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A novel *Pseudomonas putida* strain with high levels of hydantoin-converting activity, producing L-amino acids

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Abstract

Optically pure chiral amino acids and their derivatives can be efficiently synthesised by the biocatalytic conversion of 5-substituted hydantoins in reactions catalysed by stereo-selective microbial enzymes: initially a hydantoinase catalyses the cleavage of the hydantoin producing an *N*-carbamyl amino acid. In certain bacteria where an *N*-carbamyl amino acid amidohydrolase (NCAAH) is present, the *N*-carbamyl amino acid intermediate is further converted to amino acid, ammonia and CO_2. In this study we report on a novel *Pseudomonas putida* strain which exhibits high levels of hydantoin-converting activity, yielding L-amino acid products including alanine, valine, and norleucine, with bioconversion yields between 60% and 100%. The preferred substrates are generally aliphatic, but not necessarily short chain, 5-alkylhydantoins. In characterizing the enzymes from this microorganism, we have found that the NCAAH has L-selectivity, while the hydantoinase is non-stereoselective. In addition, resting cell reactions under varying conditions showed that the hydantoinase is highly active, and is not subject to substrate inhibition, or product inhibition by ammonia. The rate-limiting reaction appears to be the NCAAH-catalysed conversion of the intermediate. Metal-dependence studies suggest that the hydantoinase is dependent on the presence of magnesium and cobalt ions, and is strongly inhibited by the presence of copper ions. The relative paucity of L-selective hydantoin-hydrolysing enzyme systems, together with the high level of hydantoinase activity and the unusual substrate selectivity of this *P. putida* isolate, suggest that is has significant potential in industrial applications.

Keywords: *Pseudomonas putida*; L-amino acids; Hydantoinase

1. Introduction

Optically pure amino acids have numerous commercial applications such as the synthesis of pesticides, peptide hormones and semi-synthetic antibiotics. One method for the production of optically pure amino acids that has received considerable attention recently is the stereospecific conversion of 5-substituted hydantoins [1,2]. The enzymatic hydrolysis of 5-substituted hydantoins to their corresponding amino acids is a two-step reaction catalysed by two sequential enzymes: first a hydantoinase (E.C. 3.5.2.2) converts hydantoins to *N*-carbamyl amino acids (NCAs), and then an *N*-carbamyl amino acid amidohydrolase (NCAAH), converts NCAs to amino acids [2]. One problem in the industrial application

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of such enzyme systems is that the NCAAHs frequently have relatively low activity. In addition, the use of the microorganisms producing these enzymes in an industrial process is complicated by the fact that hydantoinases and NCAAHs typically require different conditions for optimal activity and stability. Thus the search for novel strains with improved activity remains an active field.

A wide variety of microorganisms have hydantoinase activity which enables them to produce *N*-carbamyl-D-amino acids and/or D-amino acids [3]. These include *Pseudomonas desmolyticum* producing D-phenylglycine [4], a *P. putida* [5,6], and an halophilic *Pseudomonas* strain producing *N*-carbamyl-D-phenylglycine [7]. La Pointe et al. [8] have reported several D-specific hydantoinase-producing *P. putida* strains that exhibit wide genetic diversity.

More unusual are the group of microorganisms that are able to convert hydantoins to L-amino acids. Those reported include a *Flavobacterium* sp. converting only aromatic substrates, with a non-selective hydantoinase and an L-selective NCAAH [9], and a *Bacillus brevis* with L-specific hydantoinase and NCAAH [10]. *Pseudomonas* strain NS671 is able to catalyse the conversion of (2-methylthioethyl)hydantoin to L-methionine by an ATP-dependent mechanism involving a non-selective hydantoinase, L-selective NCAAH and a racemase which converts D-hydantoins to the L-enantiomers. [11–13]. Several hydantoin-hydrolysing *Arthrobacter* isolates have been reported: *Arthrobacter* sp. strain DSM 7350 produces a non-selective hydantoinase and L-selective *N*-carbamyl amino acid amidohydrolase, but no racemase activity [14]. The hydantoin-hydrolysing enzyme system in *A. auresscens* DSM 3747 consists of an L-selective hydantoinase, an *N*-carbamyl-L-amino acid amidohydrolase and an hydantoin-racemase [15], while the enantioselectivity of an hydantoinase purified from *A. aurescens* DSM 3745, is substrate-dependent [16]. To date, the genes encoding a L-selective hydantoin-hydrolysing enzyme system have been cloned from only one bacterial strain, namely *Pseudomonas* sp. NS671 [11]. A thermophilic *B. stearothermophilus* strain, NS112A, which converted DL-5-(2-methylthioethyl)hydantoin to L-methionine by an ATP-independent mechanism, was also reported [17] and *P. putida* strain 77, was found to possess an *N*-methylhydantoin amidohydrolase with L-stereospecificity [18]. Thus there is considerable variation in the characteristics of the L-specific enzyme systems. In particular, the presence of racemases is variable, and the stereoselectivity of systems may arise from the activity of either the hydantoinase or NCAAH component or it may be substrate-dependent [19].

Of several bacterial strains exhibiting hydantoin-hydrolysing activity which we have isolated from local environmental samples [20], a strain designated RU-KM3$_S$ was selected for further investigation, since it was found to produce significantly high amounts of NCA from a range of hydantoin substrates.

The most common method for catalysing the conversion of hydantoins into amino acids is the use of resting cell reactions, where bacterial cells producing hydantoinases are incubated together with substituted hydantoin and allowed to be converted to the desired amino acid derivatives. The factors that affect the efficiency of these reactions are variable and largely unexplained, and one aim of this study was to define the conditions for optimal enzyme activity. In addition, comparison of resting cell reactions with those utilising cell extracts was conducted with the aim of developing the most efficient biocatalytic system.

2. Methods and materials

2.1. Chemicals

Hydantoin, *N*-carbamyglycine (NCG), methylhydantoin and *N*-carbamyalanine (NCA) were purchased from Sigma or Toronto Chemicals. Other 5-substituted hydantoins were synthesised by the Bucherer–Bergs method [21]. Spectrapor tubing and a molecular weight cut-off 6000–8000 was used for dialysis experiments.

2.2. Isolation and culture of bacterial cells

P. putida RU-KM3$_S$, which was among bacterial strains isolated from the environment previously, was identified by comparison of the DNA sequence of the 16S rRNA gene with 16S rRNA gene sequences in the Ribosomal Database Project as described in Ref. [22]. For the optimisation of resting

cell reactions, seed cultures were grown from a single colony in hydantoin minimal medium (per litre: 10 g glucose; 0.011 g $CaCl_2$; 0.02 g $MgCl_2$; 60 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, 0.04 g boric acid, 0.04 g $MnSO_4$, 0.02 g $(NH_4)_6Mo_2O_{24} \cdot 4H_2O$, 0.01 g KI, 0.004 g $CuSO_4$, 1% hydantoin) to saturation. These cultures were utilised to inoculate 200-ml nutrient broth supplemented with 0.1% hydantoin as an inducer to an OD_{600} of 0.02 and grown, shaking at 25°C to early stationary phase. The cells were harvested by centrifugation (7000 rpm × 10 min), washed in 0.1 M potassium phosphate buffer, pH 8.0, at 4°C, and then resuspended in an appropriate volume of the same buffer.

2.3. Biocatalytic reaction with resting cells

For resting cell assays, the reaction mixture, containing a final cell concentration of 50 mg/ml hydrated biomass in 0.1 M phosphate buffer, pH 8.0 and the specified substrate, was incubated at 40°C, 200 rpm, normally for 3 h. The reaction mixtures were then microfuged and the supernatant analysed for the presence of *N*-carbamyl amino acids and amino acids by the Ehrlich's and ninhydrin colorimetric assays, respectively [22]. Hydantoinase activity was calculated as total amount (mM) of substrate converted to the *N*-carbamyl intermediate + amino acid; NCAAH activity was calculated as the amount (mM) of amino acid produced. All reactions were conducted in triplicate and results shown are the means of the data.

2.4. Reusability of resting cells

To determine the viability of RU-KM3$_S$ cells, a semi-continuous procedure was used, under optimised conditions. Thus, cells were grown up, collected and used in four successive 3-h biocatalytic reactions. After the first 3-h reaction, the cells were collected by centrifugation, and then resuspended in fresh hydantoin solution in buffer. This was repeated over 12 h. The same experiment was conducted with 0.1% glycerol present in the reaction mixture.

2.5. Biocatalytic reaction with cell extract

For cell-free extracts, the wet cell mass was determined and the cells resuspended in phosphate buffer to give a final hydrated biomass concentration of 100 mg/ml. The resuspended cells were disrupted by a single pass through a Yeda-press (LINCA Lamon Instrumentations, Tel-Aviv) at 15 Psi, 4°C and at a flow rate of approximately 1 drop/s. The reactions were carried out by incubating 100 mg/ml crude extract (400–600 μg protein) with the hydantoin substrate (25 or 50 mM) in 0.1 M phosphate buffer, pH 8, at 40°C for a period of 1–6 h, with shaking at 200 rpm. The reaction mixtures were then microfuged, and *N*-carbamyl amino acid and amino acid production was determined and analysed as described for resting cell biocatalytic assays. In experiments to investigate the stability of the enzyme activities in the cell extracts, solutions of the extract in buffer were pre-incubated for periods up to 6 h at 40°C, after which the extract was added to 50 mM methylhydantoin solution and assayed for a period of 1 h.

2.6. Metal-dependence studies using cell-free extract

The biocatalytic assay as described above using 50 mM hydantoin as substrate was carried out in the presence of metal ions (Cu^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}) at concentration 2.5 mM, using enzyme samples which had been treated as follows: (1) Metal ion solutions added to the reaction mixture; (2) EDTA (2.5 mM) was added to the reaction mixture; (3) French-pressed extract was dialysed for 90 min against fresh buffer containing EDTA (2.5 mM), with buffer changes every 30 min, to remove metal ions as EDTA-metal complexes.

3. Results and discussion

3.1. Effects of reaction time, temperature and pH on biocatalytic activity of RU-KM3$_S$ resting cells

Resting cells were incubated with 50 mM hydantoin as substrate, under biocatalytic assay conditions as described above, but for varying lengths of time up to 24 h. The highest levels of NCG and glycine were detected after 3 h of incubation, when total conversions under these conditions were typically found to be close to 100% (Fig. 1A). In subsequent experiments, resting cell biocatalytic assays were conducted over 3 h. These activity levels, giving

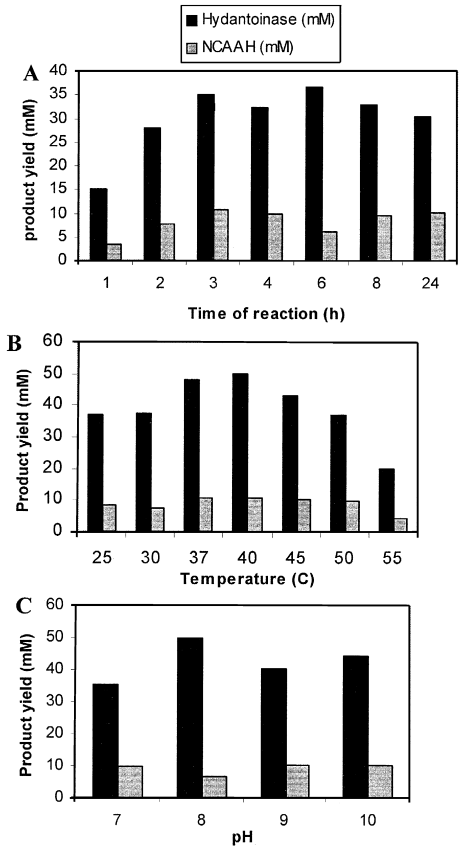

Fig. 1. Determination of optimal conditions for conversion of hydantoin to NCG and glycine by RU-KM3$_S$ resting cells. Reaction time (A), temperature (B) and pH (C). Substrate: 50 mM hydantoin; 3 h, 40°C.

yields of approximately 0.44 mg product per milligram dry cell mass, or 4.4 mg/ml, are good in comparison with other reports. For example, Yokozeki et al. [9] reported a yield of 0.7 mg/ml in the conversion of hydroxyphenylhydantoin by a pseudomonad strain under similar reaction conditions, and Sudge et al. [7] reported yields of 0.1 mg per gram dry cell mass using a halotolerant *Pseudomonas* strain.

The effect of temperature was investigated by carrying out reactions at temperatures from 25°C to 55°C. A temperature of 40°C was found to be optimal for both the hydantoinase and NCAAH enzymes (Fig. 1B). This temperature profile is typical of hydantoinase reaction systems, as reported by others [24].

To optimise the pH conditions, reactions were tested in a range of buffers between pH 7.0 and 10.0. The hydantoinase reaction gave a maximum yield at pH 8.0, while the NCAAH activity was highest at pH 9–10 (Fig. 1C). Thus the two enzymes differ with respect to their pH optima in resting cell assays. This difference is not unusual amongst hydantoinase-producers but it does provide a rational reason for consideration of cell extracts or isolated enzymes rather than whole cells in biocatalysis applications.

3.2. Effect of substrate and product concentration on biocatalytic activity of RU-KM3$_S$ resting cells

Since substrate inhibition can be an important limitation in the development of an industrial process, we investigated the effects of increasing starting concentrations of hydantoin on the conversion to NCG and glycine by RU-KM3$_S$ resting cells. At initial substrate concentrations up to 50 mM, the hydantoin was completely converted to NCG and glycine. At higher substrate concentrations, although the conversion was not complete after the 3-h reaction time, the total yield had increased (Table 1). The percentage of the *N*-carbamyl intermediate converted to amino acid decreased with increasing substrate-starting concentration, which indicates that while the productivity of the hydantoinase was not saturated at an initial substrate concentration of 100 mM, the NCAAH may be inhibited by the substrate or a reaction product.

It has been reported by others that hydantoinases and NCAAHs can be inhibited by the presence of the reaction products, NCA, amino acids or the ammonium ion [15,25,27]. To make a comparison, and in view of the results described above, the effects of addition of exogenous NCG, glycine and ammonium ions to the reaction mixture, were tested. The production of NCG was measured in each case (by subtraction of the added amount from the final amount, in the case of NCG). A clear trend of decreasing NCG production was observed as the concentration of NCG added was increased, but there was no inhibition of hydantoinase activity upon addition of glycine or ammonium ions to the biocatalytic reactions (Fig. 2). The activity of the NCAAH was unaffected by the concentration of NCG in the

Table 1
Production of NCG and glycine by RU-KM3$_S$ resting cells using different starting concentrations of substrate

Initial concentration of hydantoin (mM)	NCG produced (mM)	Glycine produced (mM) = NCAAH activity	Total hydantoin converted (mM) = hydantoinase activity	Glycine produced as a percentage of the total substrate converted (%)
25	15.43	10.829	26.26	41.2
50	39.25	10.69	49.94	21.4
75	46.17	11.49	57.66	19.9
100	70.1	9.82	79.93	12.3

Reaction conditions: 100 mg wet cell mass in 2 ml substrate solution in 0.1 M potassium phosphate buffer, pH 8, shaken at 200 rpm for 3 h, at 40°C.

medium (Fig. 3). These results indicate that the rate limiting step in the conversion of hydantoin to glycine by RU-KM3$_S$ resting cells is likely to be inhibition of the second enzyme reaction by ammonia or glycine or both, as has been reported by other groups [27,28].

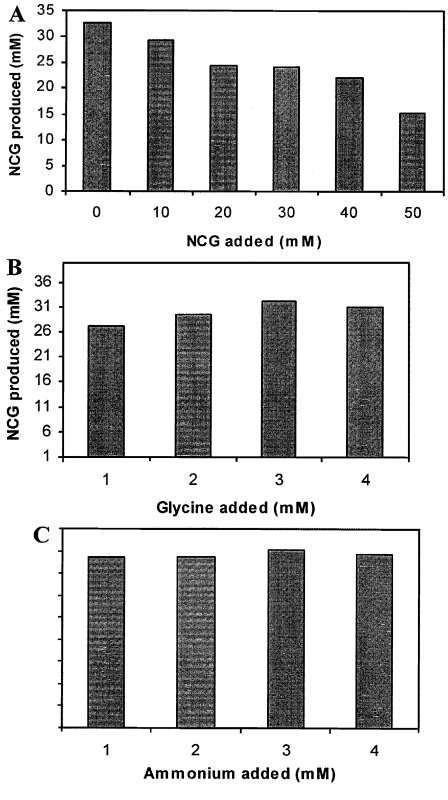

Fig. 2. Effects of increasing concentrations of reaction products on bioconversion of hydantoin to NCG by RU-KM3$_S$ cells, using added NCG (A), glycine (B) and ammonium (C).

The semi-continuous application of RU-KM3$_S$ cells was demonstrated by re-using the same batch of cells in four successive biocatalytic reactions conducted over 12 h, converting fresh portions of hydantoin substrate (Table 2). Some loss of activity was observed over the four successive reactions in the 12-h period, with 35.9 mM NCG being produced in the first reaction and 22.3 mM in the last reaction. Similarly, the activity of the NCAAH and thus the glycine production decreased from 7.9 mM to 1.66 mM, over the four successive reactions. The total yield per 3-h reaction decreased from 88% to 48%. However, over the whole 12-h period, the activities were maintained at levels sufficient to convert a total of 2.6 mmol of hydantoin per 1 g hydrated biomass, giving an overall yield of 66%.

During the resting cell biocatalytic reactions, no nutrients are available to the cells [23], and thus the maintenance of biocatalytic activity will depend largely on rate of cell death. However, Kim et al. [26] reported that glycerol stabilised D-hydantoinase activity in a *Pseudomonas* strain. In the case of RU-KM3$_S$, the presence of 0.1% glycerol in the reaction mixture used for successive reactions re-

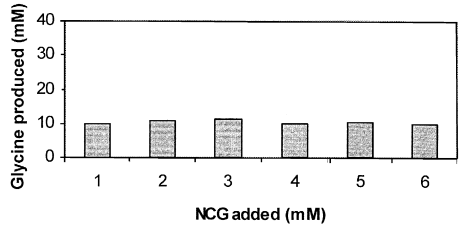

Fig. 3. Effect of increasing concentrations of NCG on glycine production from hydantoin by RU-KM3$_S$ cells.

Table 2
The reusability of RU-KM3$_S$ cells and the effect of glycerol in repeated resting cell reactions

Biocatalytic reaction	0% glycerol			0.1% glycerol		
	NCG (mM)	Glycine (mM)	Total hydantoin converted(mM)	NCG (mM)	Glycine (mM)	Total hydantoin converted(mM)
1	35.97	7.94	43.91	32.73	3.2	35.93
2	30.88	6.42	37.30	31.17	1.44	32.61
3	24.3	2.16	26.46	32.08	2.36	34.44
4	22.33	1.66	23.99	30.99	2.44	33.43
Total (12 h)		18.18	131.66		9.44	136.41

Reaction conditions: 100 mg wet cell mass initially added to 2 ml substrate solution (50 mM) for biocatalytic reaction. After each 3 h, cells were removed and added to a fresh substrate solution.
NCG: *N*-carbamylglycine.

sulted in stabilisation of the activity of the hydantoinase but not the NCAAH (Table 2).

3.3. Optimum reaction conditions for cell-free extracts of RU-KM3$_S$

Methylhydantoin was found to be readily converted by RU-KM3$_S$ cell-free extracts, and therefore, the optimal reaction time for the crude extract of RU-KM3$_S$ was investigated using DL-5-methylhydantoin as the substrate. The maximum yield was achieved after 4 h, with respect to both the hydantoinase and the NCAAH (Fig. 4A). The stability of the activity in cell-free extracts was investigated by incubating samples of the extract for various time periods before addition of the extract to the substrate solutions, and decreased hydantoinase activity was observed over 6 h (Fig. 4B). The nature of this loss of activity was investigated by the addition of the antioxidant dithiothreotol (DTT) and protease inhibitor phenylmethylsulfonyl fluoride (PMSF), preincubation for up to 4 h and then biocatalytic reactions for 1 h with methylhydantoin as substrate. Results indicate little effect by the presence of DTT

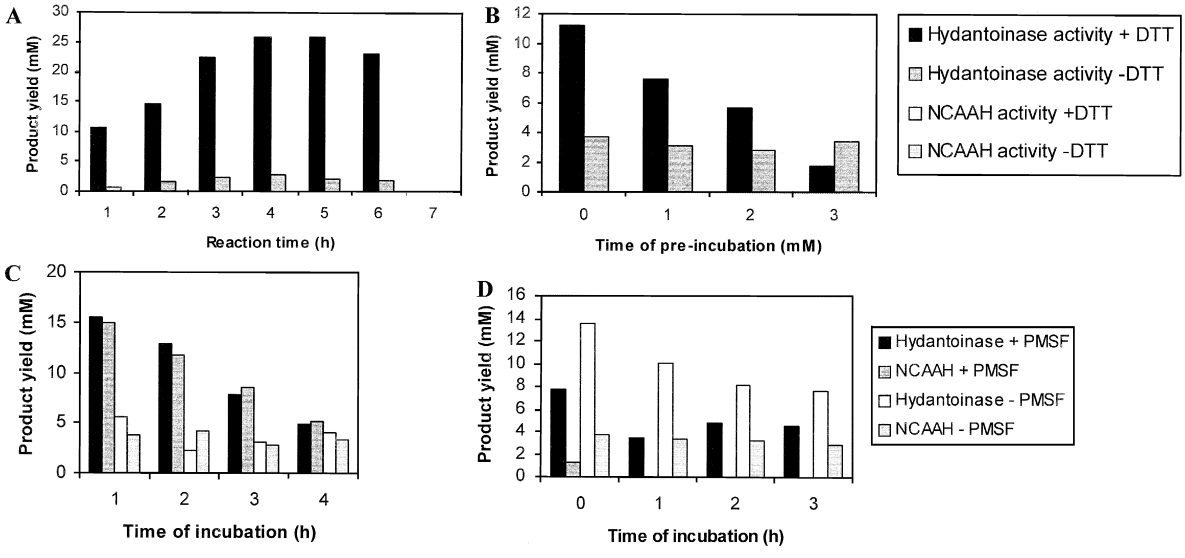

Fig. 4. Determination of optimal conditions for conversion of methylhydantoin to NCA and alanine by RU-KM3$_S$ cell extract: time of biocatalytic reaction (A); stability before reaction (B); effect of addition of DTT (C); effect of addition of PMSF (D).

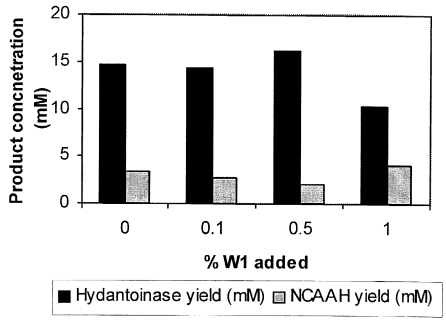

Fig. 5. Effect of addition of W1 on activity of RU-KM3$_S$ enzyme activities.

(Fig. 4C). In contrast, there was a marked decrease in both hydantoinase and NCAAH activity in the presence of PMSF (Fig. 4D). The average specific activity of RU-KM3$_S$ cell-free extracts under optimised reaction conditions was calculated to be 4.78 U/mg, on the basis of crude total protein concentration and activity measured in hydantoinase activity units of 1 μmol product formed per minute.

3.4. The effect of W1 on the biocatalytic activity of RU-KM3$_S$ crude extracts

Detergent W1 is a cell membrane-disrupting agent, and it was used in this study to determine whether its presence would increase the activity of the enzymes by releasing them from the cell debris and thus making them more readily accessible to the substrate. The results showed that at a concentration of 0.5%, the presence of W1 in the extraction buffer caused a small increase in the activity of the hydantoinase enzyme, and at 1%, a small increase in the activity of the NCAAH (Fig. 5).

3.5. Stereoselectivity of the hydantoinase and NCAAH enzymes of RU-KM3$_S$

The stereoselectivities of the hydantoinase and NCAAH enzymes are an important consideration, and in this study, they were determined by analysing the ability of the respective enzymes to convert optically pure substrates into the corresponding amino acids. This method is based on the premise that a D-selective enzyme, for example, would not be able to hydrolyse L-substrates efficiently and vice versa [16]. The total conversions of D-, L-, and D/L-methylhydantoin, respectively, to NCA were found to be approximately equivalent for all three substrates (Table 3), but there were significant differences in the amounts of the amino acid product measured. Thus, when D-methylhydantoin was used as substrate, the amount of NCA measured was very similar to that produced from DL-methylhydantoin, but the amount of alanine produced was significantly lower. Conversely, when L-methylhydantoin was used, more of the NCA produced was converted to alanine. This suggests that the activity of the hydantoinase enzyme is equivalent regardless of the stereospecificity of the substrate, but that the NCAAH activity of RU-KM3$_S$ is L-stereoselective. Thus, a greater amount of alanine was produced from L-methylhydantoin as compared with that produced from D-methylhydantoin. Watabe et al. [11] have reported similar results for a *Pseudomonas* strain producing L-methionine, but to our knowledge, an L-specific NCAAH enzyme found in a *P. putida* strain is novel.

3.6. Substrate selectivity of RU-KM3$_S$ hydantoinase

The conversion of a range of substrates was investigated to determine the nature of the preferred sub-

Table 3
Stereoselectivity of RU-KM3$_S$ enzymes in conversion of DL-, D-, and L-methylhydantoin

Substrates (50 mM)	NCA (mM)	Alanine (mM)	Total NCA converted (mM)	Ratio NCA: alanine
D/L-MH	24.49	2.57	27.06	9.6
D-MH	28.71	0.72	29.43	39.9
L-MH	19.48	4.57	24.05	4.2

Reaction conditions: resting cells resuspended in 0.1 M phosphate buffer(pH 8) and incubated for 3 h at 40°C with respective substrates NCA: *N*-carbamylalanine; D/L-MH: Equimolar mixture of D- andL-methylhydantoin;D-MH; L-MH: D- and L-methylhydantoin.

strates for RU-KM3$_S$ resting cells and cell extracts (Table 4). While hydantoin and methylhydantoin had previously been found to be converted in high yields by whole cells and cell extracts of RU-KM3$_S$ [20], high yields of several *N*-carbamyl amino acid derivatives and amino acids were obtained. Interestingly, different results were obtained using whole cells and cell-free extracts, which suggests the influence of transport-related factors in the productivity of the whole cell biocatalytic reactions, and further supports the investigation of cell-free extracts as biocatalysts for this reaction system.

N-carbamylvaline, *N*-carbamylleucine and *N*-carbamylnorleucine were produced at particularly high levels in biocatalytic reactions using both resting cells and cell free extracts, while cell free extracts produced significantly higher levels of *N*-carbamylhydroxyphenylglycine when compared with that produced by resting cells. The high yields of valine, leucine and *t*-leucine produced by cell-free extracts (100.70, 97.89 and 113.38 relative to glycine production, respectively) indicate that the preferred substrates for the hydantoin-hydrolysing enzyme system from this microorganism are aliphatic, but not necessarily short chain, hydantoins. These non-aromatic amino acids have considerable commercial value [29] and their production using RU-KM3$_S$ hydantoinase would seem to be feasible. The aromatic substrate hydroxyphenylhydantoin, used produce hydroxyphenylglycine, was not converted by whole cells. In contrast, in reactions using cell-free extracts, hydroxyphenylhydantoin was converted to hydroxyphenylglycine in high yield (110.56 relative to glycine production).

3.7. Effect of metal ions on the biocatalytic activity of RU-KM3$_S$ cells and extracts

A number of hydantoin-converting enzyme systems have been reported to exhibit metal ion dependence. In particular, it has been reported that the presence Mn^{2+} ions increased the activity, while Zn^{2+} ions resulted in inhibition of the hydantoinase in *Pseudomonas* sp. AS-1120 [25]. Similarly, Ogawa et al. [18] reported the requirement of Mg^{2+}, Mn^{2+} or Co^{2+} for hydantoinase activity in a *P. putida* strain, and inhibition of the same strain by Cu^{2+} and Fe^{2+}.

In preliminary experiments, solutions of metal ions were added directly to the reaction mixtures. The addition of Mg^{2+} ions resulted in an increase in

Table 4
Substrate selectivity of RU-KM3$_S$ cell-free extracts

Conditions	Substrate	Relative hydantoinase activity		Relative NCAAH activity		% Total substrate conversion
Resting cells	Hydantoin	NCG	100	Glycine	100	25.76
	Methylhydantoin	NCA	135.87	Alanine	44.62	35.00
	Isopropylhydantoin	NCV	183.70	Valine	41.15	47.32
	n-Butylhydantoin	NCL	222.20	Leucine	41.92	57.24
	Isobutylhydantoin	NCNL	209.63	Norleucine	47.31	54.00
	t-Butylhydantoin	NCTL	35.40	*t*-Leucine	7.69	9.12
	Hydroxyphenylhydantoin	NCHPG	60.56	HPG	15.38	15.60
Cell free extracts	Hydantoin	NCG	100	Glycine	100	31.12
	Methylhydantoin	NCA	121.77	Alanine	38.03	38.04
	Isopropylhydantoin	NCV	76.44	Valine	100.70	23.88
	n-Butylhydantoin	NCL	229.58	Leucine	97.89	71.72
	Isobutylhydantoin	NCNL	45.84	Norleucine	0.00	14.32
	t-Butylhydantoin	NCTL	134.57	*t*-Leucine	113.38	42.04
	Hydroxyphenylhydantoin	NCHPG	200.64	HPG	110.56	62.68

Reaction conditions: substrate concentration of 25 mM; reaction time 3h.
NCG: *N*-carbamylglycine; NCA, *N*-carbamylalanine; NCV: *N*-carbamylvaline;NCL; *N*-carbamylleucine; NCNL: *N*-carbamyl-norleucine; NCTL: *N*-carbamyl-tert-leucine; NCHPG: *N*-carbamylhydroxyphenylglycine; HPG:Hydroxyphenylglycine.
Total substrate conversion: percentage hydantoin converted to NCG and glycine.

Table 5
Effect of removal and re-addition of metal ions on the hydantoinase activity of RU-KM3$_S$ using hydantoin (50 mM) as substrate

Cell-free extract treatment	Hydantoinase activity (*N*-carbamylglycine + glycine — mM); Addition of metal ions (2.5 mM)							
	–	Fe^{2+}	Mg^{2+}	Mn^{2+}	Zn^{2+}	Co^{2+}	Cu^{2+}	$Mg^{2+}Co^{2+}$
CFE	27.72	20.38	30.08	25.51	24.39	23.06	nd	nd
CFE + EDTA	17.12	14.29	18.31	21.03	18.52	22.01	nd	nd
dCFE	22.18	nd	nd	nd	nd	nd	nd	nd
dCFE + EDTA	19.27	14.22	21.27	26.15	22.21	26.84	6.82	28.23

CFE: Cell-free extract; CFE + EDTA: EDTA (2.5 mM) added to reaction samples;dCFE: Extract dialysed against 0.1 M phosphate buffer (pH 8); dCFE + EDTA:Extract dialysed against 0.1 M phosphate buffer (pH 8) containing EDTA(2.5 mM); nd: Not determined.

hydantoinase activity (Table 5), while addition of Fe^{2+} resulted in a significant decrease in both hydantoinase and NCAAH activity (Tables 5 and 6). The effect of adding EDTA as a competing metal ion chelator, to remove metal ions bound in the enzymes, was also examined. The presence of EDTA in crude cell extracts resulted in a significant decrease in the activities of both enzymes (Tables 5 and 6). The addition of Mn^{2+} and Co^{2+} appeared to significantly increase the activity of the hydantoinase in the presence of EDTA, with the same effect observed upon NCAAH activity. In a confirmatory experiment, the cell-free extract was also dialysed against buffer containing EDTA, and the activity in a biocatalytic assay was subsequently measured and compared with untreated control samples. Metal ions were then added to the treated samples, and the biocatalytic activities were again measured. The addition of Mg^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} were all found to restore the activity of the hydantoinase activity, and Cu^{2+} and Fe^{2+} caused further decreases in activity. Addition of Mg^{2+} and Co^{2+} together resulted in restoration of the hydantoinase activity to levels observed in untreated cell-free extracts (Table 5), and a significant concomitant increase in NCAAH activity (Table 6). These results are similar to the observations of others (described above), and in particular, Cu^{2+} ions were found to cause significant inhibition of the hydantoinase in RU-KM3$_S$.

3.8. ATP-dependence of biocatalytic activity of RU-KM3$_S$ cells

The observed increase in the activity of NCAAH in the presence of Mg^{2+} may be due to two factors, viz., the requirement of Mg^{2+} ions in the utilisation of ATP, in which case the increase in activity of the second enzyme would indicate ATP-dependence of the NCAAH, or, alternatively, the Mg^{+} ions may be directly involved in the activity of the NCAAH. ATP-dependence of hydantoinase enzymes has been reported by others, e.g., Ishikawa et al. [17] and Ogawa et al. [18]. In the present study, assays conducted in the presence of ATP at concentrations up to 6 mM did not affect the activity of the hydantoin-hydrolysing enzymes of RU-KM3$_S$ (data not shown), indicating that they are both ATP-independent.

Table 6
Effect of removal and re-addition of metal ions on the NCAAH activity of RU-KM3$_S$ using hydantoin (50 mM) as substrate

Cell-free extract treatment	NCAAH activity (glycine — mM); Addition of metal ions (2.5 mM)							
	–	Fe^{2+}	Mg^{2+}	Mn^{2+}	Zn^{2+}	Co^{2+}	Cu^{2+}	$Mg^{2+}Co^{2+}$
CFE	12.09	7.27	12.95	10.8	11.42	9.74	nd	nd
CFE + EDTA	4.68	5.12	7.16	5.04	5.89	8.36	nd	nd
dCFE	5.34	nd	nd	nd	nd	nd	nd	nd
dCFE + EDTA	4.8	2.83	4.01	8.02	3.58	9.15	1.35	9.35

CFE: Cell-free extract; CFE + EDTA: EDTA added to reaction samples; dCFE:Extract dialysed against 0.1 M phosphate buffer (pH 8); dCFE + EDTA: Extractdialysed against 0.1 M phosphate buffer (pH 8) containing 2.5 mM EDTA;nd: Not determined.

4. Conclusions

This investigation demonstrates the presence, in *P. putida* RU-KM3$_S$, of a highly active hydantoin-hydrolysing enzyme system possessing hydantoinase and NCAAH activity. The presence of L-selective NCAAH activity in a pseudomonad is particulary useful with regard to the potential application of the strain for an industrial biotransformation process in view of the broad substrate selectivity we have observed. In our investigation, the production of L-leucine, L-nor-leucine, L-*tert*-leucine have been demonstrated at relatively high yields, suggesting that strain RU-KM3$_S$ would be a candidate for the biocatalytic production of these high-value amino acids.

P. putida RU-KM3$_S$ is among several hydantoin-hydrolysing bacterial strains, which include other *Pseudomonas* isolates and *Agrobacterium* spp. currently under investigation in our laboratories (Refs. [20,22], unpublished data). The enzymes from each of these strains show great diversity in both substrate- and stereoselectivity. The high productivity, broad substrate- and non-stereo-selective nature of the RU-KM3$_S$ hydantoinase, in combination with NCAAHs from the same or other bacterial systems at our disposal, raises the possibility of highly productive biocatalytic systems that can produce a wide range of L- or D-amino acid products.

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References

[1] J. Ogawa, S. Shimizu, TIBTECH 17 (1999) 13.
[2] C. Syldatk, A. Läufer, R. Müller, H. Höke, Adv. Biochem. Eng./Biotechnol. 41 (1990) 29.
[3] G.-J. Kim, H.-S. Kim, Biochem. J. 330 (1998) 295.
[4] D.V. Gokhale, K.B. Bastawde, S.G. Patil, U.R. Kalkore, R.R. Joshi, T. Ravindranachan, B.G. Gaikwad, V.V. Jogland, S. Nene, Enzyme Microb. Technol. 18 (1996) 353.
[5] H.R. Chien, W.-H. Hsu, Biotechnol. Tech. 10 (1996) 879–882.
[6] H.R. Chein, Y.-L. Jih, W.-Y. Yang, W.-H. Hsa, Biochem. Biophys. Acta 1395 (1998) 68.
[7] S.S. Sudge, K.B. Bastawde, D.V. Gokhale, U.R. Kalkote, T. Ravindanathan, Appl. Microbiol. Biotechnol. 49 (1998) 594.
[8] G. La Pointe, S. Viau, D. LeBlanc, N. Robert, A. Morin, Appl. Environ. Microbiol. 60 (1994) 888.
[9] K. Yokozeki, S. Nakamori, C. Eguchi, K. Yamada, K. Mitsugi, Agric. Biol. Chem. 51 (1987) 355.
[10] A. Yamashiro, K. Yokozeki, H. Kano, K. Kubota, Agric. Biol. Chem. 52 (1988) 2851.
[11] K. Watabe, T. Ishikawa, Y. Mukohara, H. Nakamura, J. Bacteriol. 174 (1992) 962.
[12] K. Watabe, T. Ishikawa, Y. Mukuhara, H. Nakamura, J. Bacteriol. 174 (1992) 7989.
[13] T. Ishikawa, K. Watabe, Y. Mukohara, S. Kobayashi, H. Nakamura, Biosci., Biotechnol., Biochem. 57 (1993) 982.
[14] T. Wagner, B. Hantke, F. Wagner, J. Biotechnol. 46 (1996) 63.
[15] C. Syldatk, V. Mackowiak, H. Hoke, C. Gross, G. Dombach, F. Wagner, J. Biotechnol. 14 (1990) 345.
[16] O. May, M. Siemann, M. Pietzsch, M. Kiess, P. Mattes, C. Syldak, J. Biotechnol. 61 (1998) 1.
[17] T. Ishikawa, Y. Mukohara, K. Watabe, S. Kobayashi, H. Nakamura, Biosci., Biotechnol., Biochem. 58 (1994) 265.
[18] J. Ogawa, J. MinKim, W. Nirdnoy, Y. Amano, H. Yamada, S. Shimizu, Eur. J. Biochem. 229 (1995) 284.
[19] C. Syldatk, O. May, J. Altenbuchner, R. Mattes, M. Siemann, Appl. Microbiol. Biotechnol. 51 (1999) 293.
[20] S. Burton, R. Dorrington, C. Hartley, S. Kirchmann, K. Buchanan, V. Phehane, J. Mol. Catal. (Enzymic) 5 (1998) 301.
[21] H.T. Bucherer, W.J. Steiner, J. Pract. Chem. 140 (1934) 291.
[22] C. Hartley, S. Kirchmann, S. Burton, R. Dorrington, Biotech. Lett. 20 (1998) 707.
[23] P. Chevalier, D. Roy, A. Morin, Appl. Microbiol. Biotechnol. 30 (1989) 452.
[24] A. Morin, D. LeBlanc, A. Paleczek, W. Hummel, M.R. Kula, J. Biotechnol. 16 (1990) 37.
[25] S. Runser, N. Chinski, E. Ohleyer, Appl. Microbiol. Biotechnol. 33 (1990) 382.
[26] D.M. Kim, G.J. Kim, H.S. Kim, Biotechnol. Lett. 16 (1994) 11.
[27] R. Olivieri, E. Fascetti, L. Angelini, L. Degen, Biotechnol. Bioeng. XXIII (1981) 2173.
[28] G. Kim, H. Kim, Enzyme Microbiol. Technol. 17 (1995) 63.
[29] A.S. Bommarius, M. Schwarm, K. Stingl, M. Kottenhahn, K. Drauz, Tetrahedron Asymmetry 6 (1997) 2851.