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D-*p*-Hydroxyphenylglycine production from DL-5-*p*-hydroxyphenylhydantoin by *Agrobacterium* sp.

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Summary. A bacterium that stereospecifically produces D-*p*-hydroxyphenylglycine (D-PHPG) from DL-5-*p*-hydroxyphenylhydantoin (DL-5-PHPH) was isolated from soil and identified as *Agrobacterium* sp. IP-I 671. The hydantoinase and the *N*-carbamyl-amino acid amidohydrolase involved in this biotransformation process were both strictly D-stereospecific. Their biosynthesis was found to be inducible by addition of 2-thiouracil to the cultivation media, or to a lesser extent by uracil. The amidohydrolase activity of *Agrobacterium* sp. was strongly inhibited by ammonium ions co-produced with D-PHPG, whereas the hydantoinase activity under the same conditions was unaffected. Optimum temperature and pH were respectively 55° C and 10 for the partially purified hydantoinase, 45° C and 6.75 when resting cells were used. Biotransformations under these slightly acidic conditions allowed to complete conversion of 30 g/l DL-5-PHPH into 25 g/l of D-PHPG (molar yield 96%) and involved enzymatic racemization of DL-5-PHPH.

Introduction

D-Phenylglycine and D-*p*-hydroxyphenylglycine (D-PHPG) are valuable synthons for the production of semisynthetic penicillins and cephalosporins such as ampicillin and amoxicillin. DL-5-Substituted hydantoins are starting materials for the chemical synthesis of DL-amino acids. However, this chemical process is cumbersome as the chemical hydrolysis to amino acids requires racemate resolution to obtain optically pure amino acids.

Dihydropyrimidinase (E.C 3.5.2.2 hydantoinase), an enzyme that is widely distributed in nature, catalyses the hydrolytic ring cleavage of dihydropyrimidines to *N*-carbamyl-β-amino acids and of DL-5-substituted hydantoins to *N*-carbamyl amino acids (Wallach and Grisolia 1957). Several optically active amino acids, including D-hydroxyphenylglycine (Olivieri et al. 1981; Yokozeki et al. 1987), are produced by using this enzymatic route, starting from the corresponding racemic 5-substituted hydantoins. The latter can be cheaply synthesised from aldehydes (Bucherer and Steiner 1934), and they easily undergo spontaneous racemization under mildly alkaline conditions. Consequently, enzymatic cleavage and simultaneous racemization lead to high yields in production of the corresponding asymmetric *N*-carbamyl amino acids, which can be converted to free amino acids either by chemical methods (Takahashi et al. 1979) or by a second enzymatic step catalysed by an *N*-carbamyl-amino acid amidohydrolase (amidohydrolase) (Olivieri et al. 1979). Few microorganisms are known to produce both hydantoinase and amidohydrolase, thus permitting a fully enzymatic transformation of the racemic hydantoin to the optically active amino acid in a one-step process (for a review, see Syldtak et al. 1986).

The present paper reports the isolation of a novel *Agrobacterium* species, showing a strong ability to stereospecifically transform DL-5-*p*-hydroxyphenylhydantoin (DL-5-PHPH) into D-PHPG by this direct route. Improvement of the D-amino acid production by resting cells was investigated through enhancement of the hydantoinase and aminohydrolase biosynthesis during fermentation. The optimal conditions for the bioconversion were determined. Substrate stereospecificity of this enzyme system and the effects of product inhibition are also reported.

Material and methods

Chemicals. Uracil, uric acid, 2-imidazolidinethione, thymine, 5,5-dimethylhydantoin and D-(−)-*p*-hydroxyphenylglycine were purchased from Janssen Chimica (Pantin, France). The D-, L-, or DL-5-substituted hydantoins and their corresponding *N*-carbamyl D-, L- or DL-amino acids were obtained from Société Française Hoechst (Stains, France), and DL-5-methylthioethylhydantoin from Société Rhône-Poulenc (Vitry-zur-Seine, France). All other chemicals used were of the best analytical grade available.

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Screening methods. The isolation of the microorganisms has been performed on a selective medium containing (in g/l): glycerol, 5; KH_2PO_4, 3; $MgSO_4$, 1; $MnSO_4 \cdot H_2O$, 0.02; $FeSO_4 \cdot 5H_2O$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.02; $CaCO_3$, 0.02; NaCl, 1; DL-5-isopropyl hydantoin, 3; cycloheximide, 50 µg. Liquid samples from soil were spread on agar petri dishes of the above medium. Once colonies appeared, each one was grown in the same liquid medium, and then re-isolated on the agar medium until a pure colony was obtained. The microorganism isolated for its capacity to hydrolyse DL-PHPH to D-PHPG was taxonomically and biochemically identified by the Collection de l'Institut Pasteur (Paris, France).

Microorganisms and cultivation media. A Gram-negative bacterium, strictly aerobic, isolated from soil, *Agrobacterium* sp. IP I-671 was used in all experiments. Medium I contained (in g/l): glycerol, 10; peptone, 10; yeast extract, 10; KH_2PO_4, 1; $MgSO_4$, 0.5; uracil, 0.1; agar, 14, in distilled water, and was adjusted to pH 7.0 with NaOH. Medium II contained (in g/l): sucrose, 10; peptone, 5; yeast extract, 5; KH_2PO_4, 1; NaCl, 0.5; $MgSO_4$, 0.1; $MnSO_4 \cdot 1H_2O$, 0.1; uracil, 0.5, in distilled water, and was adjusted to pH 7.0 with NaOH. This medium was used for the preparation of resting cells. Medium III had the same composition as medium II, except that it contained no uracil. Variations of this basic medium were used in order to determine the optimal conditions for growth and enzyme production. The following procedure was used: a loopful of cells of the microorganism grown on an agar plate of medium I was used first to inoculate 50 ml medium II in a 250-ml flask. After aerobic cultivation for 20 h at 30° C, a loopfull of this culture was inoculated in 250 ml medium III in a 1-L flask. After further cultivation for 20 h at 30° C, cells were harvested and washed twice with cold 0.1 *M* potassium phosphate buffer (pH 7.0). Activities of the cells in *N*-carbamyl-D-PHPG or D-PHPG production and the biomass were determined as described below.

Preparation of resting cells. Medium II (50 ml) in 250-ml flasks was inoculated with a loopful of *Agrobacterium* sp. subcultured on agar plates of medium I. The bacterium was aerobically cultivated at 30° C for 20 h on a rotary shaker (100 rpm). This seed culture (10 ml) was added to 250 ml of the same medium II and incubated as above for a further 20 h. The latter culture constituted the inoculum for 5 l medium II in a 7-l fermentor (Setric Génie Industriel (Toulouse, France). Biomass was produced aerobically (aeration rate, 0.5 v/v per minute; agitation rate, 400 rpm) at 30° C and the pH was maintained constant at 7.0 by NaOH addition. Cells were harvested by centrifugation (5000 *g*, 30 min) at the end of the log phase (16–18 h) where both hydantoinase and amidohydrolase actvities of the bacterium were found to be the highest. The cells were finally washed twice in a cold 0.1 *M* potassium phosphate buffer (pH 7.0).

Reaction with resting cells. Unless otherwise stated, a standard assay mixture consisted of 250 mg DL-5-PHPH and 250 mg cells (dry weight) in 25 ml of 0.1 *M* potassium phosphate buffer (pH 8.0), as most of the hydantoins are known to racemize easily under slightly alkaline conditions (Dinelli et al. 1975). The reaction was carried out at 40° C for 6 h with moderate stirring and nitrogen sparging to prevent oxidation of the hydantoin. Other details of the assay are given in each table. The activities of the cells in *N*-carbamyl-D-PHPG (hydantoinase activity) or D-PHPG (amidohydrolase activity) production were expressed in units per mg (U/mg) of dry cell mass. One unit was defined as the number of µmoles of *N*-carbamyl-D-PHPG or D-PHPG formed per hour.

Analytical methods. For the quantitative determination of the D-PHPG or *N*-carbamyl-D-PHPG formed and the DL-5-PHPH which remained in the bioconversion broth, aliquots were withdrawn, centrifuged (5000 *g*, 5 min) and the supernatant diluted with water. Analyses were performed by HPLC, using a C18 column (silica ODS-Hypersil, Société Française Chromato Colonne, Neuilly-Plaisance, France); column size: 4.6 × 250 mm; mobile phase: $H_2O/CH_3CN/H_3PO_4$ 85% (95/5/0.01 by vol); flow rate: 1 ml/min; and detector: UV detector (Milton-Roy, Paris) at 210 nm. Biomass concentration was estimated by optical density (OD) measurement at 610 nm (OD 0.1; 2.1×10^8 cells/ml); OD was calibrated against dry weight. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Product identification. The bioconversion broth was centrifuged and the supernatant was applied to preparative silica gel thin-layer chromatography (TLC) plates, using as developing solvents 1-butanol/acetic acid/water (65/15/20) or 1-propanol/ammonia (70/30). The *N*-carbamyl-D-PHPG formed was detected with Ehrlich's reagent (10% 4-dimethylaminobenzaldehyde in 6 *M* HCl), and the D-PHPG was visualized with ninhydrin. The gel bands corresponding to these two products were collected and extracted with 50% aqueous ethanol, followed by recrystallization after concentration. Identification of *N*-carbamyl-D-PHPG and D-PHPG was also achieved by comparison of infrared (IR) and ^{1}H-nuclear magnetic resonance (NMR) spectra with those of the authentic products. The optical purity of the products was calculated from the specific optical rotation values given in literature (Eagles et al. 1971). The enantiomeric excess was determined by HPLC chromatography (system above) using a chiral support (column Chiraline LO 151, Société Française chromato Colonne): column dimension, 4.5 × 150 mm; mobile phase, hexane/2-propanol (95:5 by vol); flow rate, 1 ml/min.

Enzyme preparation. The cells prepared as described for the resting cells, were resuspended in 0.05 *M* TRIS HCl – 0.025 *M* NaCl, pH 7.5 (dilution buffer) to give a 10%–20% (w/v) cell suspension. They were disrupted by passing them twice through a French pressure cell (1000 psi). The crude extract was clarified by centrifugation (10 000 *g*, 30 min), and 0.1 vol of 2% (w/v) protamine sulphate solution (pH 6.0) was added to the extract, which was centrifuged again. The supernatant was fractionated by adding solid ammonium sulphate and the precipitate obtained at 60% saturation was dissolved in a minimal amount of dilution buffer. The solution was dialysed against the dilution buffer and then applied to DEAE-Trisacryl M (IBF-Biotechnics, Villeneuve-la-Garenne, France, 26 × 700 mm column) equilibrated in the same buffer. After washing the column, the enzyme could be eluted at a concentration of 0.05 *M* NaCl.

Assay of the partially purified hydantoinase. The enzyme assay was performed according to a modification of the method of Takahashi et al. (1978). Unless otherwise stated the reaction mixture contained 10 µmol DL-5-PHPH (mol. wt. 192), 50 µmol TRIS-HCl buffer, pH 8.0, and the partially purified enzyme (0.3 mg protein) in a total volume of 1 ml. The reaction mixture was incubated for 30 min at 40° C in a water bath; the reaction was stopped by addition of 50 µl of trichloroacetic acid (40% w/v). The precipitated proteins were removed by centrifugation and the concentration of *N*-carbamyl-PHPG in the supernatant determined as described by Takahashi et al. (1978). The specific activity of the hydantoinase was expressed as nkat · mg^{-1} protein. One katal (kat) was defined as the amount of enzyme required to produce 1 mol *N*-carbamyl-PHPG (mol. wt. 210) in 1 s under the conditions of assay. A blank with acid-denatured enzyme was performed for all samples.

Results

Isolation and identification of D-PHPG producing microorganisms

By using a medium in which D,L-isopropylhydantoin was the sole nitrogen source, eight bacterial strains were isolated from soil samples. Among these strains, one was a good D-PHPG producer. That strain has been identified as an *Agrobacterium* sp. by the Collec-

tion de l'Institut Pasteur. The taxonomic and biochemical characteristics of this bacterium are very similar to those of *A. tumefaciens* CIP 67.1, but the protein profile of both microorganisms as shown by sodium dodecyl sulphate-polyacrylamide gel electrophoresis is slightly different.

Isolation and identification of the reaction product

To investigate the configuration of the PHPG produced, the following procedure was used: 500 mg (dry weight) washed cells were re-suspended in 50 ml of 0.1 *M* potassium phosphate buffer (pH 8.0) containing 500 mg of each of the substrates listed in Table 1. After the reaction mixture had been incubated at 40° C for 30 h, the reaction product was isolated. *N*-Carbamyl-PHPG was isolated from a similar bioconversion broth buffered at pH 9.5 (with 0.1 *M* TRIS-HCl buffer) incubated at 50° C. The colourless crystals obtained showed the same patterns of ^{1}H-NMR and IR spectra and the same R_f value on silica-gel TLC as those of authentic-*N*-carbamyl-D-PHPG or D-PHPG. The absolute configuration of these two products when investigated by chiral chromatography, was shown to be the D-enantiomer in both cases; enantiomeric excess of the isolated *N*-carbamyl-D-PHPG was found to be ≥97% of the D-form. From these results, the products of enzymatic hydrolysis of DL-5-PHPH by *Agrobacterium* sp. were confirmed to be the D-forms of *N*-carbamyl-PHPG and PHPG.

Effects of temperature and pH on the activity of resting cells

In order to optimize the biotransformation, we investigated first the temperature and pH optima for both the specific production of D-PHPG and the complete conversion of DL-5-PHPH (*N*-carbamyl-D-PHPG plus D-PHPG produced). The optimum temperature for amino acid production was 40°–45° C, while the optimum for

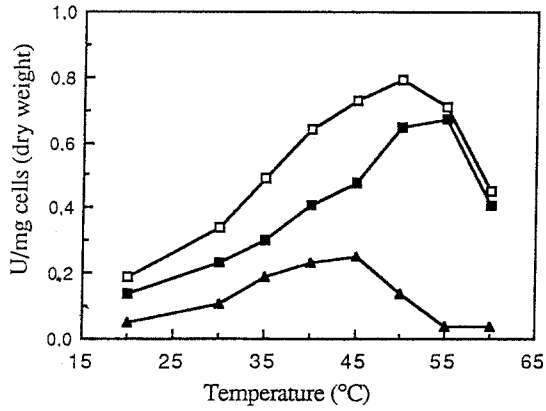

Fig. 1. Effect of temperature on the activity of resting cells: ▲, D-*p*-hydroxyphenylglycine (D-PHPG); ■, D-*N*-carbamyl-PHPG; □, total conversion of DL-5-*p*-hydroxyphenylhydantoin (DL-5-PHPH). U = units

the overall conversion of DL-5-PHPH was 50° C. At 50° C the D-PHPG produced represented less than 20% of the hydantoin transformed (Fig. 1). Concerning pH conditions, the best kinetics for the amino acid production was at pH 6.5–6.75. This slightly acidic pH optimum is lower than those reported in similar biotransformations with *A. radiobacter* (Olivieri et al. 1981) or *Pseudomonas* sp. (Yokozeki et al. 1987). At pH 9.0, where the maximum hydrolysis of DL-5-PHPH into *N*-carbamyl-D-PHPG was achieved, very little amidohydrolase activity remained (Fig. 2).

The acidic pH optimum indicated the mechanism involved in the racemization of DL-5-PHPH. Under the conditions of the bioconversion (as described in the experimental section), except for buffering at pH 6.75 and adding no bacteria, neither the D- nor the L-form of 5-PHPH were racemized (data not shown), unlike at pH 8.0 where both compounds racemized (half-life of stereoisomer: ca. 20 min). Spontaneous racemization of D- or L-*N*-carbamyl-PHPG respectively did not occur whatever the pH. Resting cells of *Agrobacterium* sp. were also unable to hydrolyse *N*-carbamyl-L-PHPG irrespective of the pH (Table 1).

Table 1. Isolation and identification of the reaction product

Substrate (500 mg)	PHPG isolated[a] (mg)	Molar yield[b] (in %)	$[\alpha]_D^{25}$ isolate[c]	Optical purity isolate in %[d]	ee (in %)[e]
DL-5-PHPH	335	77	−151°	98	96
D-5-PHPH	315	72	−151°	98	93
L-5-PHPH	357	81	−150°	97	95
N-Carbamyl-DL-PHPG	113	28	−149°	97	92
N-Carbamyl-D-PHPG	270	68	−153°	99	97
N-Carbamyl-L-PHPG	NR[f]	—	—	—	—

PHPG = *p*-hydroxyphenylglycine; PHPH = *p*-hydroxyphenylhydantoin

[a] Values obtained after recrystallization of the isolates in 70% ethanol

[b] Calculated from the respective mol. wt.: PHPH (192); *N*-carbamyl-PHPG (210); PHPG (167)

[c] For all the isolates C = 1, in 1 *N* HCl

[d] Calculated from the optical rotation of authentic D-PHPG: $[\alpha]_D^{27.5} = -154°$ (C = 0.6, in 1 *N* HCl)

[e] ee: enantiomeric excess in D-PHPG determined by HPLC chromatography on chiral stationary phase

[f] NR: no reaction. Assays were made with reaction mixtures buffered at pH 7.0; pH 7.5; pH 8.0; and pH 9.0

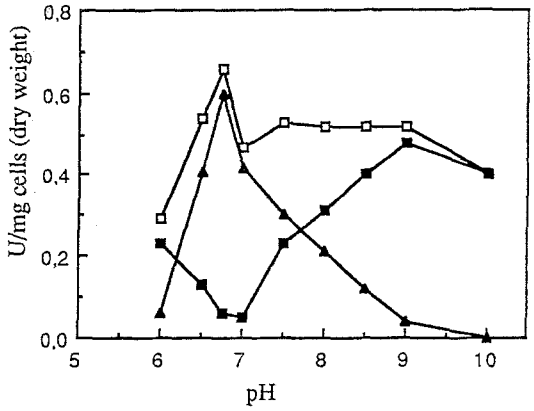

Fig. 2. Effect of pH on the activity of resting cells. Resting cells were incubated under standard conditions at the pH indicated in the figure: pH 5.0–8.0, 0.1 *M* phosphate buffer; pH 8.0–9.0, 0.1 *M* TRIS-HCl buffer; pH 9.0–10.0 carbonate buffer: ▲, D-PHPG; ■, D-*N*-carbamyl-PHPG; □, total conversion of DL-5-PHPH

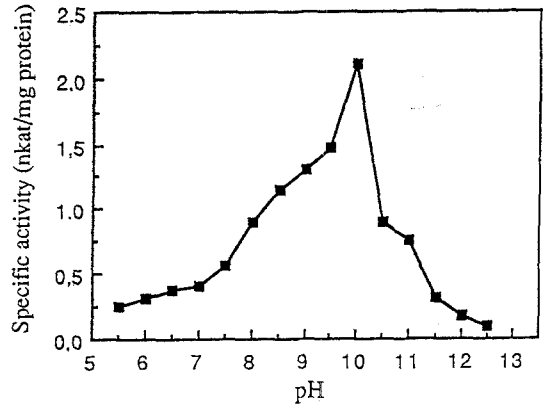

Fig. 4. Effect of pH on hydantoinase activity: ■, D-*N*-carbamyl-PHPG. The enzyme was incubated under standard conditions at the pH values indicated in the figure: pH 5.0–7.5, 0.05 *M* phosphate buffer; pH 8.0–9.0, 0.05 *M* TRIS-HCl buffer; pH 9.0–12.5, 0.05 *M* carbonate buffer

Effect of temperature and pH on the activity of the partially purified hydantoinase

The optimal temperature for the hydrolysis of DL-5-PHPH into *N*-carbamyl-D-PHPG by the partially purified enzyme was 55°–60° C (Fig. 3). The optimal pH was close to 10 (Fig. 4). This value is similar to the optimum pH for *N*-carbamyl-D-PHPG production by resting cells (Fig. 2). When a hydantoinase assay was performed as described in Materials and methods, with no DL-5-PHPH as substrate, but either 50 µmol of D-, L-, or DL-*N*-carbamyl-PHPG, no degradation of these compounds was observed after 30 min incubation. Buffering the assay with TRIS-maleate buffer, pH 6.75, did not lead to any hydrolysis of these products.

Effect of the ammonium ions on the activity of resting cells

For an *Agrobacterium* catalysing a similar reaction, it has been shown that the ammonium ions co-produced with D-PHPG led to a pH-dependent ammonia inhibition of the amino acid formation (Olivieri et al. 1981).

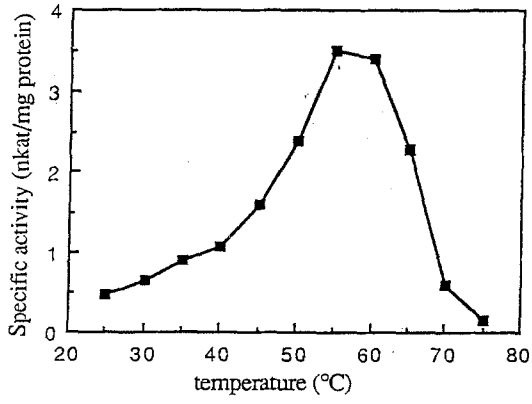

Fig. 3. Effect of temperature on hydantoinase activity: ■, D-*N*-carbamyl-PHPG

To investigate this effect with our *Agrobacterium* sp., NH_4Cl was added to the bioconversion broth at different pHs and different concentrations (Table 2). The results obtained match with those reported by Olivieri et al. (1981): a strong inhibition effect for the amidohydrolase activity was observed. At pH 6.75 (optimum for the amino acid formation) this effect was less pronounced by comparison to pH 8.0 or 9.5. At these alkaline pHs a huge decrease in amino acid production was observed. In the meantime hydantoinase activity remained unaffected.

Optimization of medium composition for resting cell preparation

In order to define the best medium for biosynthesis of the hydantoinase and the amidohydrolase during fermentation, components in the cultivation medium were examined using variations of medium III. None of the combinations in the carbon and nitrogen sources significantly promoted cell growth or the activity of either of these enzymes.

As the induction of these hydantoin-hydrolysing enzymes by addition of 5-substituted hydantoins to the cultivation medium has been reported previously (Syldatk et al. 1986; Yokozeki et al. 1987), the possible induction effects of several DL-5-substituted hydantoins were investigated. Among them, only DL-5-methylthioethylhydantoin enhanced somewhat the activity of both enzymes (Table 3). Investigations made with structural analogues of the 5-substituted hydantoins showed that uracil and dihydrouracil had the same effect as DL-5-methylthiohydantoin. In *Agrobacterium* sp., 2-thiouracil had the best inductive effect on the hydantoinase and amidohydrolase enzymes (fivefold increase in the activities of both enzymes). Stepwise addition of either uracil, dihydrouracil or 2-thiouracil to medium III during fermentation did not further improve the level of induction. From these results, medium IV was chosen as the best medium for the enzyme formation and thus was employed in the following experiment.

Table 2. Effect of ammonium ions on the activity of resting cells

pH	NH_4^+ (m*M*)[a]	*N*-Carbamyl-D-PHPG produced (U/mg cells)	D-PHPG produced (U/mg cells)	DL-5-PHPH converted[b] (U/mg cells)
6.75	0	0.10	0.53	0.63
	100	0.12	0.48	0.60
	200	0.17	0.37	0.54
	400	0.30	0.31	0.61
8.0	0	0.33	0.20	0.53
	100	0.32	0.15	0.47
	200	0.44	0.12	0.56
	400	0.46	0.08	0.54
9.5[c]	0	0.54	0.10	0.55
	100	0.48	0.07	0.55
	200	0.54	0.06	0.60
	400	0.55	0.04	0.59

[a] As NH_4Cl added to resting cell reaction mixture
[b] Calculated as *N*-carbamyl-D-PHPG plus D-PHPG produced in the standard resting cell assay
[c] In TRIS-HCl buffer
U = units

Table 3. Effect of hydantoin derivatives and structural analogues on formation of enzyme activity

Compounds (2 m*M*)	*N*-Carbamyl-D-PHPG (U/mg cells)	D-PHPG (U/mg cells)	DL-5-PHPH converted[a] (U/mg cells)
None	0.14	0.21	0.35
DL-5-Methylthioethylhydantoin	0.39	0.48	0.87
DL-5-Benzylhydantoin	0.25	0.29	0.54
DL-5-*p*-Hydroxyphenylhydantoin	0.24	0.28	0.52
DL-5-*sec*-Butylhydantoin	0.23	0.26	0.49
DL-5-Isobutylhydantoin	0.22	0.25	0.47
DL-5-Isopropylhydantoin	0.22	0.24	0.46
DL-5-Cyanoethylhydantoin	0.20	0.23	0.43
Hydantoin	0.14	0.16	0.30
5,5-Dimethylhydantoin	0.14	0.16	0.30
DL-5-Carboxyethylhydantoin	0.13	0.12	0.25
2-Thiouracil	0.69	0.96	1.65
Uracil	0.42	0.62	1.04
Dihydrouracil	0.27	0.57	0.84
Dihydroorotic acid	0.21	0.35	0.56
Dihydrothymine	0.20	0.34	0.54
Allantoin	0.23	0.30	0.53
Thymine	0.24	0.29	0.53
6-Azauracil	0.25	0.29	0.54
2-Thiohydantoin	0.21	0.27	0.48
2-Imidazolidone	0.25	0.27	0.52
Uric acid	0.17	0.24	0.41

[a] Calculated as *N*-carbamyl-D-PHPG plus D-PHPG produced in the standard resting cell assay

Time course of D-PHPG production by Agrobacterium sp.

A typical bioconversion pattern for the production of D-PHPG from DL-5-PHPH in the presence of washed cells, the D-PHPG producing activity of which had been induced by 2-thiouracil, is shown in Fig. 5. Under the optimal conditions (pH 6.7, 45° C) 30 g/l DL-5-PHPH were completely consumed in less than 20 h and 25 g/l D-PHPG asymmetrically produced (molar yield 96%). *N*-Carbamyl-D-PHPG never exceeded a concentration of 6 g/l and represented less than 2% of the hydantoin hydrolysis product when the bioconversion went to completion.

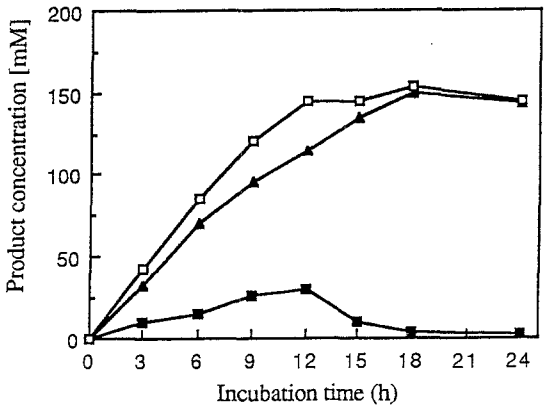

Fig. 5. Time course of D-PHPG production by resting cells of *Agrobacterium* sp. under optimal conditions: ▲, D-PHPG; ■, D-*N*-carbamyl-PHPG; □, total conversion of DL-5-PHPH

Discussion

In the past, several papers have dealt with the enzymatic hydrolysis of DL-5-substituted hydantoins, either to produce different *N*-carbamyl-amino acids (Morin et al. 1986) or to obtain in a one-step process their corresponding D- or L-amino acids (Syldatk et al. 1986). Recent developments of this enzymatic route to optically pure amino acids establish the possibility of using immobilized cells with fairly good hydantoinase activity in different kinds of enzymatic reactors (Chevalier et al. 1989).

There are only a few reports on the direct production of D-PHPG from DL-5-PHPH. From the work of Olivieri et al. (1981) it is known that this production may be achieved using resting cells of *A. radiobacter*. More recently Yokozeki et al. (1987) obtained similar results with a *Pseudomonas* strain. A bacterial strain able to produce directly and enantioselectively D-PHPG from DL-5-PHPH has now been isolated and identified as an *Agrobacterium* sp. Improvement of the D-amino acid production by resting cells could be achieved through optimization of the growth medium composition. Addition of 2-thiouracil, a structural analogue of the DL-5-substituted hydantoins led to a five-fold increase in the specific activities of the amidohydrolase and the hydantoinase. These enzymes in *Agrobacterium* sp. are strictly D-specific and have very different pH optima (pH 6.75 and pH 10.0, respectively).

Investigations with a partially purified hydantoinase showed that *N*-carbamyl-PHPG was the final product of the hydantoinase reaction with DL-5-PHPH. Further decarbamylation into the free amino acid is due to a distinct *N*-carbamyl-amino acid amidohydrolase, showing different features from those of the hydantoinase. These findings are in good agreement with earlier reports for these two hydrolases in different microorganisms (Syldatk et al. 1986). The problem of different pH optima, encountered in a one-step bioconversion process, has been pointed out by Möller et al. (1988). From our studies with the resting cells of *Agrobacter* sp., 45° C and pH 6.75 appeared to be a good average for temperature and pH, as it is possible to get a complete conversion of the racemic DL-5-PHPH into D-PHPG after short incubation times. Bioconversion under acidic conditions indicated the existence of an essential 5-substituted hydantoin racemase. This enzyme is responsible for rapid racemization of the hydantoin molecule at pH 6.75, as no chemical racemization is observed at this pH. The presence of the racemase in this microorganism is an interesting characteristic. Dinelli et al. (1975) showed that the rates of chemical racemization of several 5-substituted hydantoins depended mostly on the nature of the substituent in the 5-position of the hydantoin ring, and this rate might become the limiting step in the production of some amino acids.

Few data are available concerning a potential inhibition of hydantoin degradation towards the corresponding amino acid by the products of the reaction. By assuming no effect of chloride ions and under the conditions of pH and temperature used, both NH_3 and NH_4^+ forms are present in the broth. At pH 6.75, where only NH_4^+ ions should occur, the amidohydrolase activity is less affected by the NH_4^+ concentration. At pH 8.0 or 9.5, the hydantoinase activity remained constant at different concentrations of NH_4Cl, whereas the amidohydrolase activity was found to strongly decrease both with increase in pH and NH_4Cl concentration. These results point out the fact that only the NH_3 form inhibits amino acid formation. Further experiments by monitoring the true NH_3 concentration in the bioconversion broth will be required to confirm this.

In conclusion, the features of the enzymatic system responsible for the cleavage of the hydantoin in *Agrobacterium* sp. might be a competitive industrial tool to get high yields of D-PHPG (see patents Société Française Hoechst 1987, 1988). Currently, attempts are underway to further purify the hydantoinase in this bacterium. The production of other D-amino acids is also being investigated since both hydrolases in *Agrobacterium* sp. exhibited a broad substrate specificity.

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