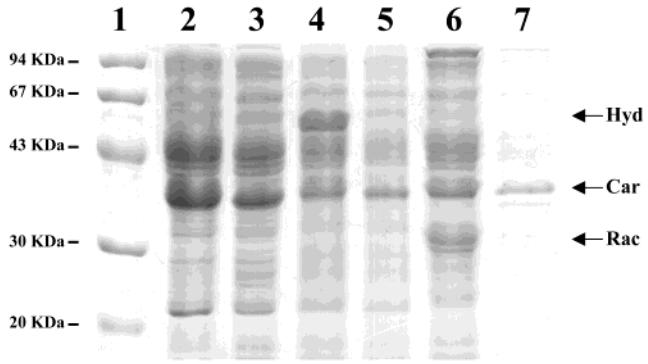

Figure 2. SDS−PAGE analysis (15%) of *E. coli* BL21 crude extracts harboring the plasmids for the D-hydantoinase (pSER11), D-carbamoylase (pJMC38), and hydantoin racemase (pSER17). Lane 1, low molecular weight marker; lanes 2 and 3 *E. coli* BL21 pJMC38 supernatant and pellet, respectively; lanes 4 and 5 *E. coli* BL21 pSER11 supernatant and pellet, respectively; lanes 6 and 7 *E. coli* BL21 pSER17 supernatant and pellet, respectively. In all cases the induction was for 4 h.

The expression of the D-hydantoinase and D-carbamoylase genes in different cells was analyzed on SDS−PAGE (Figure 2). The expression levels of D-hydantoinase in BL21 pSER11 and D-carbamoylase in BL21 pJMC38 were estimated as a 10% and 15% of the whole soluble proteins. The insoluble fractions (pellet) showed the

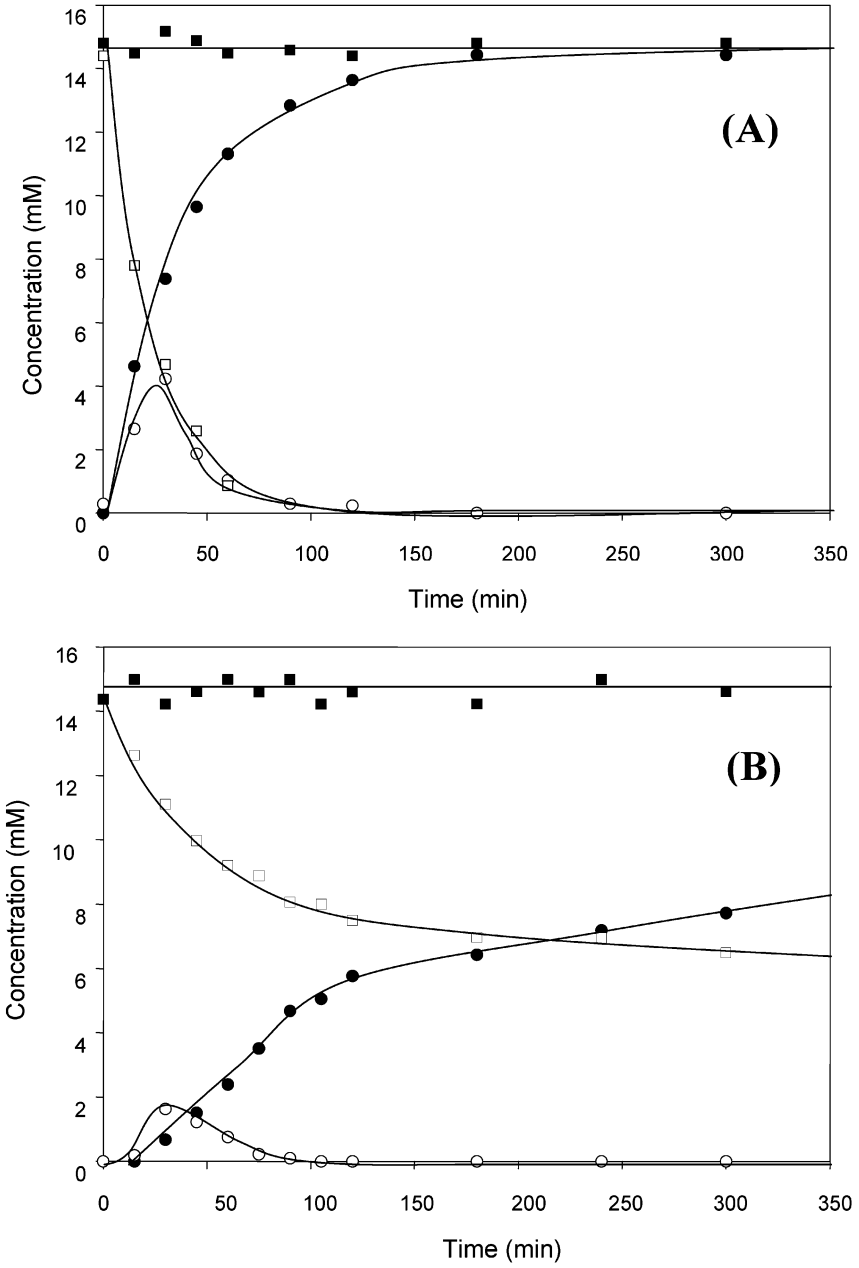

Figure 3. Reaction profile of D-methionine production from D,L-MTEH using the triple (A) and the double (B) system. Free *E. coli* cells containing separately expressed enzymes were resuspended with 100 mM phosphate buffer (pH 8) to an OD_{600} of 10. Initial substrate concentration was 15 mM: (●) D-methionine; (○) *N*-carbamoyl D-methionine; (□) D,L-MTEH; (■) sum of all three.

presence of protein bands in D-carbamoylase but not in D-hydantoinase.

Whole Cell Reaction Using D-Hydantoinase, D-Carbamoylase, and Hydantoin Racemase Enzymes. To study the activity of the obtained hydantoin racemase, a reaction system using whole cells was developed. This triple system contained separately expressed D-hydantoinase, D-carbamoylase, and hydantoin racemase enzymes added in the same ratio in the reaction tube (see Materials and Methods). Simultaneously, a double reaction system with only separately expressed D-hydantoinase and D-carbamoylase enzymes was created in order to ascertain the necessity of hydantoin racemase for complete conversion.

Complete substrate conversion was observed in the triple reaction system (Figure 3a) using different D,L-5-monosubstituted hydantoins (D,L-BH; D,L-EH; D,L-IMH; D,L-IPH; D,L-MTEH). When the double system with only D-hydantoinase and D-carbamoylase enzymes was used for the reaction with the same substrates, the conversion reaction stopped after consuming 50% of the hydantoins (Figure 3b). The only exception to this was with D,L-pHPH substrate, in which case conversion was total as a result of its very fast spontaneous racemization.

Temperature and pH dependency are known to affect the kinetic properties of the conversion of D,L-5-monosubstituted hydantoins into the corresponding D-amino acids. For this reason both parameters were studied in the whole cell system. Previous studies have described different optimal pH values for D-hydantoinase and D-carbamoylase enzymes (Olivieri et al., 1981; Kim and Kim, 1995; Louwrier and Knowles, 1996; Chao et al., 1999a,b; Park et al., 2000). To calculate the optimal pH value for the whole cell reaction, D-methionine yield was studied in a pH range of 6.5−9.5. Three different buffers were used, phosphate buffer from 6.5 to 7.5, Tris buffer from 7.5 to 9, and glycine/NaOH buffer at 9 and 9.5. When common pH values were analyzed, no differences

were observed in the conversion percentages of the three buffers. The whole cell system showed maximum activity at pH 8, with total conversion from D,L-MTEH to D-methionine after 60 min (Figure 3a). Higher pH values (8.5−9.5) produced total conversion in the same time, probably because spontaneous hydantoin racemization occurred. This phenomenon was corroborated by conversions of over 50% at these pH values, using the double reaction system.

The optimum temperature for the whole cell system was around 50 °C, reaching 100% conversion in 30 min at pH 8. There was a correlation between temperature and enzyme activity up to 50 °C, whereas higher temperatures produced lower conversion percentages of D-methionine from D,L-MTEH. Similar results have been reported for recombinant systems with D-hydantoinase and D-carbamoylase genes where the thermostability of these enzymes was analyzed (Chao et al., 1999a,b; Park et al., 2000).

Discussion

The production of optically pure D-amino acids from D,L-5-monosubstituted hydantoins using the hydantoinase process and enzymatic decarbamoylation by recombinant systems has been reported, but these studies used D,L-5-*p*-hydroxy-phenylhydantoin (D,L-pHPH) (Grifantini et al., 1998; Chao et al., 1999b; Park et al., 2000). D,L-pHPH has a very high velocity of chemical racemization allowing total conversion to D-amino acid. However, when total conversion is desired using substrate with a low velocity of chemical racemization, it can only be produced by using hydantoin racemase together with D-hydantoinase and D-carbamoylase.

The *A. tumefaciens* hydantoin racemase showed significant amino acid sequence identity when compared with those previously described. Moreover, two cysteine residues at positions 79 and 184 that were probably involved in the catalytic center of the protein (Wiese et al., 2000) were highly conserved within the studied hydantoin racemases. The molecular weight of the *A. tumefaciens* hydantoin racemase monomer after loading on SDS−PAGE (31 kDa) was very similar to those of *Pseudomonas* sp. NS671 and *Arthrobacter aurescens* DSM3747 (32 and 31 kDa, respectively). Moreover, the calculated molecular mass of the *A. tumefaciens* hydantoin racemase monomer (25.4 kDa) was very similar to those of *Pseudomonas* sp. NS671 and *Arthrobacter aurescens* DSM3747 (27.1 and 25.1 kDa, respectively) and for the three enzymes shorter than the denatured monomer on SDS−PAGE. These differences between the calculated molecular masses and the apparent ones for the hydantoin racemases may be due to a molten globule state, where the enzymes in SDS−PAGE conditions are not fully unfolded (Ewbank and Creighton, 1991). However, the hydantoin racemase identity percentage between *A. tumefaciens* and *Agrobacterium* sp. IP I-671 was identical to that between *A. tumefaciens* and *Arthrobacter aurescens* DSM 3747, when higher identity would be expected for the former, as they belong to the same genus. In addition to this, a recent work has described that the D-hydantoinase-process genes in *Agrobacterium* sp. IP I-671 and *A. radiobacter* NRRL B11291 are located on large plasmids of sizes similar to the Ti plasmids (Hils et al., 2001), but the *A. tumefaciens* C58 hydantoin racemase is included in the linear chromosome (GenBank accession nos. NC003063, NC003305). Furthermore, all the known genetic organizations of the hydantoin utilization genes are very similar, with hydantoinase, carbamoylase, and hydantoin racemase grouped together in one cluster, while the *A. tumefaciens* C58 hydantoin racemase was located far from the hydantoinase and carbamoylase genes. These data indicate that this hydantoin racemase may be involved in several metabolic pathways.

After combining the hydantoin racemase from *A. tumefaciens* C58 together with D-hydantoinase and D-carbamoylase from *A. radiobacter* NRRL B11291 a complete biocatalysis system was created. This system allowed complete conversion of D,L-5-monosubstituted hydantoins to D-amino acids in a very short reaction time. Previously, hydantoin racemases from *Pseudomonas* sp. NS671 and *Arthrobacter aurescens* DSM3747 have been biochemically characterized and used in a whole cell system for the production of L-amino acids (Watabe et al., 1992a,b; Wiese et al., 2000; Wilms et al., 2001). This is the first recombinant system for the production of D-amino acids from precursors with very low spontaneous racemization. Moreover, the system showed high substrate specificity, and it was effective toward both aliphatic and aromatic D,L-5-monosubstituted hydantoins, producing a complete conversion to the corresponding D-amino acids. As expected, complete conversion of D,L-pHPH to D-pHPG was produced using both triple and double reaction systems.

The whole cell system is the alternative to using immobilized enzymes for D-amino acid production from D,L-5-monosubstituted hydantoins. To provide an economically feasible process, cost-effective production of the biocatalysis system is a prerequisite. In this study, a catalytic system was used in which the three enzymes were separately expressed and then combined in the same activity ratio for the reaction. Recent studies have reported that D-amino acid production from hydantoins by *A. radiobacter* was higher in cells with intact cell membranes than in toluene-treated cells (Lee et al., 2001). However, in our work no differences in activity were detected after toluene cell permeabilization. Activity of the enzymes belonging to the whole cell system is strongly influenced by the pH and the temperature of the reaction, considerably influencing the yield and the amount of D-amino acid produced. pH values above the optimum produced the same conversion percentage but also accumulation of the *N*-carbamoyl-D-amino acid. The enzyme reaction rate increased higher temperatures, but after the optimum temperature activity decreased, probably as a result of the deactivation of D-carbamoylase. These data suggest that D-carbamoylase is more labile against high temperatures and pH, and it is the limiting factor of the whole reaction. These results were corroborated using the double reaction system and were in accordance with previous studies where the double system and D-carbamoylase alone were analyzed (Louwrier and Knowles, 1996; Chao et al., 1999a,b; Park et al., 2000).

In this work a complete enzymatic process is presented for the production of D-amino acid from any D,L-5-monosubstituted hydantoin. Regardless of the spontaneous racemization rate, after inclusion of a novel hydantoin racemase from *A. tumefaciens* total conversion and 100% optically pure D-amino acid can be obtained. The hydantoinase process is an economically attractive method for the production of many unnatural amino acids that are components of potential pharmaceuticals (Bommarius et al., 2001). The inclusion of the hydantoin racemase enzyme in the D-hydantoinase process will allow the production of these potential pharmaceuticals based on natural and unnatural D-amino acids.

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