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Production of enantiomerically pure amino acids: characterisation of South African hydantoinases and hydantoinase-producing bacteria

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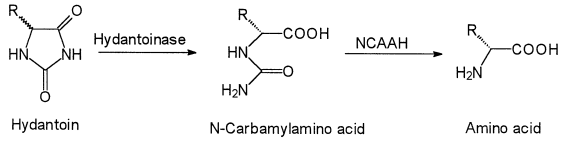
Abstract

Chiral amino acid derivatives can be synthesised by the biocatalytic conversion of substituted hydantoins using microbial enzymes or resting cells: a hydantoinase performs the ring-opening cleavage of the hydantoin to produce an *N*-carbamyl-amino acid and *N*-carbamylamidohydrolase then converts this intermediate to the amino acid, ammonia and CO_2. The hydantoinases from four locally isolated bacterial strains are currently being characterised in terms of conditions for optimal enzyme activity assay, and to demonstrate the effects of pH, temperature, metal ions, protease inhibitors, surfactants, and anti-oxidants on hydantoinase activity in crude extracts. Typically, pH 8, 50°C, and 0.3 mM Cu^{2+} were found to be optimal. Disruption of cells using a detergent or membrane freeze-fracture resulted in increased activities, suggesting that the hydantoinase enzymes may be membrane bound. It was also found that three *Pseudomonas* strains exhibited higher activities than the *Agrobacterium* strain, in terms of hydantoin conversion, with % conversion of hydantoins to *N*-carbamyl-amino acids from 66% to 2%. Comparisons of hydantoinase and amidohydrolase activity in resting cells and in cell extracts also show marked differences in activity profile for different strains, e.g., strain RU-KM1 exhibited hydantoinase activity in whole cells and cell extracts, but amidohydrolase activity only in cell extracts, while RU-OR showed higher amidohydrolase activity than hydantoinase activity.

Keywords: Hydantoinase; Amino acids; Biotransformations

1. Introduction



Hydantoin N-Carbamylamino acid Amino acid

Chiral amino acids and their derivatives are important starting materials in the production of pharmaceuticals such as semi-synthetic antibiotics, and as nutritional supplements. They can be efficiently synthesised by biocatalytic conversion of substituted hydantoins, in reactions catalysed by microbial enzymes, viz., hydantoinase and *N*-carbamylamino acid amidohydrolase (NCAAH) as shown above [1,2]. Our objective is to identify local hydantoinase-producing organisms, to characterise their biocatalytic ac-

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tivity, and to develop stereoselective amino acid production systems utilising them. Four strains were selected for characterisation, after an extensive screening programme: *Pseudomonas putida* strains RU-KM1; RU-KM3$_S$; RU-KM3$_L$ and *Agrobacterium tumefaciens* strain RU-OR.

2. Experimental

2.1. Batch culture

Cells grown on hydantoin minimal medium plates were cultured in nutrient broth with 1% hydantoin as inducer, or hydantoin minimal medium, at 25°C, 200 rpm, 20 h.

2.2. Cell extracts

Cells were separated by centrifugation, washed and resuspended in phosphate buffer; French pressed at 15 MPa, 0°C, in presence of protease inhibitor and in some cases, the polyoxyethylene ether detergent W1 (Sigma).

2.3. Activity assays

Assays were conducted using whole cells (100 mg wet mass ml^{-1} reaction mixture (20 mg ml^{-1} for RU-OR), reaction time 6 h) or French pressed extracts (400–600 μg protein ml^{-1}, reaction time 1 h). For hydantoinase activity, using the respective hydantoin as substrate, *N*-carbamylamino acid products were quantified using Ehrlich's reagent or HPLC. For NCAAH activity, using *N*-carbamylamino acids as substrates, amino acid products were quantified using ninhydrin assay or HPLC. Overall activity was quantified by using hydantoin substrates and quantifying amino acids produced. Biocatalytic activity was measured in terms of production of the intermediate *N*-carbamylamino acids, and of the amino acids, from the substrates shown below, using colorimetric assays and HPLC. 1 Unit = 1 μmol product mg^{-1} protein min^{-1}.

2.4. Freeze-fracture

French pressed cell extracts were freeze dried overnight and stored under liquid nitrogen. The dry powder was thawed and used at a concentration of 0.1 mg ml^{-1} [3].

3. Results

3.1. Characterisation of local strains

The growth of the strains was correlated with the development of enzyme activity, using whole cells and enzyme extracts. Growth curves were obtained for whole cells of strains RU-OR, RU-KM1, and RU-KM3$_L$, grown in complete and in minimal media. Stationary phase was reached after approximately 25 h in the case of RU-KM1, but after 60 h for RU-OR grown in minimal medium.

3.2. Enzyme activity of resting cells

The enzyme activity developed by whole cells of the strains was measured in resting cell reactions. Hydantoinase and NCAAH activities were

Table 1
Substrate specificity for four strains measured using resting cell reactions

Strain	Substrate					
	HYD		MH		HPH	
	Product (mM)					
	NCG	GLY	NCA	ALA	NCHPG	HPG
RU-KM3$_L$	33.6	12.6	9.2	0.27	3.02	0.56
RU-KM1	10.5	0.26	12.7	0.5	13.8	0.30
RU-OR	0.94	2.84	6.15	1.83	1.88	7.7
RU-KM3$_S$	30.0	8.6	21.1	2.0	3.4	0.1

Product expressed as μmol ml^{-1} after whole cell assay for 6 h at 40°C, pH 8.0, using cells harvested at early stationary phase, with initial substrate concentration 50 mM.
Key: NCG—*N*-carbamylglycine, GLY—glycine, NCA—*N*-carbamylalanine, ALA—alanine, NCHPG—*N*-carbamylhydroxyphenylglycine, HPG—hydroxyphenylglycine, HYD—hydantoin, MH—methylhydantoin, HPH—hydroxyphenylhydantoin.

assayed during growth in batch culture, measuring the concentrations of *N*-carbamylglycine (NCG) and glycine produced (Table 1). In whole cell assays, strain RU-KM1 was initially found to show low NCAAH activity, but high hydantoinase activity. However, later results obtained using cell extracts showed that the NCAAH activity was present. Strain RU-OR exhibits high glycine production, and as a result, the NCG yields appear low and somewhat erratic. RU-$KM3_S$ gives high yields of NCG and glycine, and is being investigated further. RU-$KM3_L$ also shows high hydantoinase and NCAAH activity in whole cell reactions, and this strain exhibits thermostability.

3.3. Inducers of enzyme activity

Hydantoinase and NCAAH activity have been reported in the literature to be inducible [1,4], and therefore we investigated the effects on hydantoinase and NCAAH activity in resting cell reactions with each of the four strains. Hydantoin was found to be a good inducer for strains RU-KM1, RU-$KM3_L$ and RU-$KM3_S$, while dihydrouracil and thiouracil also induced RU-$KM3_S$, and thiouracil was the most effective inducer for RU-OR.

3.4. Substrate specificity of selected bacterial strains

A variety of substrates can potentially be converted by hydantoinase-producing strains. In particular, variations are reported between substrate preferences for aromatic and non-aromatic substrates. *p*-Hydroxyphenylhydantoin is an important substrate because it can be converted to *p*-hydroxyphenylglycine, which is the starting material in the production of the semi-synthetic antibiotic amoxycillin. Optimal substrates, as indicated by highest hydantoinase activities, were as follows: RU-OR and RU-KM1: Hydroxyphenylhydantoin; RU-$KM3_S$: Hydantoin; RU-$KM3_L$: Methylhydantoin (see Table 1).

3.5. Stereoselectivity in four local strains

The stereospecificity of the strains is an important consideration, since D-amino acids are often sought as synthetic intermediates, while L-amino acids are commonly produced as nutrient additives. Chiral TLC and HPLC were used to identify the chirality of the products from resting cell reactions using D,L-methylhydantoin, D-methylhydantoin and L-methylhydantoin as substrates. The results indicate D-selective enzymes in strains RU-KM1 and RU-OR, while those of RU-$KM3_L$ and RU-$KM3_S$ are L-specific. While the presence of a racemase enzyme is not excluded in any of these strains, it was not detected under the experimental conditions used.

3.6. Characterisation of strain RU-OR using resting cell reactions

Strain RU-OR was selected for further study because it appeared to have high NCAAH activity and thus potentially could produce high yields of amino acid products. The biocatalytic potential of strain RU-OR was investigated with the aim of optimising the productivity of the conversion of hydantoins to amino acids. The results obtained using resting cell reactions showed an optimum pH of 9 and optimum temperature of 40–60°C.

3.7. Development of enzyme activity in cell extracts

In order to characterise the enzymes from three bacterial strains, cell extracts were obtained by French pressing cultures of the strains. Substrate conversion was measured over the growth period of the strains, to ascertain optimal harvesting times. For RU-KM1 and RU-OR grown in complete medium, the maximum hydantoinase and NCAAH activity were obtained after approximately 20 h, at the end of log phase.

3.8. Location of hydantoin-hydrolysing activity in cells

In preliminary research into methods for the isolation of purified enzymes from strains RU-OR, RU-KM1 and RU-KM3$_L$, we have found indications of the novel property that the enzymes appear to be membrane-bound. Experiments to measure the enzyme activities in cell extracts which included, or excluded, the cell debris after French pressing showed that the activity was found to be far higher in extracts where the cell debris was not removed by centrifugation, suggesting that the enzymes are membrane bound. In addition, the inclusion of detergent W1 (which is used specifically to disrupt cell membranes) in the extracts gave an increase in hydantoinase activity in the case of RU-OR. We observed that 75% more NCG was produced after detergent treatment, as compared with whole cell reaction yields (Table 2). Furthermore, the use of a freeze-fracture technique to disrupt membrane bilayers [3] caused a 43% increase in the hydantoinase activity, but no increase in NCAAH activity. In the case of RU-KM3$_L$, sonication of the cell extract was found to increase the reaction yields by approximately 40% over those obtained using equivalent amounts of non-disrupted cells.

3.9. Optimisation of conditions for activity in cell extracts of strains RU-KM1 and RU-OR

RU-KM1 was initially selected for comparison with RU-OR, since RU-KM1 apparently had low NCAAH activity, as exhibited by resting cell reaction, and thus gave high yields of the intermediate *N*-carbamylamino acid products. However, marked NCAAH activity is observed in cell extracts. The comparison between isolated enzyme activity and that of whole cells is important in choices relating to industrial processes. The aim of this investigation was to determine the optimal conditions for hydantoin conversion in French pressed cell extracts, in order to compare enzyme activity with whole cell reactions using strains RU-KM1 and RU-OR. Results shown were obtained using extracts obtained by French pressing cells grown in complete medium. Optimised conditions for RU-KM1 hydantoinase, giving specific activity 0.40 μmol mg^{-1} min^{-1}, were: pH 7–9; 50°C; 0.1% W1; 0.3 mM Cu^{2+} and for RU-KM1 NCAAH (specific activity 0.142 μmol mg^{-1}

Table 2
Product yields obtained from 50 mM hydantoin in reactions using whole cells and cell extracts, to show effects of cell disruption and detergent treatment

Strain	Conditions	*N*-carbamyl-glycine produced (mM)	Glycine produced (mM)
RU-KM1	whole cells	13.2	0.25
	cell extract with cell debris	13.8	4.28
	cell extract without cell debris	3.06	2.14
RU-OR	whole cells	0.51	7.54
	cell extract with cell debris	4.06	4.67
	cell extract without cell debris	1.55	1.55
RU-KM3$_S$	whole cells	22.5	14.7
	cell extract with cell debris	24.1	13.2
	cell extract without cell debris	nd	nd
RU-KM3$_L$	whole cells	35.8	11.1
	cell extract with cell debris	31.4	7.78
	cell extract without cell debris	2.67	0.00

min^{-1}): pH 10; 50°C; 0.1% W1; 0.1 mM Cu^{2+}. For RU-OR glycine production (specific activity 0.141 μmol mg^{-1} min^{-1}) optimal conditions were: pH 10; $>$ 50°C; 0.5% W1; 0.2 mM Cu^{2+}.

3.10. Characterisation of RU-KM3$_L$ activity

Strain RU-KM3$_L$ was observed to exhibit good biocatalytic activity at temperatures above 40°C, and therefore we investigated the possibility of obtaining thermostable hydantoin-converting enzymes from this strain. In addition, this strain differs from the other selected strains, in that it shows L-stereoselectivity, and in cell extracts, the preferred substrate is methylhydantoin. Optimum conditions for activity in cell extracts of RU-KM3$_L$ have been found to be: pH 9–10, temperature 50°C, sonication of the French pressed extract for 3 min, retention of the cell debris, and reaction time 3 h. Specific activity of hydantoinase under these conditions, using methylhydantoin substrate: 0.09 μM mg^{-1} min^{-1}.

3.11. Stabilisation of RU-KM3$_L$ enzyme extracts

One objective of our study of RU-KM3$_L$ is to immobilise the enzyme for application in organic media, and in order to achieve this, we investigated the stability of the crude enzyme extract after freeze drying. The highest activity was retained by freeze drying the extract in 0.5 mM buffer in the presence of 50% sucrose.

4. Conclusions

The bacterial strains selected for investigation exhibit a widely varying set of properties and clearly, it is necessary to characterise each before a biotransformation process can be established. The results of our investigations have indicated that although strain RU-OR has lower activity levels than some other strains, it has the capacity to fully convert the intermediate *N*-carbamylamino acid present in a reaction mixture as a result of hydantoin substrate hydrolysis, to the corresponding amino acid, which is an advantage over many other hydantoin-converting strains where NCAAH activity is low. Further investigations of the thermostability of the enzymes and purification of the enzymes, to facilitate kinetic characterisation, are underway. In expanding the substrate range of the reaction, the thermostability and solvent tolerance of the enzymes is important, particularly with respect to non-water soluble substrates. The enzyme activities of RU-KM3$_L$ are favourable in comparison with, for example, RU-KM3$_S$ which gives high activity but is not tolerant of high temperatures. We are presently investigating the activity of RU-KM3$_L$ enzyme extracts in the presence of organic solvents. While the conversion of hydantoins to *N*-carbamylamino acids is the essential first step in the production of the amino acid products, the second (NCAAH) enzyme activities appear to be the more varied in their activity and optimal reaction conditions among bacterial strains [5–8], and are expected to prove more interesting subjects of further study.

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