THE ISOLATION AND CHARACTERISATION OF THERMOSTABLE HYDANTOINASES FROM HYDANTOINASE-PRODUCING BACTERIA

THESIS

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ABSTRACT

In order to characterise thermostable hydantoin-hydrolysing enzymes from bacteria, locally-isolated thermophilic organisms were screened for the ability to convert hydantoin to *N*-carbamylglycine at 55°C using the hydantoinase enzyme. Cell disruption of a selected strain, RU-20-15, was conducted by French pressing to release enzyme from within the cell. In all of the experiments conducted, the amounts of product were low. In view of the low yields of products formed by the thermophiles, a previously-isolated Gram negative strain, RU-KM3$_L$, was selected from a number of mesophiles by screening for hydantoinase and carbamylase activity over a 40-55°C temperature range. Hydantoin conversion at 40°C using crude extract from pressed cells of this organism was similar to conversion at 50°C, and therefore subsequent assays were conducted at the higher temperature.

The growth kinetics of RU-KM3$_L$ cells were studied and the enzyme activities of the extracts were compared in complete and chemically-defined media. The results suggested that the optimal time to harvest cells was at early stationary phase, when using complete medium for culture of cells; the specific activity of enzyme extracts produced by culture in complete medium was higher than that obtained in chemically-defined medium. 5-methylhydantoin was shown to be the preferred substrate for both the hydantoinase and carbamylase enzymes in the crude extract of RU-KM3$_L$. The substrate specificity of the hydantoinase and carbamylase enzymes of the crude RU-KM3$_L$ extract was observed to be altered in the presence of increasing amounts of hydantoin, 5,5-dihydrouracil (DHU) and 5-thiouracil (TU) as inducers, showing selectivity for 5-methylhydantoin over hydantoin at inducer concentrations of 0.1 to 1%. A limiting effect on the hydrolysis of 5-methylhydantoin was observed

when DHU and 5,5-dimethylhydantoin (DMH) were used as inducers, while the limiting effect on hydantoin specificity was observed when DHU and TU were used as inducers. The limiting effect was observed to be dependent upon the concentration of inducer, and was not observed when hydantoin was used as an inducer. The optimal time for assay of the hydantoinase enzyme in crude extract preparations at 50°C was observed to be 3h. Alkaline conditions were shown to be optimal for both the hydantoinase and carbamylase enzymes of RU-$KM3_L$. Assay for enzyme activities of RU-$KM3_L$ extract in the presence of metal ions showed Mn^{2+} ions (and to a lesser extent, Co^{2+}) to activate both the hydantoinase and carbamylase activities. Cu^{2+} ions were observed to inhibit the hydantoinase enzyme.

In order to determine the location of the enzymes within the cell, cell debris from disrupted cells of RU-$KM3_L$ was removed by centrifugation. A decrease in enzyme activity in the supernatant was observed, and suggested association of the enzymes with the cell membrane. Ammonium sulfate fractionation experiments conducted on the crude extract provided further evidence for this result. Sonication of the crude enzyme extract was the only successful method for the releasing of membrane-associated enzyme.

Of a number of strategies investigated, the use of sucrose at 50% (w/v) concentration was shown to preserve the hydantoinase and carbamylase enzyme activities during lyophilisation. Furthermore, assay for these enzyme activities showed the activities to be higher after lyophilisation in the presence of sucrose. However, sucrose did not increase the thermostability of lyophilised crude enzyme extracts.

Water-miscible organic solvents at 1% concentration were shown to be inhibitory to the hydantoinase and carbamylase enzymes of RU-KM3$_L$, and the inhibition was also observed to increase with increasing concentrations of these solvents. Hydantoinase activity in the presence of water-immiscible organic solvents was shown to increase with an increase in the hydrophobicity of these solvents, but the activity observed was not significantly higher than activity in the absence of solvent when hydantoin and 5-methylhydantoin were used as substrates.

The possibility of reversing the hydantoinase enzyme reaction by water-immiscible organic solvents was investigated, and the results obtained suggested that the reaction could be reversed. It was thought that the partitioning of substrates or products into hydrophobic organic solvents could influence the reaction equilibrium, but the partitioning observed was not sufficient to affect reaction rates. Peptide synthesis was shown to have occurred in small amounts when the hydantoinase reaction was carried out in the presence of water-immiscible organic solvents.

In conclusion, the hydantoin-hydrolyzing enzyme activity of a crude extract preparation from the bacterial strain RU-KM3$_L$ was characterised at elevated temperatures, and in the presence of water-miscible and -immiscible organic solvents.

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ALA	D,L-alanine
BH	5-benzylhydantoin
$CaCl_2$	calcium chloride
CHL	chloroform
Co^{2+}	copper
Cu^{2+}	copper
$CuSO_4$	copper sulphate
D	Dextro, the direction (right) in which the plane of polarisation of polarised light is rotated
D,L-5-MH	D,L-5-(3-indolylmethyl)hydantoin
DHU	5,5-dihydrouracil
DMF	dimethylformamide
DMH	5,5-dimethylhydantoin
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fe^{2+}	iron
GLY	glycine
HEP	n-heptane
HEX	hexane
HPLC	high pressure liquid chromatography
HXL	1-hexanol
HYD	hydantoin
KCl	potassium chloride
KCN	potassium cyanide
KCN	potassium cyanide
KH_2PO_4	potassium dihydrogen phosphate

KI	potassium iodide
K_m	Michaelis constant
L	Laevo, the direction (left) in which the plane of polarisation of polarised light is rotated
L-CAM	N-carbamyl-L-methionine
Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
MHz	Megahertz
Mn^{2+}	manganese
$MnSO_4 \cdot 7H_2O$	manganese sulphate
MWCO	molecular weight cut-off
Na_2HPO_4	disodium hydrogen phosphate
NaCl	sodium chloride
$NaKC_4H_4O_6 \cdot 4H_2O$	sodium potassium tartrate
NaOH	sodium hydroxide
NCA	*N*-carbamylalanine
NCG	*N*-carbamylglycine
NCHPG	*N*-carbamyl-D,L-hydroxyphenylglycine
NH_4Cl	ammonium chloride
$(NH_4)_2CO_3$	ammonium carbonate
$(NH_4)_6MO_7O_{24} \cdot 4H_2O$	molybdate, ammonium salt tetrahydrate
$(NH_4)_2SO_4$	ammonium sulphate
Ni^{2+}	nickel
NMR	Nuclear Magnetic Resonance spectroscopy
OCT	n-octane
P	partition coefficient of a solvent between water and (1-) octanol in a two-phase system
PEG	polyethyleneglycol
PEN	n-pentane

PHE	phenylalanine
*p*HPH	D,L-*p*-hydroxyphenylhydantoin
PMSF	phenylmethane sulfonyl fluoride
SA	Specific Activity ($U.mg^{-1}$), where 1U is defined as the amount of enzyme required to produce 1µmol product in one minute under the specified conditions
SAIMR	South African Institute of Medical Research
SEM	standard error of the mean
TCA	trichloroacetic acid
TE	triethylamine
TOL	toluene
Tris	Tris (hydroxymethyl) aminomethane
TU	5-thiouracil
V_{max}	maximum reaction velocity
Zn^{2+}	zinc
$ZnSO_4$	zinc sulphate

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CHAPTER 1: INTRODUCTION

Enantiomerically pure (or chiral) amino acids are important from a commercial perspective as they are used as precursors for the synthesis of antibiotics, artificial sweeteners, insecticides and therapeutic agents. Chiral amino acids may be synthesized using chemical methods. An example is the preparation of D-(-) hydroxyphenylglycine (Eizember and Ammons, 1976 cited in Louwrier and Knowles, 1997). Disadvantages of this approach include the requirement for high amounts of energy, long racemization processes and only a 50% yield of the compound of interest (Yokozeki *et al.*, 1987a).

Biocatalytic processes for the production of enantiomerically pure amino acids are often preferred over chemical and/or chemoenzymatic methods since the former can be very competitive in large-scale production. In addition, there is a growing demand for fine chemicals that are specific in their mode of action, whose products have less toxic side-effects and are environmentally tolerable (Kamphuis *et al.*, 1990). Biocatalysts are able to perform most of the existing chemical reactions under mild conditions of temperature, pH and pressure. In addition, regio- and enantioselectivity, and specificity of biocatalysts is high, and these characteristics can be exploited in biotransformations (Polastro *et al.*, 1989). Figure 1.1 shows examples of optically pure amino acids and their structural relationship to some products derived from them (Kamphuis *et al.*, 1990).

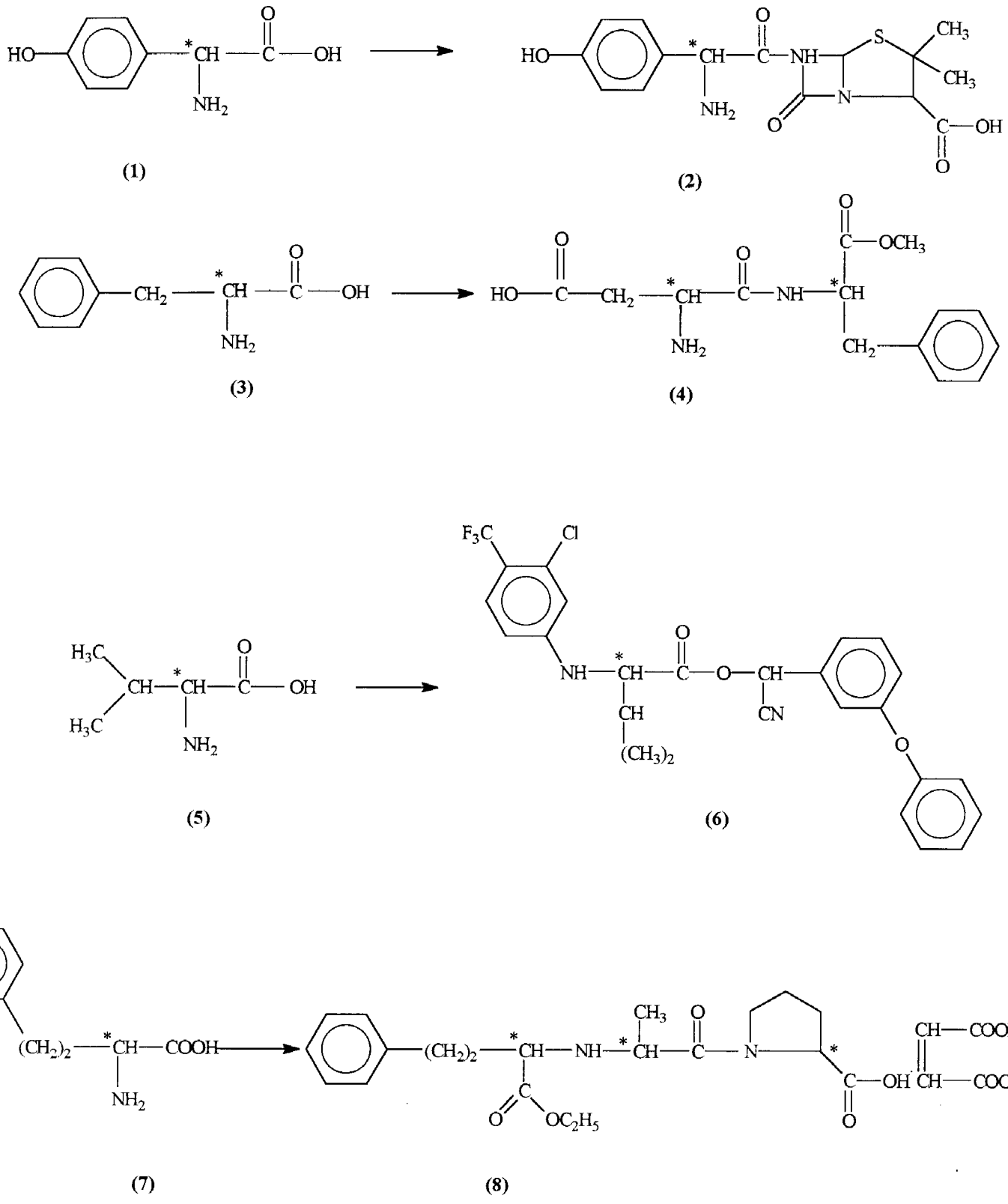


Fig 1.1 Applications of optically-pure amino acids (adapted from Kamphuis *et al.*, 1990)
(1) D-(*p*-hydroxy)phenylglycine, (2) semisynthetic penicillin/ ampicillin, (3) L-penylalanine, (4) α-aspartame, (5) D-valine, (6) Fluvalinate, (7) L-homophenylalanine, (8) Enalapril

Membrane enzyme reactors have been applied in the production of chiral α-amino acids using keto acid precursors as reviewed by Tramper (1996). Alternatively, synthesis of optically-active amino acids may be achieved by the hydantoinase reaction.

1. The hydantoinase reaction

The substrate for the hydantoinase reaction is a 5-substituted hydantoin (imidazolidine-2,4-dione or 2,4-diketotetrahydroimidazole) or a derivative thereof (Syldatk *et al.*, 1990a). The initial ring-opening step in biological hydrolysis of the substrate is catalyzed by the hydantoinase enzyme. The catalysis may be D-selective (Yokoseki and Kubota, 1987 cited in LaPointe *et al.*, 1994) or L-selective (Sano *et al.*, 1977, Nishida *et al.*, 1987, Yamashiro *et al.*, 1988, Syldatk *et al.*, 1992a, Syldatk *et al.*, 1992b cited in Louwrier and Knowles, 1997) or non-selective (Yokozeki *et al.*, 1987b cited in Louwrier and Knowles, 1997; Möller *et al.*, 1988, Watabe *et al.*, 1992a cited in LaPointe *et al.*, 1994). Stereoisomerism is due to the chiral carbon at position 5 (Figure 1.2 below) and affords the D- or L- or both forms of the intermediate, *N*-carbamylamino acid. The second cleavage step is catalyzed in an L-specific (Yokozeki et al., 1987a) or D-specific (Olivieri et al., 1979, Yokozeki et al., 1987b cited in Louwrier and Knowles, 1997) manner by the enzyme *N*-carbamyl amino acid amidohydrolase (carbamylase), which hydrolyses the intermediate to the optically pure amino acid with carbon dioxide and ammonia as by-products. Some D-hydantoinase and *N*-D-carbamylase enzymes were reported to be identical to the dihydropyrimidinase and the β-ureidopropionase enzymes whose natural functions are the degradation of cytosine and/or uracil and pyrimidine respectively (Yamada *et al.*, 1978; Syldatk *et al.*, 1988 cited in Louwrier and Knowles, 1996). A racemase enzyme, which

converts any L-hydantoin in a racemic mixture of substrate to D-hydantoin (where the hydantoinase enzyme is D-specific), may be present. It is thus theoretically possible to have complete (100%) conversion of the substrate. Spontaneous racemization of L-hydantoins has also been shown to occur by keto-enol tautomerism (Bovarnick and Clark, 1938 cited in Tsuji *et al.*, 1997) at temperatures in excess of 60°C and in alkaline conditions (Takahashi *et al.*, 1978). Figure 1.2 is an illustration of the chemical events in the hydantoinase reaction.

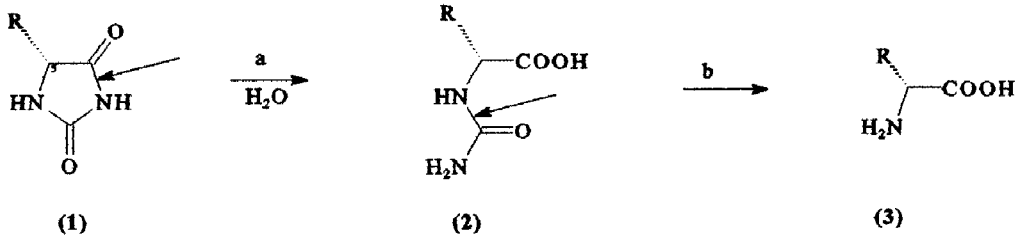


Figure 1.2 The hydantoinase reaction (Adapted from LaPointe *et al.*, 1994). (1) 5- monosubstituted hydantoin substrate, (2) intermediate (*N*-carbamyl D- or L-amino acid) (3) D- or L-amino acid. a, hydantoinase enzyme, b, carbamylase enzyme

Patents and commercial processes for producing certain amino acids exist. An example is the commercialization of D-*p*-hydroxyphenylglycine production by Kanegafuchi Chemical Industries using a hydantoinase enzyme system of bacterial origin. The successful large-scale production of D-phenylglycine and D-valine has been modelled on the Kanegafuchi process (Syldatk *et al.*, 1990a).

2. Substrates for the hydantoinase reaction

Two main methods are employed to synthesize 5-monosubstituted hydantoin derivatives. They include the Bucherer-Bergs synthesis (Bucherer and Steiner, 1934), and condensation of aldehydes with hydantoin, both of which will be described briefly.

2.1. Bucherer-Bergs synthesis

Carbonyl compounds are precursors for this synthesis. Aliphatic or aromatic aldehydes are treated with KCN (potassium cyanide) and $(NH_4)_2CO_3$ (ammonium carbonate) under mild conditions. Figure 1.3 is a schematic representation of the Bucherer-Bergs method for synthesis of hydantoin derivatives.

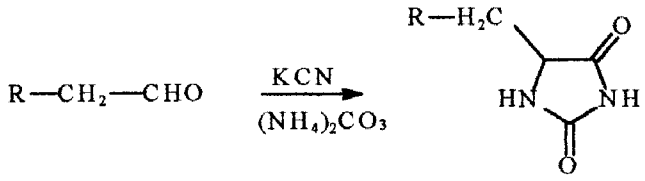


Figure 1.3 Bucherer-Bergs synthesis of 5-substituted hydantoin derivatives (Adapted from Syldatk *et al.*, 1990a)

2.2. Condensation of aldehydes with hydantoin

The 5-position of the hydantoin ring is substituted by an aldehyde. The 5-methylene group on the hydantoin ring is active, and allows for condensation with the aldehyde. Figure 1.4 shows the reaction.

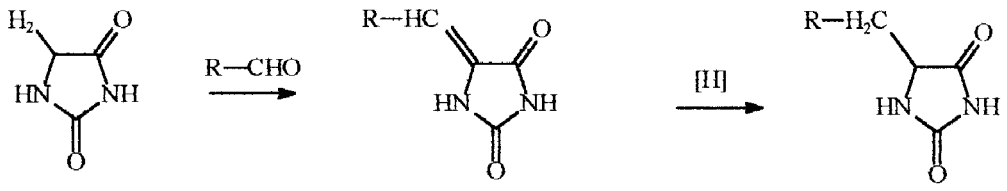


Figure 1.4 Synthesis of 5-substituted hydantoin derivatives by condensation of hydantoin with an aldehyde (Adapted from Syldatk *et al.*, 1990a)

These synthetic methods have been modified for the synthesis of other substrates. As an example, Ohashi *et al* (1981) reported the synthesis of 5-(4-hydroxyphenyl)hydantoin (*p*-HPH), a substrate for the synthesis of D-2-(4-hydroxyphenyl)glycine, which is in turn an important precursor for semi-synthetic penicillins and cephalosporins. The method involves an amidoalkylation of phenol with glyoxylic acid and urea under acid conditions. Advantages of this method include the omission of reactants sodium cyanide and 4-hydroxybenzaldehyde, which are toxic and undergo product-contaminating side-reactions respectively. An undesirable isomer, *o*-HPH was co-produced with the *p*-HPH, and methods to favour the synthesis of *p*-HPH were devised: gradual mixing of reactants, optimization of urea and phenol amounts and the use of high temperature and acid conditions.

3. Quantification of hydantoinase reaction products

The identification of hydantoin-hydrolyzing organisms requires the detection (and quantification) of

reaction products, *N*-carbamylamino acids and amino acids. Initial quantification of hydantoinase activity is generally achieved using the Ehrlich's reaction in which the reagent, acidic *p*-dimethylaminobenzaldehyde, reacts with the *N*-carbamyl amino acid to form a yellow-coloured compound. The Ninhydrin reaction is used to quantify amino acids and thus to characterize the activity of the amidohydrolase enzyme. In this reaction, ninhydrin (triketohydrindene hydrate) reacts with α-amino acids to afford a purple-coloured compound, decarboxylated amino acid, CO_2 and aldehydes, over pH range 4-8 (Plummer, 1987).

In view of the importance of the enantiomeric resolution of amino acids, chiral analysis techniques have been developed to distinguish between and quantify D- and L-amino acids. Polarimetry is one such technique (Morin *et al.*, 1986b) but is unreliable since impurities, variation in solvent type and temperature are factors that affect specific rotation and accurate determination of enantiomeric excess (Martens and Bhushan, 1989). Chromatographic methods of separation were originally achieved using GC (gas chromatography), and later using HPLC (high pressure liquid chromatography), TLC (thin layer chromatography) and CE (capillary electrophoresis) (Gil Av *et al.*,1966; Rogozhin and Davankov, 1971; Weinstein, 1984; Rogan *et al.*, 1994 cited in Marchelli *et al.*, 1996). Recent chromatographic methods have incorporated derivatization steps. Chiral stationary phases (CSP) and chiral mobile phases (CMP) that form diastereomeric complexes with the different enantiomers are used. The differences in retention times/separation are attributed to the differences in stability of these complexes. CSP's and CMP's can be incorporated in GC and HPLC. In HPLC, an additive (a chiral copper (II) complex) in the mobile phase is used with achiral columns. A number of disadvantages are afforded by these techniques, including expense and the requirement for derivatization, which in

turn may lead to racemization (Günther and Rauch, 1985 cited in Martens and Bhushan, 1989). Chiral TLC has often been the technique of choice (Lee *et al.*, 1994) as it is affordable and the organic solvents used during development of the chromatogram are volatile and do not interfere with the detection reagents. In addition, quantification of spots on TLC plates is possible using infra-red and densitometric scanners (Novak and Hercules, 1985 cited in Martens and Bhushan, 1989).

4. Screening for hydantoin-hydrolyzing microorganisms

Techniques developed for isolation of microorganisms with the ability to hydrolyze hydantoin or its derivatives were developed.

Morin *et al.*, 1986a documented the use of an overlay assay (an indirect approach) for the identification of hydantoinase-producing organisms. Inducer-supplemented plates with colonies obtained by enrichment of an isolation were overlaid with agar containing the substrate of interest. Plates were incubated and the presence of the hydantoinase enzyme was detected by the development of a yellow colour when colonies were covered with 10% acidic *p*-dimethylaminobenzaldehyde (Ehrlich's reagent). Colour development within 5-10 seconds using this "spot-test" was considered to be indicative of an efficient hydantoinase-producer.

The detection of hydantoinase-producers on solid media was further studied by Morin *et al.* (1987). Dihydrouracil was reported to be the most suitable screening substrate. The detection of false positives (positive for the plate test, negative for the assay) was suggested to be due to the non-

specificity of the Ehrlich's reagent, or inactivation of the enzyme during assaying.

Chien and Hsu (1996) modified the "spot test" or overlay assay by using microtitre-plates. Essentially, microbial cells were inoculated into microtitre plate wells containing hydantoin-supplemented medium and incubated for 18 hours. Cells were washed with dihydrouracil in buffer and incubated for an additional 30 minutes. Hydantoinase activity was detected using 5% *p*-dimethylaminobenzaldehyde added to the wells. The "spot test" of Morin *et al.* (1986a) was used in parallel on the same strains as a comparison. The microtitre plate assay was able to detect two extra D-hydantoinase-positive strains. About 18 hours were required to obtain a result in the microtitre plate assay, whereas the "spot test" required two days. Sensitivity of the "spot test" was compromised by the yellowish colour of the agar medium. In addition, colour development in some colonies interfered with that of colonies close-by, so that detection of false positives was possible. Thus the microtitre plate assay was shown to be economical over the "spot test" with respect to time and sensitivity, and application to colonies with a mutated D-hydantoinase gene was envisaged.

A number of techniques have been developed to further improve the sensitivity of screening methods. Siemann *et al.* (1993) made use of *Arthrobacter*-derived polyclonal antibodies to detect L-hydantoinase, racemase, and *N*-L-carbamyl amino acid amidohydrolase. These antibodies were used in a transfer experiment. Colonies were transferred onto cellulose membrane, lysed, washed and used in an ELISA (enzyme linked immunosorbent assay). Hydantoinase-positive colonies could be identified. It was possible to incorporate Western-blot analysis to estimate internal enzyme amounts. Thus the potential was realized for the use of serological methods in the detection and quantification

of hydantoinase-producers and enzymes respectively.

In a molecular approach, DNA probes were developed using the sequence of the D-hydantoinase gene from *Pseudomonas putida* DSM 84 (LaPointe *et al.*, 1994). The most conservative region of known D-hydantoinase genes was used. Rapid detection of these genes was made possible by DNA and colony hybridization.

In studies of cell extracts, the structure and function of proteins may be compromised by temperature, oxidation-reduction effects, radiation, solvent types, metal ions and various modifications. Enzyme-antibody complexes could be applied to stabilize proteins (Shami *et al.*, 1989 cited in Siemann *et al.*, 1994). This was a rationale for the study conducted by Siemann *et al.* (1994) where polyclonal antibodies were produced against L-hydantoinase and racemase enzymes from *Arthrobacter aurescens* DSM 3747. Stabilization of L-hydantoinase by its antiserum was increased three-fold, and that for the racemase two-fold. The activity of the racemase, in particular, remained at 75% of its initial activity over 76 hours while the relevant controls (L-hydantoinase incubated with pre-immunserum or antiracemase or phosphate buffered saline) showed a decline in activity in about 1.5 hours. The enzyme-antibody complexes could be immobilized onto a Sepharose 4B support for detection of enzyme activity even after 17 cycles of column flushing with substrate.

5. Characterization of enzyme activities

5.1. Hydantoinase activity

Several authors have identified the hydantoinase enzyme in different sources and reported on its activity in whole cells, crude enzyme extract or purified enzyme.

A *Pseudomonas fluorescens* strain DSM 84 was cultured in basal medium allowing isolation and purification of the hydantoinase enzyme (Morin *et al.*, 1986b). The enzyme was reported to consist of similarly-sized subunits. Using isopropylhydantoin as a substrate, a 30% conversion to *N*-carbamyl-D-valine was observed. The subsequent conversion to D-valine was chemically achieved. Metal requirement studies showed Fe^{2+} and Mn^{2+} to have a stimulatory effect on conversion by the purified enzyme. Cu^{2+} ions were shown to be inhibitory, and this was in agreement with Sun (1983) (cited in Morin *et al.*, 1986b) who showed the same effect for a *P. putida* strain. *p*-Hydroxymercuribenzoate was shown to inhibit activity, suggesting sulfhydryl group participation in the reaction. Temperature and pH profiles were determined using dihydrouracil as substrate. The optimum temperature for activity was determined to be 55°C, while stability was retained up to 45°C. The pH range of activity was found to be 5.5 to 8.5, with an optimum at pH 9. Takahashi *et al.* (1978) reported a similar temperature optimum, but less activity at alkaline pH for a *Pseudomonas striata* strain. Several substrates were tested, and dihydrouracil was found to be the preferred substrate, with a K_m value of 1.1×10^{-2}M. Similarity to dihydropyrimidinase (EC 3.5.2.2) was proposed by Morin *et al.* (1986b) on the basis of substrate preference and K_m value.

Syldatk *et al.* (1987) reported on the hydantoinase from a different microbial source, namely *Arthrobacter* sp. BH20. The hydantoinase enzyme was shown to be inducible by D-, L- or D,L-indolylmethylhydantoin, and had a wide substrate specificity. Stereoselectivity was not demonstrated. Mn^{2+} and Co^{2+} ions were found to be activators of enzyme activity, while Cu^{2+} and Zn^{2+} ions inhibited enzyme activity. This was comparable with the metal ion requirement results obtained by Morin *et al.* (1986b). However, substrate- and stereoselectivity, induction and metallodependence results suggested that this enzyme was dissimilar to dihydropyrimidinase (EC 3.5.2.2).

A hydantoinase from *Agrobacterium* sp. IP I-671 was partially purified and studied by Runser and Ohleyer (1990). However, substrate selectivity showed strictly D-specificity for compounds substituted at the 5-position by an aromatic group. Di-substituted substrates with a free acid group at position 5 were not hydrolyzed, making this enzyme dissimilar from dihydropyrimidinase. Ni^{2+} ions were found to activate the activity the most. The activity was retained up to 70°C, while the pH optimum was found to be at pH 10. The high temperature and pH stability were shown to be useful, since reactions could be conducted under conditions which allowed improved substrate solubility and exploitation of the benefits of chemical racemization (isomerization to substrate of correct chirality for enzymatic conversion).

Runser and Meyer (1993) purified to homogeneity the hydantoinase enzyme from *Agrobacterium* sp. IP I-671. The molecular mass and oligomeric structure data reported for this hydantoinase enzyme were similar to that reported by Takahashi *et al.* (1978), and Morin *et al.* (1986b) for *P. striata* and *P. fluorescens* respectively. The inhibitory effect by thiol-blocking agents suggested a role in the

reaction mechanism by sulfhydryl groups. The absence of catalytic hydrolysis of dihydropyrimidines by this enzyme suggests that it differs from dihydropyrimidinase (EC 3.5.2.2).

A *Pseudomonas stutzeri* strain ATCC 17588 was used as the source for partial purification of a dihydropyrimidinase enzyme (Xu and West, 1994). The results obtained were compared to those obtained for the dihydropyrimidinase enzyme from *Clostridium uracilicum* (Campbell, 1958 cited in Xu and West, 1994). The substrate preference of this enzyme was in the order: hydantoin, dihydrothymine and almost insignificantly, dihydrouracil. The pH range for activity was determined as pH 7.5 - 9, while the range of temperature was found to be 25-70°C, with an optimum at 45°C. Mg^{2+} ions slightly stimulated the enzyme activity. Cu^{2+} and Zn^{2+} ions readily inhibited the hydantoinase activity. The dihydropyrimidinase enzyme from *C. uracilicum* was different from that of *P. stutzeri* in that the former did not hydrolyse dihydrothymine or hydantoin. Their pH optima were similar, but the temperature of activity for the clostridial hydantoinase was much lower, at 30-35°C. Both pseudomonad and clostridial enzymes activities were activated by magnesium ions.

Durham and Weber (1995) used the cell-free extracts of *Agrobacterium tumefaciens* strain 47C to study its hydantoinase activity. The enzyme specificity was broad over a wide temperature range, with an optimum at 70°C. A broad pH range for activity was observed, with an optimum at pH 8 - 10.5. The preferred substrate was found to be 5,6-dihydrouracil, with benzylhydantoin being the least favoured. The reverse trend in substrate selectivity was found for *Agrobacterium* IP I-671 (Runser and Ohleyer, 1990). The D-specificity for D,L-phenylhydantoin of *A. tumefaciens* was shown to be the same as that reported by Runser and Ohleyer (1990) for *Agrobacterium* sp. IP I-671. Metal ions

did not enhance activity, and neither did EDTA (ethylenediaminetetraacetic acid) supplementation indicate metal dependence. This was different to the data previously reported for other hydantoinases. However, the stability under high alkaline and temperature conditions was similar to that reported by Runser and Ohleyer (1990) for *Agrobacterium* sp. IP I-671.

Ogawa *et al.* (1995a) proposed that two different cyclic ureide-hydrolyzing enzymes occur in *Blastobacter* sp. A17p-4. It was suggested that one be referred to as an imidase enzyme, different to a previously-reported cyclic amide-hydrolyzing dihydropyrimidinase isolated from rat liver (Yang *et al.*, 1993 cited in Ogawa *et al.*, 1995a). The difference between the two was based on substrate specificity. The cyclic amide-hydrolyzing enzyme did not hydrolyze 5-substituted hydantoins with D-stereospecificity, which was similar to the dihydropyrimidinase reported by Takahashi *et al.*, 1978; Yamada *et al.*, 1980 and Olivieri *et al.*, 1981 cited in Ogawa *et al.*, 1995a). Ogawa *et al.* (1997) purified the imidase enzyme identified in a previous communication (Ogawa *et al.*, 1995a) and cyclic imide substrate selectivity was shown to be reproducible. Moreover, strict cyclic imide substrate preference was shown over analogues (cyclic acid anhydrides and amides) of the substrate. Substituted imides and ureides were not hydrolyzed, as had been shown previously (Ogawa *et al.*, 1995a). Steric hindrance was suggested to be a cause of this specificity. The enzyme activity could be reversibly inhibited by the sulfhydryl reagent *p*-chloromercuribenzoate and irreversibly by *N*-bromosuccinimide. Co^{2+} ions were found to be activators of the enzyme activity, and this had not been documented before for hydantoinase enzymes.

The use of resting cells for the production of D(-)*N*-carbamoylphenylglycine by a hydantoinase

enzyme was reported for the first time by Gokhale *et al.* (1996), using a *Pseudomonas desmolyticum* NCIM 2112 strain that showed optimum activity at pH 9.5 and at 30°C respectively. Activity under alkaline conditions had also been shown for other hydantoinases (Runser and Ohleyer, 1990; Xu and West 1994; Durham and Weber, 1995).

The L-hydantoinase of *Arthrobacter aurescens* DSM 3745 was purified and crystallized for x-ray diffraction studies (May *et al.*, 1996). This will allow for the determination of tertiary structure in subsequent studies, and the reaction mechanism and relationship to other hydantoin-hydrolyzing enzymes can be elucidated in due course.

5.2. The racemase enzyme

A racemase would convert the D-hydantoin isomer of a racemic mixture to the L-hydantoin, for conversion *N*-carbamyl-L-amino acid and L-amino acid. This would potentially result in total conversion of the substrate into product. Such activity was demonstrated by the enzyme system of a *Pseudomonas* sp. NS671 (Ishikawa *et al.*, 1993 cited in Ishikawa *et al*, 1997).The genes involved in this conversion have been cloned into *Escherichia coli* and sequenced from a plasmid pHN671 of the *Pseudomonas* strain (Watabe *et al.*, 1992a). The open reading frames of the smaller DNA insert identified were named *hyu*A, *hyu*B and *hyu*C (*hy*dantoin *u*tilization). The gene products were tested for their functional role, and *hyu*A and *hyu*B were found to be involved in the conversion of D- and L-5-substituted hydantoins to the *N*-carbamyl-D- and L-amino acids respectively, while the *hyu*C gene product was suggested to be involved in the conversion of *N*-carbamyl-L-amino acids to L-amino acids.

Identification and sequencing of the larger DNA insert, pHPB14, was performed by Watabe *et al.* (1992b). An additional open reading frame *hyu*E was identified, and was shown to be involved in the conversion of D-5-substituted hydantoins to L-amino acids, suggesting racemase activity.

Watabe *et al* (1992c) purified and characterized the gene product of *hyu*E. The gene was inserted into a derivative of pUC18, pkPN17, in an *E. coli* strain, JM103. The enzyme (a racemase) was reported to be a hexamer, with optimal pH and temperature 9.5 and 45°C respectively for hydantoinase activity. Mn^{2+} and Co^{2+} ions enhanced the enzyme activity, while Cu^{2+} and Zn^{2+} inhibited the activity considerably. The preferred substrates were the D- and L-isomers of 5-(2-methylthioethyl)hydantoin and 5-isobutylhydantoin. 5-L-methylhydantoin was preferred over the D-isomer, while both isomers of 5-isopropylhydantoin were inhibitory. This was demonstrated by pre-incubation of the enzyme with these compounds, followed by determination of residual activity on the preferred substrate, 5-(2-methylthioethyl)hydantoin. The 5-isopropylhydantoin was hydrolyzed in the presence of the preferred substrate, 5-(2-methylthioethyl)hydantoin, suggesting protection of the enzyme by 5-(2-methylthioethyl)hydantoin from inactivation by 5-isopropylhydantoin. Further analysis of this protective effect confirmed that divalent sulfur-containing compounds protected the enzyme from inhibition by 5-isopropylhydantoin. This was the first documentation of the purification and characterization of the activity of the racemase enzyme.

5.3. The *N*-carbamyl amino acid amidohydrolase enzyme (carbamylase)

Ogawa *et al* (1993) used a cell-free extract of *Comamonas* sp. E222C to purify *N*-carbamyl-D-amino

acid amidohydrolase. The D-isomers of substrates with hydrophobic groups (*N*-carbamyl-D-phenylalanine, -D,L-methionine, -D,L-norleucine, and -D,L-tryptophan) were preferred over the L-isomers. Fe^{2+}, Fe^{3+}, Al^{2+}, Sn^{2+} and Pb^{2+} ions slightly activated the enzyme activity. Inhibition by sulfhydryl reagents was demonstrated, which is similar to other hydantoinases (Morin *et al.*, 1986b; Runser and Meyer, 1993) and later Ogawa *et al.* (1997). The enzyme stability was maintained over pH 7-9, and below 40°C. However, activity was optimum at pH 8-9, and maximum activity was measured at 40°C.

A different organism, *Alcaligenes xylosoxidans* was used for the purification and characterization of the activity of an *N*-carbamyl-L-amino acid amidohydrolase by Ogawa *et al.* (1995b). The homodimeric enzyme was L-specific for a variety of substrates, *N*-carbamyl-L-valine being the preferred substrate. Mn^{2+}, Co^{2+} and Ni^{2+} ions activated the enzyme considerably. Sulfhydryl reagents and sodium propionate (an inhibitor of β-ureidopropionase) significantly inhibited the amidohydrolase activity. This seemed to suggest similarity with the amidohydrolase of *Comamonas* sp. (Ogawa *et al.*, 1993) in that both enzymes are possibly related to β-ureidopropionase (EC 3.5.1.6). The N-terminal sequence was determined to be similar to that of *Comamonas* sp. (Ogawa *et al.*, 1993) and *Blastobacter* sp. (Ogawa *et al.*, 1994b cited in Ogawa *et al.*, 1995b). The pH range for activity was 6-9.5, with an optimum at pH 8-8.3. Optimum temperature for activity was reported as 30°C, which is comparable to that of *Comamonas* sp. (Ogawa *et al.*, 1993).

Ishikawa *et al.* (1996) studied the purified form of the *N*-carbamylase-L-amino acid amidohydrolase (*hyu*C product) from *Pseudomonas* strain NS671. The plasmid pDST38 (a derivative of pUC18)

contained the gene for the enzyme which was expressed in and purified from *E. coli* JM103. The homodimeric enzyme showed a requirement for Mn^{2+}, Co^{2+} and Ni^{2+} ions and this finding was identical with that reported for the same enzyme in *A. xylosoxidans* (Ogawa *et al.*, 1995b). A pH of 7.5 and temperature of 40°C were optimal for activity, and were comparable with the optimum pH and temperature conditions of the amidohydrolase of *Comamonas* sp. (Ogawa *et al.*, 1993). The substrate specificity was found to be wide, with preferred hydrolysis of L-isomers. Inhibition by ATP and *p*-mercuribenzoate was reported, the latter observation having been made previously for amidohydrolases from *Comamonas* sp., and *A. xylosoxidans* (Ogawa *et al.*, 1993; Ogawa *et al.*, 1995b).

Louwrier and Knowles (1996) purified the carbamylase enzyme from an *Agrobacterium* sp. The homodimer was strictly D-specific, and *N*-carbamyl-D-methionine was reported to be the preferred substrate. In addition, hydrolysis of aliphatic and aromatic *N*-carbamyl amino acids was shown. The activity was measured over pH range 5.8-9, and an optimum was found at pH 7.3-7.4. The temperature range for activity was 25-80°C, with an optimum at 70°C. Metal ions did not activate the activity, and possible sulfhydryl group involvement was demonstrated by irreversible inhibition of enzyme activity when the enzyme was incubated with thiol a reagent. This inhibition was observed even in the presence of excess substrate as a protective agent. Dependence on reducing agents was shown: the absence of DTT (dithiothreitol) resulted in inhibition of the enzyme. The authors suggested rapid oxidation of sulfhydryl groups by oxygen to be the cause of inhibition. $(NH_4)_2SO_4$ (ammonium sulphate) and NH_4Cl (ammonium chloride) were both inhibitory, possibly by a product inhibition mechanism. The authors proposed that the enzyme from this organism was novel as it

differs from the ureidopropionase of *C. uracilicum* (Campbell, 1960 cited in Louwrier and Knowles, 1996) with respect to lower temperature range for activity and sensitivity to ureidopropionase inhibitors.

Some bacterial strains have been shown to possess both the hydantoinase and the carbamylase enzymes so that the complete conversion of substrates through to the corresponding amino acid is possible.

An organism with both the hydantoinase and the carbamoylase enzyme is *Agrobacterium radiobacter* NRRL B11291 (Olivieri *et al.*, 1981). Whole cells were used in this investigation, except where mechanical disruption/ transport studies were conducted. Broad substrate specificity was reported. D,L-phenylhydantoin conversion (hydantoinase activity) was optimal at pH 9, while the conversion of the intermediate (carbamoylase activity) was observed over the pH range 7.5-9. Optimum temperatures for the hydantoinase and carbamoylase enzymes were found to be 60°C and 50°C respectively. Ammonia derivatives and sodium chloride were inhibitors of both enzymes, and this was found to be more pronounced for the carbamoylase enzyme under alkaline conditions. Transport studies using *N*-carbamyl-D-phenylglycine indicated that toluene-treated or disrupted cells produced more D-phenylglycine than intact cells. Limited permeability of the cells to the *N*-carbamyl derivatives was suggested as a possible explanation for this observation. Using D,L-5-phenylhydantoin, the opposite effect was observed; intact cells produced more amino acid than the toluene-treated/disrupted cells. The availability of intermediate, *N*-carbamylamino acid, to the carbamoylase enzyme within the intact cells was proposed to explain this. Upon cell membrane disruption, the

intermediate would be diluted within the reaction volume so that conversion to amino acid would be limited.

Syldatk *et al.* (1990b) isolated an organism capable of converting D,L-5-(3-indolylmethyl)hydantoin (D,L-5-IMH) to L-tryptophan. This organism was designated the name *Arthrobacter* sp. DSM 3747. The enzyme system was shown to be inducible by supplementation of growth medium with D,L-5-IMH. Induction by this substrate had previously only been shown in the genera *Arthrobacter* and *Flavobacterium* (Syldatk *et al.*, 1987; Yokozeki *et al.*, 1987b). Mn^{2+} ions were shown to be required for the synthesis of the hydrolyzing enzymes, as had been observed for the hydantoinase enzyme of *A. aurescens* BH20 (Syldatk *et al.*, 1987). However, ammonium sulphate was reported to be a suitable nitrogen source for enzyme induction, and whereas this was not the case for the *Agrobacterium* sp. of Louwrier and Knowles (1996). Mn^{2+} ions and D,L-5-IMH were found to effect an increase in cell growth and enzyme specific activity, provided they were not added at the beginning of culture, but rather 8-10 hours into fermentation. In this way, maximum substrate conversion could be achieved by a high cell mass (obtained under Mn^{2+}-free conditions). A non-utilisable analogue of D,L-5-IMH, D,L-5-(3-indolylmethyl)-3-*N*-methylhydantoin, was used as an inducer at the onset of cultivation, and was shown to increase the enzyme specific activity, and not to decrease biomass. This raised the possibility of culture supplementation with Mn^{2+} and D,L-5-IMH after 8-10 hours of inducer-free cultivation as a means of simultaneously obtaining rapid cell growth and higher specific activity or enzyme efficiency.

Other ways of increasing enzyme efficiency have been evaluated. The production of D-*p*-

hydroxyphenylglycine from hydroxyphenylhydantoin by *Agrobacterium* sp. I-671 was investigated (Kim and Kim, 1994) since both enzymes were present in this organism. Ammonium ions which are by-products of the reaction were found to be inhibitors of the carbamoylase enzyme, and an efficient means of ammonium ion removal was developed to drive the reaction in the direction of amino acid synthesis. This was achieved with a re-usable ammonium ion adsorbent, AD 300 NS, and the yield of D-hydroxyphenylglycine was increased from 50 to 98%.

Only *Bacillus* and *Pseudomonas* genera had been previously reported as being capable of conversion of D,L-5-(2-methylthioethyl)hydantoin to L-methionine, and thus *Arthrobacter* sp. DSM 9771 was evaluated for capability of effecting the same conversion (Wagner *et al.*, 1996). A mutant of the parent strain, *Arthrobacter* DSM 7330, was selected on the basis of its ability to constitutively express hydantoin-hydrolyzing enzymes when grown on glucose and *N*-carbamyl-L-methionine (L-CAM) as sole carbon and nitrogen sources. This was necessary because the preferred inducer, D,L-5-indolylmethyl-3-methylhydantoin (N3-IMH) had previously been found to inhibit growth of the parent strain. The parent strain did not produce significant amounts of the enzymes of interest, while the mutant did. In addition, the mutant showed higher hydantoinase activity on D,L-5-(2-methylthioethyl)hydantoin and growth rate even in the absence of inducer. However the carbamoylase activity of the mutant was lower than that of the parent strain.

The hydantoinase enzyme of the parent strain DSM 7330 was previously shown to be non-stereospecific, affording both D- and L-isomers of the *N*-carbamyl amino acid, *N*-carbamyl methionine, whereas the carbamoylase enzyme of this strain was specific for L-CAM (Völkel and

Wagner, 1995 cited in Wagner *et al.*, 1996). In this case, racemisation of D-CAM, and subsequent enzymic conversion to L-methionine were thought to be the rate-limiting steps of the reaction. The racemisation was proposed to occur when most of the L-CAM had been converted to L-methionine. At this point (after 2 hours) the carbamoylase pH optimum shifted from 8 to 7.5, so that enzymic conversion of D-CAM occurs 3 times faster than at pH 8.5 (Wagner *et al.*, 1996).

Tsuji *et al.* (1997) reported the chemical conversion of D,L-5-(*p*-trimethylsilylphenylmethyl)hydantoin to *N*-carbamyl-D-*p*-trimethylsilylphenylalanine. Enzymic conversion was not possible, since the bulky trimethylsilyl moiety did not allow for microbial hydrolysis. Further conversion of *N*-carbamyl-D-*p*-trimethylsilylphenylalanine to D-*p*-trimethylsilylphenylalanine was catalysed by the partially-purified *N*-carbamyl-D-amino acid amidohydrolase of *Blastobacter* A17p-4. This was important because the final product could confer novel properties to penicillins and cephalosporins, by virtue of its hydrophobicity, and the larger atomic radius and electronegativity of silicon as compared to carbon (Colvin, 1981 cited in Tsuji *et al.*, 1997). A pH of 8 was optimal for the amidohydrolase activity, and high optical purity was observed in the product.

A mechanism for the complete conversion of D,L-substituted hydantoins to L-amino acids by all three enzymes (namely the hydantoinase, racemase and *N*-carbamylase enzymes) in *Pseudomonas* sp. strain NS671 was proposed (Ishikawa *et al.*, 1997). The mechanism proposed distinguishes the hydantoinase of this organism from all others reported in that it cannot be classed as strictly D-specific, strictly L-specific, or non-specific. The L-form of D- and L-5-(2-methylthioethyl)hydantoin was hydrolyzed preferentially, and the D-form of the substrate was hydrolyzed after nearly complete

hydrolysis of the L-form. When the D-form of the substrate was used, both D- and L-forms of the intermediate (*N*-carbamyl-methionine) were detected. The conclusion was that the same L-specific hydantoinase could hydrolyse D-hydantoins, although spontaneous racemisation of D- to L-substrate was suspected. The mechanism is schematically represented in Figure 1.5.

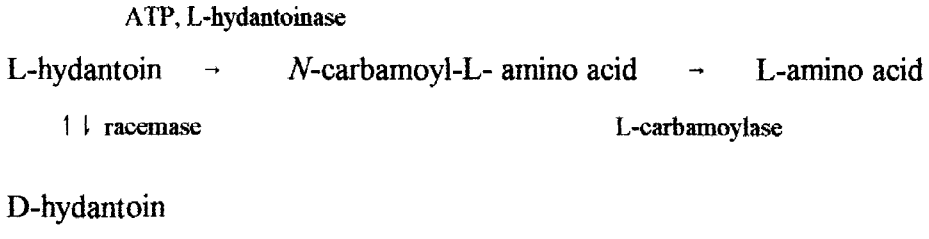


Figure 1.5 Proposed mechanism of hydantoin hydrolysis in *Pseudomonas* sp. NS671 (Adapted from Ishikawa *et al.*, 1997).

6. Immobilisation as a strategy for stabilization of enzymes

The instability of the carbamylase enzyme in comparison with the D-hydantoinase (Ogawa *et al.*, 1993, Ogawa *et al.*, 1994b cited in Lee and Lin, 1996; Ogawa *et al.*, 1995b) prompted the development of methods to stabilize hydantoin-hydrolyzing enzymes for use at elevated temperatures. One of these methods is immobilisation of enzyme or whole cells on a support of some kind while maintaining full catalytic activity. An added advantage would be that the immobilized biocatalyst could then be used in a continuous system. Several methods of immobilization could be used, including carrier-binding, cross-linking and entrapment. Entrapment is a commonly used method for immobilization. Enzymes or cells are entrapped within the lattice of a polymer matrix, or enclosed in semipermeable membranes. Several matrices can be used, alginate and polyacrylamide being

common (Chibata *et al.*, 1986). Entrapment in calcium alginate gel is simple, and requires the use of mild conditions which are unlikely to compromise enzyme activity (Tramper, 1985). The use of an immobilized biocatalyst has the added advantage of permitting easy recovery of the biocatalyst by filtration or centrifugation for re-use (Tramper, 1985).

6.1 Application of immobilisation in hydantoin-hydrolyzing systems

Several authors have reported the immobilization of hydantoin-hydrolyzing cells or enzymes. Cells of *Pseudomonas putida* DSM 84 were immobilized in alginate beads and in microporous hollow-fibre cartridges (Chevalier *et al.*, 1989). Conversion rates of batch and continuous systems were compared, and in batch cultures, immobilized and non-immobilized cells were found to have similar activity. Immobilization and the effect on activity in continuous systems was also investigated. *N*-carbamyl-β-alanine production by cells in a cell growth-limiting medium was observed, ie, hydantoinase activity could be detected in non-growing cells. In addition, simultaneous tests for cell viability were positive. This facilitated the investigation of continuous production in upflow packed bed columns using growth-limitation since, plugging of columns by non-growing cells would not occur. Bed damage owing to bead disruption and swelling was observed after 24 hours of reaction. Hollow-fibre cartridges were used in an alternative study, and were found to be undesirable for conversion of substrate due to membrane damage, and leakage of biocatalyst into the lumen (confirmed by scanning electron microscopy). Of the two continuous systems evaluated, the packed bed volume system was found to be preferable and to have potential for use in scale-up studies.

Pseudomonas sp. KBEL 101 was isolated from soil, and cells were immobilized within polyacrylamide gel (Kim and Kim, 1994). The 50-hour half-life of the immobilised biocatalyst was not considered satisfactory. The authors then investigated methods of increasing the operational stability of the biocatalyst for production of D-hydroxyphenylglycine from D,L-5-hydroxyphenylhydantoin. Nitrogen and carbon sources were evaluated for their stabilizing effect on the biocatalyst. Glycerol as a carbon source was found to have a stabilizing effect for up to 7 days, and a sharp decline in activity thereafter was attributed to the decrease in cell viability. Nitrogen sources, ammonium sulphate and yeast extract, were evaluated, and ammonium sulphate was found to have a stabilizing effect equivalent to that of glycerol at the same concentration. However, yeast extract increased the half-life of the immobilized biocatalyst to 25 days, and the components of the yeast extract (vitamins, fatty acids, amino acids) were suggested to be responsible for this maintenance of cell viability.

As is evident from the preceding review, the literature clearly confirms that there is a significant diversity amongst mesophilic hydantoin-hydrolyzing enzyme systems with respect to inducer requirement, substrate- and stereospecificity, pH and temperature optima, purified enzyme characteristics (subunit organization, size), and inhibitor effects. Despite these differences, an important similarity occurs for some systems in that they exhibit maximum activity at temperatures in excess of 50°C (Takahashi *et al.*, 1978; Olivieri *et al.*, 1981; Morin *et al.*, 1986b; Runser and Ohleyer, 1990; Durham and Weber,1995; Louwrier and Knowles, 1996). Thus in addition to mesophiles, literature on the conversion of hydantoin substrates to enantiomerically pure amino acids by thermophiles occurs. The significance of pursuing the search for thermostable enzymes is discussed

below, preceding a survey of the thermostable hydantoin-hydrolyzing enzymes that have been documented.

7. Characteristics of proteins from thermophiles

There are a number of advantages in exploiting thermostable enzymes. Reactions may be carried out at elevated temperatures, owing to the thermostability of enzymes. The elevated temperatures may lead to increased reactant solubility and diffusion capacity, and reduction in medium viscosity. Volatile enzyme-inhibitory products may be evaporated off. Some thermostable enzymes can catalyze different reactions, and this permits the use of "multifunctional" enzymes in complex reaction systems at elevated temperatures (Parvaresh *et al.*, 1990).

Proteins in thermophiles are generally more stable than those in mesophiles, and this may be due to subunit interaction (Jaenicke, 1996), secondary or supersecondary structure, domain packaging and stabilization by association with carbohydrate groups and nucleic acids. Domain boundaries and extensions at the carboxy- or amino termini (Trinkl *et al.*, 1994 cited in Jaenicke, 1996) are also proposed as contributors to protein stability.

Cellular components of thermophiles either show high intrinsic stability, or they may rapidly synthesize whatever component is being diminished under the prevailing conditions. The characteristically high intrinsic stability exhibited by proteins in thermophiles in turn allows for resistance to proteolysis and denaturants at modest concentrations (Jaenicke, 1991 cited in Jaenicke,

1996) as well as to organic solvents (Parvaresh *et al.*, 1990). The differences between the stability properties of proteins in extremophiles and non-extremophiles are not remarkable. Added stability is due to slightly more hydrogen bonds, ion pairs and/or hydrophobic interactions in the case of extremophiles (Jaenicke, 1991 cited in Jaenicke, 1996).

8. Thermostability in hydantoin-hydrolyzing enzymes

Screening for thermophilic organisms with a particular enzymatic activity requires isolation of the organisms from areas with elevated temperatures as a characteristic feature. An example of such an area would be compost heaps, where microbial activity results in heat generation giving temperatures of up to 80°C (Bandu *et al.*, 1988). The organisms responsible for composting would be appropriately referred to as thermotolerant: they can survive and grow at temperatures 25-60°C (Davis *et al.*, 1992a). This is in contrast to obligate mesophiles that do not actively grow at 60°C, but survive at such temperatures (Davis *et al.*, 1992b).

Lee *et al.* (1994) were the first to report the isolation of a D-hydantoinase produced by a thermophilic *Bacillus* SD-1 species. Isolates were obtained from a variety of locations, and enrichment was conducted at 55°C. Colonies from the enrichment cultures were cultivated at 55°C, and assays for the hydantoinase activity were conducted at the same temperature. The thermostability of the hydantoinase enzyme was retained up to 70°C, and selection of the *Bacillus* strain over other strains for further study was based on the high thermostability observed. The hydantoinase enzyme was strictly D-specific. Activity occurred over pH range 6.5-10.5, with the optimum at pH 8. The optimal

temperature for activity was determined to be 70°C, and the thermostability of this organism was reported to be greater than those documented previously (Runser and Meyer, 1993).

The hydantoinase enzyme of *Bacillus stearothermophilus* SD-1 was purified to homogeneity (Lee *et al.*, 1995). Isolation and assay conditions were essentially the same as those used by Lee *et al.* (1994) (see above). The enzyme was reported to be a homodimer, and the activity was shown to be D-specific. The enzyme was non-inducible and was similar to that reported by Morin *et al.* (1986b) for a *Pseudomonas fluorescens* strain. Hydantoins with aromatic or aliphatic substituents were poorly hydrolyzed, and hydantoin was the preferred substrate. Mn^{2+} ions were a requirement for activity. pH and temperature were optimum for enzyme activity at 8 and 60°C respectively. The long half-life of 30 minutes at 80°C had not been observed before. No carbamylase activity was detected, and this could be attributed to its instability.

The medium composition was optimized for D-hydantoinase production by the strain *Bacillus stearothermophilus* SD-1 prior to immobilization of crude enzyme extract on DEAE-cellulose resin (Lee *et al.*, 1996b). The pH and temperature optima for activity of immobilized enzyme were found to be about 8.5, and 60°C respectively. The increased solubility of the substrate, D,L-5-hydroxyphenylhydantoin at the higher temperature was proposed as a reason for the high conversion observed. The enzyme activity was consistent over nine successive batch operations at 55°C.

The establishment of increased aqueous solubility and hence efficient conversion of D,L-5-hydroxyphenylhydantoin by *B. stearothermophilus* SD-1 justified the cloning of the gene for the D-

hydantoinase enzyme into *E. coli* DH5α (Lee *et al.*, 1996a). The specific activity of the recombinant *E. coli* was 30 times higher than of *B. stearothermophilus* SD-1.Thus *E. coli* DH5α was cultured, and a cell-free extract was used for further studies. Temperature and pH optima were determined to be 55°C and 9 respectively, both of which compare favourably with the optima for the immobilized enzyme mentioned above (Lee *et al.*, 1996b).

Finally, with respect to the *B. stearothermophilus* SD-1 hydantoinase enzyme, the primary structure and sequence have been reported (Kim *et al.*, 1997b). Comparison at the molecular level of this D-hydantoinase was made with other hydantoin-hydrolyzing enzymes, since although they are involved in the same type of reaction, their substrate and stereospecificities vary. *B. stearothermophilus* sp. NS1122A (Ishikawa *et al.*, 1994), *B. stearothermophilus* sp. SD-1 and *Pseudomonas* sp. DSM84 were shown to be related by analysis of sequence homology. A significant difference was discovered in the C-terminal regions of the hydantoinases from *B. stearothermophilus* sp. NS1122A and *B. stearothermophilus* sp. SD-1. The differences in substrate and stereospecificity were suggested to be attributable to the marked differences in the C-termini. Analysis of hydropathy profiles of the C-terminal regions of these two hydantoinases suggests that the *B. stearothermophilus* sp.SD-1 hydantoinase is hydrophilic, while that of *B. stearothermophilus* sp. NS1122A is hydrophobic. However the secondary structures were similar, and since active-site amino acid residues are conserved, a relationship was suggested.

Other groups (Ishikawa *et al.*, 1994; Mukohara *et al.*, 1994) have studied *B. stearothermophilus* NS1122A in parallel with other studies on *B. stearothermophilus* (Lee *et al.*, 1994; Lee *et al.*, 1995;

Kim *et al.*, 1997b). *B. stearothermophilus* NS1122A was chosen for further analysis based on the appreciable activity which had been reported earlier (Ishikawa *et al.*, 1994). Culture conditions for production of L-methionine were optimized, and the hydantoin-hydrolyzing enzymes were purified and characterized. Optimum temperature and pH were reported as 60°C and 9.5 respectively, with predominantly L-specificity for *N*-carbamylamino acids. The carbamylase enzyme was activated by Co^{2+}, Mn^{2+} and Ni^{2+} metal ions, showing strict L-specificity for a range of substrates. Optimum pH and temperature for the homotetrameric carbamylase enzyme were reported as 8 and 60-70°C respectively.

Mukohara *et al.* (1994) cloned and sequenced the hydantoinase gene of *B. stearothermophilus* using *E. coli* JM105 for the transformation. Expression of the hydantoinase gene resulted in an insoluble hydantoinase aggregate which was different from that reported by Kim *et al.* (1997b): the product obtained by the latter authors was soluble. Characterization of the hydantoinase from the *E. coli* recombinant was carried out, and the enzyme was found to be a homotetramer, readily inhibited by the sulfhydryl reagent, *p*-chloromercuribenzoate. Mn^{2+} and Co^{2+} ions stimulated the activity in a pH-dependent manner. pH dependence was not observed in the absence of metal ions. The oligomeric conformation was suggested to confer thermostability as the monomer was poorly thermostable. In addition, Mn^{2+} ions were suspected to promote oligomer formation, and hence the thermostability. The authors proposed that the hydrophobicity suggested by the hydropathy studies possibly contributed to thermostability as was also suggested by Kim *et al.* (1997b).

Keil *et al.* (1995) reported the characteristics of two commercially-available thermostable

hydantoinases. These were designated the names D-HYD-1 and D-HYD-2. The pH optimum was 8 for activity of both enzymes, and the activity of D-HYD-2 was observed to increase ten-fold when temperature was raised from 37 to 80°C. Both enzymes were stable up to 70°C, but stability decreased considerably at temperatures greater than 80°C. This temperature for activity was comparable to that reported by Runser and Ohleyer (1990) for *Agrobacterium* sp. IP-671. Aromatic substituted hydantoins were the preferred substrates for both enzymes, but D-HYD-2 hydrolyzed aliphatic substituted hydantoins more readily than D-HYD-1.

Lee and Lin (1996) immobilized a thermostable D-hydantoinase that had been purified from a recombinant *E. coli* strain onto an organic polymer carrier. Kinetic studies on the immobilized enzyme indicated that the binding between enzyme and substrate, D,L-*p*-hydroxyphenylhydantoin, decreased with increase in temperature, but specific activity was higher at elevated temperatures. The optimum temperature for activity was observed to be 60°C. As was reported by Lee *et al.* (1996a), higher temperatures increased solubility and thus the conversion of D,L-5-hydroxyphenylhydantoin. The biocatalyst could be used in 8 consecutive reactions at 50°C (compared to the 9 reported by Lee *et al.*, 1996b). Lower operational stability of the biocatalyst was attributed to loss of enzyme by support fouling, as confirmed by scanning electron microscopy.

Sharma and Vohra (1997) documented the thermostability and stability in high alkaline conditions of a D-hydantoinase from a mesophilic *Bacillus* sp. AR9 using crude extracts of the enzyme for characterization. The expression of the enzyme was suggested to be constitutive, with yields of 4500U/ml, which were higher than any that had been reported previously. The stereospecificity of

the hydantoinase was strictly D-specific, and unsubstituted hydantoin was the preferred substrate, which contrasts with the substrate specificity reported by Lee *et al.* (1994) for *B. stearothermophilus* SD-1.The pH optimum for activity was 9.5, which was higher than the hydantoinase previously reported for a *Pseudomonas* species (Morin *et al.*, 1990 cited in Sharma and Vohra, 1997; Yamada *et al.*, 1978; Yokozeki *et al.*, 1987a). Co^{2+} ions activated hydantoinase activity considerably as had been observed before with other hydantoinases. A half-life in excess of 12 hours at 50°C was observed, which was longer than had been reported for other organisms.

Although relatively little literature is to be found on thermophilic hydantoin-hydrolyzing organisms, the diversity amongst these organisms is comparable to that of the mesophiles. In addition, the investigation of thermostable hydantoinases is important because of their potential value in industry since reaction rates can be enhanced, and substrate solubilities improved at higher temperatures, leading to efficient conversions. Where immobilization of thermostable hydantoinases has been successful, even greater thermostability is possible.

Apart from the intrinsic properties of the enzyme that confer stability, the environment in which the enzyme is used may confer stability.

9. The effect of organic media on the activity and stability of enzymes

Biocatalysis may be achieved in nearly anhydrous or non-aqueous media. Such an environment would be provided by organic solvents in which water content is less than one percent, and is distinguished

from biphasic media which are mixtures of aqueous and water-immiscible organic solvents such as octane, and media that contain water-miscible solvents, for example, methanol and acetone. Increased stability has been observed for enzymes in non-aqueous media, for example, full activity of chymotrypsin was maintained after 6 months in anhydrous octane at 20°C, whereas the half-life for the same enzyme in aqueous media was a few days (Zaks and Klibanov, 1988).

Apart from increased general stability, there are a number of other possible advantages in the use of enzymes in organic systems. Thermal stability can be enhanced, regio- and substrate specificities can be altered and controlled, and reaction reversal may occur. The catalysis of new reactions may be observed. Enzymes can be recovered with ease from the organic medium as they are suspended, rather than dissolved. The solubility of non-polar substrates may be enhanced, and undesirable water-dependent side-reactions may be eliminated.

Some protein-bound water is required by proteins for hydrogen bonding, ionic interactions, hydrophobic interactions and van der Waals interactions (Schultz and Schirmer, 1979 in Gorman and Dordick, 1992), but the amounts are minimal. Only a monolayer of water around the protein is necessary, and this remains fixed on the protein. Stripping this essential water layer from the protein by partitioning into less hydrophobic solvents can result in loss of enzyme catalytic activity. This has been shown for yeast alcohol oxidase, mushroom polyphenol oxidase and horse liver alcohol dehydrogenase in water-miscible organic solvents (Zaks and Klibanov, 1988). In non-aqueous solvents, the activity of the enzymes was increased proportionately with increase in the water content of the medium, and less water was required to reach maximum catalysis in hydrophobic solvents than

in hydrophilic solvents. This implied that there would be less or no partitioning or quenching of water into or by water-immiscible organic solvents if these were used.

The stripping of water by solvents was illustrated by quantifying water desorbed from enzymes (Gorman and Dordick, 1992). Tritiated water was used to replace enzyme-bound water, and the enzymes were placed in water-miscible and water-immiscible solvents. Polar solvents were shown to effect more desorption of tritiated water than non-polar solvents. It has also been shown that it is the interaction of the organic solvent with the monolayer of water that affects enzyme activity, and not the interaction between solvent and enzyme (Zaks and Klibanov, 1988).

Water plays a major role in the thermoinactivation of enzymes. The effects include β-elimination to disrupt disulfide bonds, deamidation of some residues and hydrolysis of peptide bonds. These reactions would be absent in an essentially water-free medium, and thus thermostability would be favoured. This was demonstrated using porcine pancreatic lipase, which was shown to have high catalytic activity and long half-life (in excess of 12 hours) in a 0.015% water/tributyrin/ heptanol mixture at 100°C. In contrast, this enzyme was completely inhibited within seconds at 100°C in aqueous medium. Furthermore, the enzyme-catalysed transesterification rate between tributyrin and heptanol at 100°C was five times greater than at 20°C (Zaks and Klibanov, 1984). It has been proposed that the rigidity conferred by dehydration afforded the observed thermostability, or that the insolubility of the enzyme in organic solvent ensures maintenance of a conformation which is favourable for optimum activity.

Related to the protein conformation is the ionic state of the enzyme to be used in organic media. Enzymes can assume an optimal ionization state in aqueous media which is retained when they are lyophilized from aqueous media and used in organic media (Zaks and Klibanov, 1985 cited in Deetz and Rozzell, 1988). This enhances the catalytic activity of the enzyme in organic solvents, and is convenient since pH cannot easily be measured or altered in organic solvents (Dordick, 1989). Thus lyophilization of enzymes from aqueous buffer at optimum pH is usually carried out before use in organic solvents. There are two alternatives to this lyophilization. One is the saturation of water-immiscible solvent with buffer of optimum pH, and another is the use of highly hydrophobic acid-sodium salt and base-chloride buffers: the acid or amine to salt ratio can be varied so that changes in acidity or basicity may be buffered (Blackwood *et al.*, 1994 cited in Bell *et al.*, 1995).

Consideration of the type of solvent to be used is important since enzyme stability and activity can be affected by the solvent. Protein structure may be distorted by some organic solvents, and this may lead to instability. Water-miscible solvents such as methanol and ethanol at very low concentrations may bind to the enzyme and cause protein unfolding, or compete with the substrate for binding sites (Tang, 1965 cited in Butler, 1979). Enzyme reaction rates are affected by the increase in K_m value (Butler, 1979), so that catalytic efficiency (K_{cat}/K_m or V_{max}/K_m) decreases (Dordick, 1991 cited in Blinkovsky *et al.*, 1992). An explanation for the effect of solvent on K_m proposes that the substrate is desolvated and binds to enzyme. If the substrate has a low affinity for the solvent, K_m will be low because substrate-enzyme affinity is enhanced. Conversely, K_m will be high if the substrate has a higher affinity for the solvent than for the enzyme. K_m varies for systems using different solvents, but generally, polar substrates will have a higher K_m in polar solvents (where association with polar

solvents is preferred), and a low K_m in non-polar solvents (where association with enzyme is preferred) (Bell *et al.*, 1995).

Solvents may also interact with the substrates of the reaction. An example is chloroform, which quenches phenoxy radicals that are important for initiation of phenol oxidation by the peroxidase enzyme (Kazandjian *et al.*, 1986 cited in Dordick, 1989). In addition, the course of the reaction may be altered by the organic solvent. For example, polar products may remain in the vicinity of the enzyme, and may not be extracted into the non-polar solvent. A likely consequence of this is concentration of the products around the enzyme, and this could result in product inhibition.

A number of models have been formulated to assist in choice of solvent because the range of solvents that could be used is large. One of these models involves the term log P, and is a measure of solvent hydrophobicity, where P is the partition coefficient of a particular solvent between water and (1-)octanol in a two-phase system (Laane *et al.*, 1987 in Tramper, 1996). The log P concept allows for the prediction of solvent toxicity to the enzyme: solvents with logP<2 are toxic to most enzymes as they readily disrupt the protein-water monolayer association. Solvents with log P between 2 and 4 have unpredictable toxicity but disruption is generally less. Most solvents with log P>4 are non-toxic, as disruption does not occur (Laane, 1987).

9.1 Enzyme stability enhancement in organic solvents

Work has been done on improving biocatalyst operational stability in the presence of organic solvents,

and was achieved by modification of the enzyme, the use of suitable support material, or the use of additives.

Modifying enzymes can involve coupling to compounds which have both hydrophobic and hydrophilic groups, for example polyethylene glycol (PEG) . The coupling could be carried out in aqueous medium in which both the enzyme and PEG are soluble, and the removal of water by lyophilization would allow solubilization of the modified enzyme in solvents such as benzene and toluene. An advantage of this modification is that limitation of mass-transfer of substrates and products between solvent and biocatalyst would be eradicated. However, there is the possibility of enzyme inactivation by the chemical modification (Mattiasson and Adlercreutz, 1991).

The sulfhydrogenase enzyme from the hyperthermophile *Pyrococcus furiosus* was modified by attachment to PEG-*p*-nitrophenylcarbonate to determine the effect of the modification on enzyme activity in toluene (Woodward and Kaufman, 1996). Hydrogen sulfide evolution (indicating enzyme activity) in toluene was ten-fold higher for the modified hydrogenase than for the unmodified enzyme. The enhanced solubility of sulfur in toluene was suggested as an explanation for this result. However, PEG is unsuitable for coupling to enzyme for increase in operational stability because it is not amphiphilic, and short-chained PEG molecules (PEG-1500) do not dissolve in organic solvents (Jene *et al.*, 1997). Modification of biocatalysts with larger PEG molecules (PEG-5000) is an alternative (Takahashi *et al.*, 1984 cited in Jene *et al.*, 1997) but could inactivate the biocatalyst possibly owing to the steric bulkiness which would be conferred. An alternative to PEG would be nonionic surfactants such as brij35 (polyoxyethylene ether). The hydrophilic polyoxyethylene part would keep

the essential water layer around the protein of interest intact and allow for solubility in aqueous media, while the hydrophobic alkyl tail would facilitate solubility in organic solvent. The activity of catalase was shown to have increased 200-fold more after this type of modification, and the thermostability of commercially-available brij35-modified bovine-liver catalase was increased 280-fold at 50°C in organic solvent, compared to aqueous buffer. This activity was shown to be proportional to solvent hydrophobicity (Jene *et al.*, 1997).

A number of different supports are available for immobilization of enzymes for use in organic solvent systems. Reslow *et al.* (1988) immobilized bovine pancreatic α-chymotrypsin and horse liver dehydrogenase on a series of support materials which were classed on the basis of aquaphilicity (a measure of partitioning of water between solid support and water-saturated diisopropyl ether), or simply the ratio water on support:water in solvent. The results obtained for the activity measurements indicated that support materials with low aquaphilicity are suitable for esterification and hydrolysis applications. Competition for water between the enzyme and the support was demonstrated, indicating that in the design of reaction systems, it is important to take water-binding capacities into account. With less than 1% water in the system, the high aquaphilicity of PEG and glycerol, for example, keep the biocatalyst hydrated, thus not compromising the activity. However, the nature of the enzyme reaction and biocatalyst have to be considered in conjunction with the support material.

Additives such as ethyl cellulose, ethylene glycol and glycerol may serve to make biocatalysts resistant to denaturation (Laane, 1987). Salts such as KCl were shown to increase the activity of subtilisin Carlsberg and α-chymotrypsin suspended in a wide variety of organic solvents (Khmelnitsky *et al.*,

1994). The proposed functions of the salt include protection of the enzyme from inactivation by organic solvents, and maintenance of enzyme structure during lyophilization, which together increase enzyme activity. Griebenow and Klibanov (1997) showed the same stabilisation effect on subtilisin Carlsberg using KCl, and suggested that the KCl induced the preservation of the enzyme secondary structure during lyophilization, and hence the increased activity in organic medium.

In an investigation into the mechanism of stabilization and activation of enzymes in organic media by additives (Triantafyllou *et al.*, 1997), various buffer salts used as co-lyophilization additives were shown to increase transesterification of chymotrypsin in proportion to their concentrations. However, high concentrations of sodium phosphate inhibited the transesterification activity, possibly because the mode of association of sodium phosphate with water could affect the water content around the protein. The presence of KCl and Tris-HCl were shown to increase the activity of chymotrypsin in peptide synthesis, and the effect of additives on transesterification by *Candida antarctica* lipase was similar to that on chymotrypsin. Sodium phosphate and sorbitol at increasing concentrations were found to make chymotrypsin more prone to thermoinactivation. This was suggested to be because as the hydration level increased, protein flexibility increased, and thermoinactivation would then be increased.

9.2 Applications of enzymes in organic solvents

A few examples serve to illustrate how enzymes have been utilized in organic solvent systems, and how these biocatalytic systems differ from aqueous-medium systems.

9.2.1 Stereospecific esterifications

Lipases are often used in organic solvents to effect the resolution of racemic mixtures. An example is the application of the enzyme from *Candida cylindracea* in the esterification of (±) menthol in the presence of lauric acid and heptane. (-)Menthyllaurate in 95%ee was obtained (Langrand *et al.*, 1986 cited in Dordick, 1989). Lipases have also been used to selectively esterify R-isomers of 2-halocarboxylic acids in butanol/hexane (Kirchner *et al.*, 1985 cited in Klibanov, 1986). The two chiral acids could be used in their pure form for the synthesis of herbicides. Sakurai *et al.* (1988) studied the transesterification of 2-chloroethylesters of *N*-acetyl D- or L-alanine with propanol by the protease, subtilisin Carlsberg. It was proposed that the binding of the D-isomer to the enzyme is favoured in very hydrophobic solvents, and this substrate displaces water from the active site. This would be thermodynamically favourable for enzyme-substrate complex formation. However, the alternative binding of the L-isomer displaces more water from the active site, and this would be energetically unfavourable for the formation of the enzyme-substrate complex, so that the D-enantiomer reactivity is greater than that of the L-enantiomer.

9.2.2 Regioselectivity

Regioselectivity by the lipase from *Pseudomonas cepacia* could be altered in acylation reactions by organic solvents (MacManus and Vulfson, 1997). The substrate for acylation was a trityl group containing glucopyranoside, which afforded products in ratios which were influenced by the type of

solvent used. The monosaccharide moiety of the substrate was assumed to be acylated at the active site but this assumption was not made for the trityl moiety owing to its bulkiness. In a very hydrophobic solvent, association of the trityl group with the solvent would occur, so that acylation at the 2-hydroxyl group occurs. In the presence of a hydrophilic solvent, the trityl group would prefer association with the hydrophobic cleft of the active site so as to effect 3-acylation of the monosaccharide moiety.

9.2.3 Substrate specificity

By placing enzymes in organic solvents, rigidity is conferred upon the enzyme structure so that bulky substrates are not accommodated, ie., loss in flexibility is conferred. This changes the substrate specificity as shown for wet and dry preparations of porcine pancreatic lipase (Zaks and Klibanov, 1984). Both preparations catalyzed transesterification between tributyrin and smaller and secondary alcohols, while bulky tertiary alcohols were substrates for the wet preparation only.

Zaks and Klibanov (1986) used α-chymotrypsin to illustrate changes in substrate specificity effected by organic hydrophobic solvents. Hydrophilic substrates were shown to have low K_m values (high affinity for enzyme) and therefore efficient transesterification when placed in the hydrophobic solvent, octane. However, hydrolysis of the ester substrates was shown to be much less efficient for the hydrophilic solvents, as compared to the hydrophobic solvents.

9.2.4 Reversal of reactions

It is possible to favour condensation reactions over hydrolyses using water-immiscible organic solvents. Enzymes that usually hydrolyse substrates are capable of catalyzing the synthesis. One application is the synthesis of the dipeptide sweetener, Aspartame®, which was achieved using thermolysin suspended in ethyl acetate (Ooshima *et al.*, 1985 cited in Dordick, 1989).

9.2.5 Catalysis of new reactions

Lipases have been shown to catalyse new reactions such as esterifications and aminolysis in organic media. These reactions would not be observed in aqueous solvents where hydrolysis would dominate (Zaks and Klibanov, 1985 cited in Klibanov, 1989).

9.3 Application of hydantoinases in organic media

Water-miscible organic solvents have been used for the hydantoinase reaction (Kim and Kim, 1993). Whole cells of soil-isolated bacterial strain number KBEL 101 were immobilized within polyacrylamide gel, and the immobilized biocatalyst was used for the conversion of D,L-*p*-hydroxyphenylhydantoin to D-*p*-hydroxyphenylglycine in 5% (v/v) DMSO (dimethylsulfoxide). The higher conversion observed with this solvent over all other solvents tested was possibly due to higher substrate solubility; D,L-*p*-hydroxyphenylhydantoin solubility is poor in aqueous solvent. Although maximum hydantoinase catalytic activity was observed in 5% DMF (dimethylformamide), DMSO was

more suitable with regard to the **stability** of the biocatalyst. Experiments were thus conducted in 5% DMSO which afforded a 99% conversion. The rate of conversion declined after 4 successive batch operations, and the authors concluded that although conversion was satisfactory, it would be necessary to improve the operational stability of the immobilized biocatalyst in this particular system.

10. Objectives of the present study

In view of the information in this literature survey, it was concluded that the study of hydantoinase activity in organic solvents at elevated temperatures had potential for an industrial application. Therefore, the isolation of microorganisms with thermostable hydantoin-hydrolyzing activity would be conducted, as well as the characterization of enzyme activity in both water-miscible and water-immiscible organic solvents to determine the most suitable medium with respect to enzymic conversion at elevated temperature.

Based on this, the following objectives were proposed:

- The isolation of thermophilic hydantoin-hydrolysing organisms.

- The characterisation of enzyme activity in the form of crude extract in relation to induction conditions/growth kinetics, assay conditions (for example pH, temperature, incubation time), substrate specificity, and metal ion effects.

•The partial purification of either the hydantoinase enzyme or the carbamylase enzyme from a suitable strain with a view to characterizing crude enzyme activity in water-miscible organic solvents, and non-aqueous media.

•Enhancing the biocatalyst thermostability in organic media, for reactions to produce enantiomerically pure amino acids.

CHAPTER 2: SCREENING FOR THERMOPHILIC HYDANTOINASE-PRODUCING STRAINS

Screening for thermophilic hydantoinase-producing organisms was conducted in order to find thermostable hydantoinase enzymes, and requires that the sources of sampling be characterized by temperatures in excess of 50°C. In this study, such sources included compost heaps, decomposing pine bark, hot springs and sewage sludge. The isolation of hydantoinase-producing organisms was based primarily on induction of enzyme activity in shake flask experiments, and the measurement of the degree of hydantoin conversion to *N*-carbamylglycine (NCG) by whole cells was conducted using a modification of the method reported by Yamada *et al.* (1978) (section 2.1.4, Appendix). In addition, resting cell biocatalytic reaction conditions were varied. Some of the strains were selected for inducer-preference studies, based on the overlay assay results (section 2.2; Morin *et al.*, 1986a), and the growth kinetics for a selected strain were established.

Materials and methods

2.1 Chemicals

Hydantoin, *N*-carbamylglycine (NCG), 5,5-dihydrouracil (DHU) and glycine (GLY) were purchased from Sigma Chemicals. All other reagents were of analytical grade.

2.2 Isolation of hydantoinase-producing strains

A total of 16 isolates were sampled from the sites mentioned above, affording 263 strains, 78 of

which were assayed as in section 2.1.4 (Appendix) for hydantoin-hydrolyzing capability after a satisfactory overlay result.

2.3 Induction of hydantoinase activity in strains

100ml sterile basal medium supplemented with 1% hydantoin as sole nitrogen source was inoculated with a spoonful of isolate, and enriched for 6-24 hours at 55°C on an orbital shaker at 200rpm. The enriched broth was allowed to settle, and 1ml of the broth was diluted in series in 0.85% saline. 100µl aliquots of diluted cells were plated onto basal medium plates supplemented with 1% hydantoin, and incubated at 55°C. The colonies formed were transferred to nutrient agar plates supplemented with 1% hydantoin, and incubated at 55°C for 24-36 hours. The plates were overlaid with agar containing hydantoin and incubated for 6 hours at 55°C. Hydantoin-hydrolyzing activity in the colonies was indicated by the formation of a yellow colour when a drop of acidic 10% *p*-dimethylaminobenzaldehyde solution was placed onto the colonies (Morin *et al.*, 1986a). Colonies with detectable enzyme activity by the overlay test were selected for further study, i.e., induction in nutrient broth or basal medium, supplemented with hydantoin as sole nitrogen source. Resting cell reactions (biocatalytic assays) were then conducted on these strains, using 100mg cells per reaction. Hydantoinase activity was determined colorimetrically as in section 2.1.4, Appendix.

2.4 Plate induction studies using selected hydantoinase-producing strains

Strains RU-20-15, RU-22-5, RU-23-3, RU-27-10, RU-27-15, RU-32-1, RU-32-2, RU-33-13 and RU-34-2 were plated onto nutrient agar plates supplemented with 0.1% hydantoin, thiouracil, or dihydrouracil, and incubated for 24 hours at 55°C. Plates were overlaid with agar containing

hydantoin and incubated for 6 hours at 55°C. The overlay assay was conducted on these plates.

2.5 Growth analysis of RU-32-2

Cells of RU-32-2, cultured in basal medium supplemented 0.1% dihydrouracil, were inoculated into nutrient broth supplemented with 0.1% dihydrouracil to give an initial optical density of 0.02. Optical density readings of cells cultured at 55°C were then taken at 2-hour intervals. The growth analysis was repeated, harvesting cells by centrifugation at 8000rpm for 10 minutes at 4°C, at early log, late log/early stationary, and late stationary phases. These samples of cells were suspended in 0.1M cold potassium phosphate buffer, pH8, using 100mg cells per reaction and assayed in the presence of 50mM hydantoin for 6 hours at 55°C. The NCG and GLY produced were detected by colorimetric methods as in sections 2.1.4 and 2.1.3, Appendix.

2.6 Variation of assay conditions

2.6.1 Scale-down of the resting cell reaction, and use of Tris-HCl buffer.

Aliquots of nutrient broth (10ml) supplemented with 1% hydantoin, were each inoculated with a loopful of cells of RU-21-5, RU-22-6, or RU-KM1, previously cultured on basal medium plates supplemented with 1% hydantoin, and cultures were induced over 16 hours at 55°C. A sample of each culture (1.5ml) was microfuged for 3 minutes. The supernatant was discarded, the pellet resuspended in 0.5ml 0.1M cold Tris-HCl buffer, pH8, and incubated for 4 hours at 55°C in the case of RU-21-5 and RU-22-6, or 40°C for the previously-isolated mesophile RU-KM1, in the presence of 50mM hydantoin (added as 0.5ml 100mM solution). Microcentrifugation afforded a supernatant that was assayed colorimetrically as in section 2.1.4 (Appendix) for NCG production.

2.6.2 Omission of shaking during incubation

Strains RU-20-15 and RU-22-3 were cultured on nutrient agar plates supplemented with 1% hydantoin, overnight at 55°C. Cells were scraped from the plates and suspended in 1.5ml 0.1M cold Tris-HCl buffer, pH8. Microcentrifugation of each culture afforded a pellet that was resuspended in 750µl buffer and washed. Microcentrifugation, resuspension and washing were repeated, and the final pellet was resuspended in 250µl buffer and incubated at 55°C in the presence of 50mM hydantoin (added as an equal volume of 100mM) for 4 hours. After incubation, the samples were microfuged and the supernatant assayed for the presence of NCG as in section 2.1.4, Appendix.

2.6.3 Cell disruption experiments

Nutrient broth (150ml) supplemented with 1% hydantoin was inoculated with RU-20-15 previously cultured on basal medium plates supplemented with 1% hydantoin, and cultures were incubated for 16 hours at 55°C. The cells were harvested by centrifugation at 8000rpm for 10 minutes at 4°C, and resuspended in 0.1M cold potassium phosphate buffer, pH8, using 40mg cells per reaction. The cells suspension was disrupted by up to 3 passes through a French Press (Colinca Scientific Instruments) at 15MPa. 1ml aliquots of extract were assayed at 55°C for 3 hours in the presence of an equal volume of 100mM hydantoin pre-warmed to 75°C. After incubation, centrifugation afforded a supernatant that was assayed for the presence of NCG as in section 2.1.4, Appendix. Protein concentrations were determined by the Bradford method (Bradford, 1976) using Bovine Serum Albumin as a standard (section 2.1.2, Appendix). Specific activity (SA) was expressed as units per milligram protein, where one unit (U) was defined as the amount of enzyme required to produce 1µmol of product in one minute under the specified conditions.

Results

2.3 Induction of hydantoinase activity in strains

Induction of strains was conducted over different lengths of time to determine which strain(s) had hydantoin-hydrolyzing activity.

2.3.1 Induction of hydantionase activity in strains, over 16 hours

The results obtained when selected strains were induced for 16 hours using 1% hydantoin as inducer are shown in Table 2.3.1.

Table 2.3.1 NCG production by selected strains after 16 hours induction

Strain	NCG (μmoles/ml)
RU-20-4	0
RU-20-7	0.67
RU-20-12	0.56 (±0.431)
RU-20-15	1.48 (±0.73)
RU-32-1	0.05 (±.024)

Strain induction was carried out for 16 hours in nutrient broth supplemented with 1% hydantoin. Resting cell assays were conducted over 6 hours at 55°C in the presence of 50mM hydantoin. NCG (*N*-carbamyl glycine). These data represent the mean (±SEM) of triplicate determinations.

2.3.2 Induction of hydantionase activity in strains, over 24 hours

The results obtained when selected strains were induced for 24 hours using 1% hydantoin as inducer are shown in Table 2.3.2.

Table 2.3.2 NCG production by selected strains after 24 hours induction

strain	NCG produced (μmoles/ml)		
	assay time (h)		
	4	**6**	**24**
RU-20-7		0	
RU-20-15		1.1 (±0.067)	
RU-22-6		1.12 (±0.892)	
RU-23-3		4.46 (±0.491)	
RU-27-3		0.13 (±0.076)	
RU-27-4		1.08 (±.036)	
RU-23-4	0.28 (±.0165)		
RU-24-16	0.04 (±0.025)		
RU-27-5	0		
RU-27-5	0		
RU-27-2		1.64 (±0.073)	
RU-27-10	0	7.59 (±0.355)	
RU-27-11	0	0.61 (±0.177)	
RU-27-15	0.1 (±.053)		0.79 (±0.398)
RU-33-2		0.48 (±0.435)	0
RU-33-4		0.18 (±0.124)	0
RU-33-6		0.88 (±0.82)	0

Strains were induced for 24 hours in nutrient broth supplemented with 1% hydantoin except RU27-2, RU27-10 and RU27-11 (induced in basal medium supplemented with 1% hydantoin and assayed for 6 hours). Assays were conducted at 55°C in the presence of 50mM hydantoin for the times indicated. NCG (*N*-carbamylglycine). These data represent the mean (±SEM) of triplicate determinations.

2.3.3 Induction of hydantionase activity in strains, over 36 hours

The results obtained when selected strains were induced for 36 hours using 1% hydantoin as inducer are shown in Table 2.3.3.

Table 2.3.3 NCG production by selected strains after 36 hours induction

strain	NCG produced (μmoles/ml)	
	assay time (h)	
	6	**24**
RU-20-15	1.09 (±0.101)	
RU-22-3	0.34 (±0.19)	
RU-27-1	0.29 (±0.077)	
RU-27-2	0.32 (±0.163)	
RU-34-1	0.42 (±0.211)	
RU-34-2	0	1.01 (±0.582)
RU-34-6		0.14 (±.07)
RU-34-18	0	0.08 (±0.049)
RU-22-5	0	
RU-33-13	0.36 (±0.18)	
RU-27-10	0.27 (±.146)	2.27 (±1.147)
RU-27-15	0.36 (±0.18)	

Strains were induced for 36 hours in nutrient broth supplemented with 1% hydantoin. Assays were conducted at 55°C in the presence of 50mM hydantoin for the times indicated. NCG (*N*-carbamylglycine). These data represent the mean (±SEM) of triplicate determinations.

2.3.4 Induction of hydantionase activity in strains, over 48 hours

The results obtained when selected strains were induced for 48 hours using 1% hydantoin as inducer are shown in Table 2.3.4.

Table 2.3.4 NCG production by selected strains after 48 hours induction

strain	NCG produced (μmoles/ml)		
	assay time (h)		
	4	**6**	**24**
RU-28-1	0.09 (±0.051)		1.37 (±0.688)
RU-33-1		0.12 (±0.078)	0
RU-33-3			0
RU-33-5		0	0
RU-33-7		0	0
RU-33-8		0	0
RU-33-9		0	0
RU-33-10		0.25 (±0.128)	0.1 (±0.052)
RU-33-11		0.64 (±0.322)	
RU-33-12		0.95 (±0.476)	0.62 (±.0312)
RU-33-13		0	0

Strains were induced for 48 hours in nutrient broth supplemented with 1% hydantoin. Assays were conducted at 55°C for the times indicated, in the presence of 50mM hydantoin. NCG (*N*-carbamylglycine). These data represent the mean (±SEM) of triplicate determinations.

2.3.5 Induction of hydantionase activity in strains, over 96 hours

The results obtained when selected strains were induced for 96 hours using 1% hydantoin as inducer are shown in Table 2.3.5.

Table 2.3.5 NCG production by selected strains after 96 hours induction

strain	NCG produced (μmoles/ml)				
	assay time (h)				
	4	**6**	**8**	**24**	**48**
RU-27-2			0.31(±0.17)	1.77(±0.89)	1.97(±0.99)
RU-27-10	1.29(±0.71)		2.38(±1.19)	2.88(±1.45)	
RU-33-14		0		0	

Cell induction was conducted for 96 hours in basal medium supplemented with 1% and 0.01% hydantoin and yeast extract respectively, except for strain RU-33-14 induced in nutrient broth supplemented with 1% hydantoin. Assays were conducted at 55°C for the times indicated, in the presence of 50mM hydantoin. NCG (*N*-carbamylglycine). These data represent the mean (±SEM) of triplicate determinations.

2.4 Plate induction studies using selected hydantoinase-producing strains

A list of 9 strains was compiled from the 78 strains assayed. Strains were selected on the basis of the amounts of NCG produced in whole cell assays. Further inducer studies were conducted on these 9 strains to determine inducer preference. The overlay assay indicated that dihydrouracil was the preferred inducer, especially for the strain RU-32-2, as the colour intensity of NCG produced was greatest for this strain. The growth analysis of RU-32-2 was thus studied to determine the optimal time at which the hydantoin-hydrolyzing enzymes are produced. The data is shown in the following section.

2.5 Growth analysis of RU-32-2

Figure 2.5 depicts the growth kinetics of strain RU-32-2 cultured in nutrient broth supplemented with dihydrouracil, and Table 2.5 shows the variation in enzyme activity over the same period.

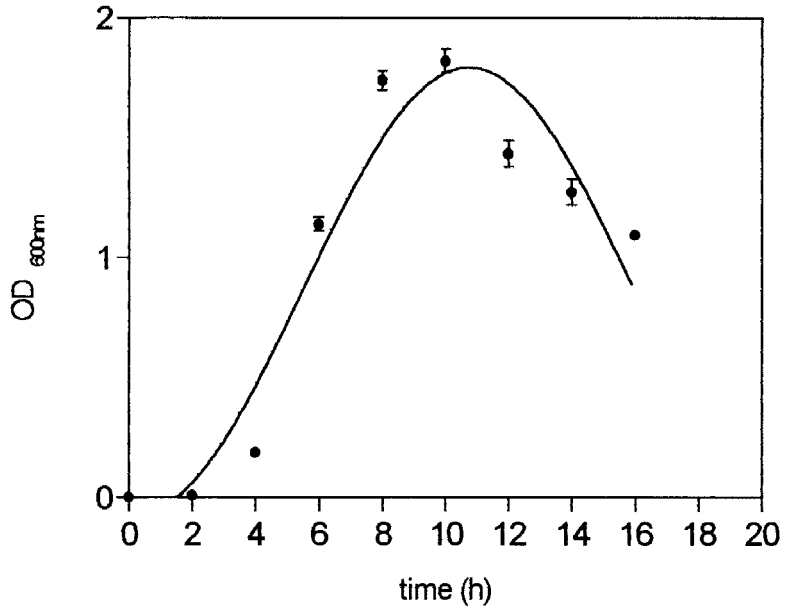


Fig 2.5 Growth kinetics of RU-32-2
Induction was conducted for 16 hours in nutrient broth supplemented with 0.1% dihydrouracil. The substrate used was 50mM hydantoin. These data represent the mean (±SEM) of duplicate determinations.

Table 2.5 NCG and GLY production by RU-32-2 induced with dihydrouracil over 16 hours.

time (h)	NCG(μmoles/ml)	GLY(μmoles/ml)
6	0.25 (±0.228)	0.33 (±0.052)
8	0.91 (±0.865)	0.47 (±0.023)
16	0.39 (±0.154)	0.04 (±0.025)

Induction was conducted for 16 hours in nutrient broth supplemented with 0.1% dihydrouracil. The substrate used was 50mM hydantoin. NCG (*N*-carbamylglycine), GLY (glycine). These data represent the mean (±SEM) of triplicate determinations.

2.6 Variation of assay conditions

Assay conditions were varied in experiments with selected organisms, as it was thought that this may improve conversion.

2.6.1 Scale-down of the resting cell reaction, and use of Tris-HCl buffer.

It was proposed that potassium phosphate buffer be replaced by Tris-HCl buffer as the former may not have been suitable for the enzyme system. The resting cell reaction was scaled-down to allow for the rapid screening of new isolates. The results for the effect of changing the buffer system and scaling-down of the whole cell biocatalytic assay to allow for rapid screening of a large number of isolates, are shown in Figure 2.6.1.

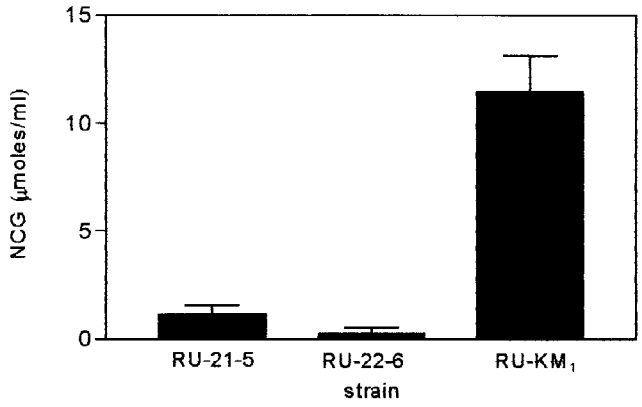


Fig 2.6.1 Hydantoinase activity of selected strains measured in the presence of Tris-HCl buffer using a scaled-down biocatalytic assay
RU-KM1 is a control mesophile assayed at 40°C. NCG (*N*-carbamylglycine). These data represent the mean (±SEM) of triplicate determinations.

2.6.2 Omission of shaking during incubation

It was proposed that shaking of reaction mixture during incubation was inhibitory to the enzyme activity possibly due to high enzyme instability. Therefore enzyme activity was measured in cultures which were not shaken during the resting cell reaction, the results of which are shown in Table 2.6.2.

Table 2.6.2 Biocatalytic assay of cells without shaking.

strain	NCG produced (µmoles/ml)
RU-20-15	2.06 (±0.482)
RU-22-3	0.5 (±0.022)

NCG (*N*-carbamylglycine. These data represent the mean (±SEM) of triplicate determinations.

2.6.3 Cell disruption experiments

It was thought that poor hydantoin-hydrolyzing activity may be due to inaccessibility of the substrate to the enzyme located within the cell, and/or inability of the product to exit the cells. A French Press was used as a means to disrupt cells in order to expose the substrate to the enzyme, or allow for detection of the products. Table 2.6.3 shows the result of substrate conversion by the crude extract of strain RU-20-15.

Table 2.6.3 The effect of cell disruption by French Press on substrate conversion and product detection by strain RU-20-15

Number of passes	NCG produced (μmoles/ml)	Specific activity (μmoles product.mg^{-1}protein.min^{-1})
0	1.36 (±0.09)	0.0062 (±0.0004)
1	1.27 (±0.39)	0.005 (±0.00156)
2	1.21 (±0.07)	0.0043 (±0.00023)
3	1.08 (±0.11)	0.0032 (±0.00036)

NCG (*N*-carbamylglycine). SA (specific activity. These data represent the mean (±SEM) of triplicate determinations.

Discussion

Although large numbers of organisms were screened for hydantoinase enzyme production (Tables 2.3.1 to 2.3.5), the amounts of NCG produced under these conditions were not significantly high.However the data taken together suggest that induction for 24h and assay for hydantoinase activity over a period of 6h generally results in the production of more NCG. Notable examples

are strains RU-23-3 and RU-27-10 (Table 2.3.2). Longer induction times, assay times and/or alteration to the induction medium composition did not appear to significantly improve the enzyme activities. It is proposed that the majority of organisms isolated were actually false positives: seemingly satisfactory hydantoinase-producers in the plate assay, but hydantoinase production in broth was poor.

The strains isolated were indeed thermophilic, but the hydantoinase activity detected was very low. Two strains, RU-23-3 and RU-27-10 appeared to be suitable producers of the hydantoinase enzyme, but the hydantoinase activity detected was not reproducible during experiments. It is proposed that the use of high temperatures resulted in the loss of plasmids carrying genes encoding hydantoinase enzymes, and hence the loss of these strains. It is suggested that initial enrichment at 55°C be for longer (1-2 weeks). Plates with cells from enrichment could be incubated at 55°C for several days to allow for colony development. The medium for enrichment could be modified by supplementation with cell-growth inducing compounds that would not be nitrogen sources. Hydantoin would be the only nitrogen source incorporated to select for organisms capable of its hydrolysis. It is acknowledged that hydantoin was the only screening substrate (inducer) used, and a variety of these could have been tested.

Strains were selected based on the results observed in overlay assays, and growth kinetics studies on one of these stains, RU-32-2, showed that induction in 1% dihydrouracil for 8-9 hours was optimal (Figure 2.5 and Table 2.5). After this time, optical density declined sharply. The data in Figure 2.5 is consistent with the literature in that hydantoin-hydrolyzing enzymes are generally

produced by cultures in the early stationary phase of growth. Induction of strains during isolation was for at least 16 hours, which resulted in harvesting of cells that were well into decline phase for resting cell reactions. The poor hydantoin-hydrolysing activity observed could thus be attributed to late harvesting of the cultures.

Assay conditions were varied with the aim of identifying conditions that would significantly improve hydantoin conversion to NCG (*N*-carbamyl glycine).The hydantoinase activity of a previously isolated and assayed mesophile, RU-KM1 was satisfactorily reproducible under the varied conditions (Figure 2.6.1). The use of Tris-HCl buffer as an alternative to potassium phosphate buffer did not have any effect on RU-KM1 activity, and thus potassium phosphate buffer was not considered to be limiting to the enzyme activity of the isolates. The resting cell reaction was successfully scaled-down for screening of a large number of isolates, and the result obtained using the organism RU-KM1as an internal control demonstrated the applicability of this method.The microtitre plate assay (Chien and Hsu, 1996) appears to be a superior technique for rapid screening owing to less time consumed, the larger number of strains that can be tested and sensitivity, and could be applied.

Auxostats or chemostats for continuous enrichment and cultivation of desirable strains independent of the initial population could be used. A number of selective pressures (temperature, medium composition) could be simultaneously applied, a disadvantage being that months of continuous cultivation may be required.

Omission of shaking during resting cell reactions did not significantly improve hydantoin conversion by new isolates (Table 2.6.2), and thus poor conversion was not attributable to enzyme inhibition by shaking during assaying. Therefore shaking was included in the subsequent assays for enzyme activity.

It appeared that cell disruption by French Press did not significantly improve the enzyme-substrate contact, or detection of product liberated (Table 2.6.3). A decrease in NCG production was observed with the number of passes, and this may be due to hydantoinase enzyme instability.

Conclusion

The results of the screening were considered to be unpromising as the amounts of hydantoin converted to NCG were very low. This was the case even when resting cell reaction conditions were varied. It is suggested that low activity observed is due to an innate low enzyme exhibited by the strains isolated.

The approach taken would not easily fulfil the objective of obtaining thermostable hydantoin-hydrolyzing enzymes. An alternative line of research involved enhancing the thermostability of hydantoinase and carbamylase enzymes of a previously-isolated mesophilic organism by altering reaction medium composition. It was envisaged that the conditions under which the hydantoinase and carbamylase enzymes of a crude extract of a mesophile would hydrolyse substrate could be optimized, and compared with respect to substrate specificity and thermostability, in particular,

with those obtained in non-aqueous systems.

CHAPTER 3: OPTIMIZATION OF BIOTRANSFORMATION CONDITIONS FOR CRUDE EXTRACT OF RU-KM3$_L$

In the screening for thermophilic bacterial organisms capable of hydantoin hydrolysis, the isolates obtained showed low levels of conversion compared with mesophiles previously characterized in our laboratory. Therefore, a previously-isolated mesophilic strain, RU-KM3$_L$, was selected after comparison of hydantoinase activity at elevated temperatures with other mesophiles. The current chapter deals with the optimization of reaction conditions for RU-KM3$_L$ crude extract, with the objective of enhancing the hydantoinase activity of RU-KM3$_L$ for application at elevated temperatures.

Materials and methods

3.1 Chemicals, microorganism, crude extract preparation, and enzyme activity assay

3.1.1 Chemicals

Hydantoin, 5,5-dimethylhydantoin (DMH), 5,5-dihydrouracil (DHU), 5-thiouracil (TU), *N*-carbamylglycine (NCG), *N*-carbamyl-D,L-alanine (NCA), glycine (GLY), and D,L-alanine (ALA) were purchased from Sigma Chemicals. 5-methylhydantoin (MH) was synthesized by the Bucherer-Bergs method (section 3, Appendix). D, L-*p*-hydroxyphenylhydantoin (*p*HPH) and *N*-carbamyl-D,L-hydroxyphenylglycine (NCHPG) were synthesised as reported by Ohashi *et al.* (1981). All other reagents were of analytical grade.

3.1.2 Microorganism and culture conditions

A mesophilic Gram negative rod was identified as *Pseudomonas putida* through biochemical analysis at the SAIMR, Johannesburg, South Africa, and genetically by Matcher (1997). The strain was desiganted the name RU-KM3$_L$, and was initially isolated as a mixed culture from soil (MacLeod, 1994). Purification and initial characterisation of this was conducted by Gardner (1995), and Hartley (1995). The culture medium consisted of nutrient broth or basal medium supplemented with inducer at different concentrations. The basal medium (hydantoin minimal medium) was prepared as in section 1.1, Appendix. Induction medium (100ml) in 500ml volumetric flasks was inoculated either from a seed (starter) culture at stationary phase in basal medium , or by a loopful of cells cultured on basal medium plates. The seed culture was used to inoculate nutrient broth to give an initial cell optical density (600nm) of 0.02. Induction was carried out with shaking at 200rpm on an orbital shaker at 25°C.

3.1.3 Preparation of crude enzyme extract

Cells were harvested after induction (as above) for a specific length of time, and the medium was removed by centrifugation at 8000rpm for 10 minutes at 4°C. Cells were resuspended and washed twice in cold buffer. The cells of the final pellet were suspended in buffer, and disrupted by passing once through a French Press cylinder at 15MPa at 0°C. The extract was made up to 1/4 of the induction volume, and was used in this form unless otherwise specified.

3.1.4 Assay of enzyme activity

Enzyme activity determination was carried out on the supernatant of the reaction mixture.The method

to determine hydantoinase activity was based on that reported by Yamada *et al* (1978) (section 2.1.4, Appendix). *N*-carbamylase activity was determined by the Ninhydrin method for amino acid quantitation as described in section 2.1.3, Appendix. Protein concentrations were determined by the Bradford method using Bovine Serum Albumin as a standard (as described in section 2.1.2, Appendix).

The specific activity (SA) was expressed as units per milligram of protein, where one unit (U) was defined as the amount of enzyme required to produce 1μmol of product in one minute under the specified conditions. The data is presented as specific activity, unless otherwise stated.

3.2 Comparison of hydantoinase and carbamylase activities in mesophiles

3.2.1 Conversion of hydantoin by mesophilic strains, at 40°C

A loopful of each of the mesophiles RU-KM3$_L$, RU-KM1, RU-KM3$_S$, RU-184$_S$, RU-OGB and RU-OR from a culture collection were grown on basal medium plates supplemented with 1% hydantoin at 25°C, and inoculated into 200ml nutrient broth supplemented with 0.1% hydantoin. Induction was carried out for at least 24 hours at 25°C. The cells were harvested by centrifugation and resuspended in 15ml 0.1M cold potassium phosphate buffer, pH8. The protease inhibitor PMSF (phenylmethane sulfonyl fluoride) was added to the extract to a final concentration of 1mM prior to disruption by French Press. The crude extract obtained was made up to 25ml with buffer. 1ml aliquots of each extract were incubated with an equal volume of 100mM hydantoin and PMSF at 1mM concentration for 1 hour at 40°C. The remainder of the crude extract was frozen at -40°C. NCG (*N*-carbamyl

glycine), GLY (glycine) and protein concentration were determined as in section 3.1.4.

3.2.2 Substrate conversion by mesophiles at elevated temperatures

Samples of each mesophile extract that had been frozen at -40°C were thawed and 1ml aliquots were incubated with an equal volume of 100mM hydantoin. PMSF was added to a final concentration of 1mM. Samples of the reaction mixtures were incubated for 1 hour at 40, 45, 50 or 55°C respectively. NCG, GLY and protein concentrations were determined as in section 3.1.4.

3.3 The effect of PMSF on enzyme activity of RU-KM3$_L$ crude extract

A stationary phase seed culture of RUKM3$_L$ cultured at 25°C was inoculated into 200ml nutrient broth supplemented with 0.1% hydantoin. Cells were harvested at early stationary phase by centrifugation and resuspended in 15ml 0.1M cold potassium phosphate buffer, pH8. Cell disruption was effected by French Press, and the crude extract was made up to 25ml with buffer. 1ml portions of the extract were incubated with equal volumes of 100mM hydantoin for 1h in the presence and absence respectively of 1mM PMSF. NCG, GLY and protein concentration were determined as in section 3.1.4.

3.4 Growth kinetics for RU-KM3$_L$

Stationary phase seed culture, obtained as in section 3.3, was inoculated into 100ml nutrient broth or basal medium supplemented with 1% hydantoin (so that the amount of inducer would not be limiting over the induction time), to give an initial optical density of 0.02. At certain time intervals, cells were harvested by centrifugation and resuspended in 7.5ml 0.1M cold potassium phosphate

buffer, pH8. The French Press was used to disrupt the cells and the crude extract made up to 12.5ml with buffer. 1ml portions of the extract were assayed by addition of an equal volume of 100mM hydantoin for an hour at 50°C. NCG, GLY and protein concentration were determined as in section 3.1.4.

3.5 Substrate specificity of the crude extract of strain RU-KM3$_L$

RU-KM3$_L$ was cultured to early stationary phase in basal medium as in section 3.3, and this culture was inoculated in to 200ml nutrient broth supplemented in 1% hydantoin to give an initial optical density of 0.02. Cells were induced to early stationary phase, harvested by centrifugation and resuspended in 15ml 0.1M cold potassium phosphate buffer, pH8. The cells were disrupted by French pressing, and the crude extract was made up to 25ml with buffer. 1ml portions of the extract were incubated with equal volumes of 30mM *p*-HPH, or 100mM hydantoin, or 100mM 5-methylhydantoin (MH) for 1h at 50°C.The concentrations of the respective products and protein were determined as in section 3.1.4.

3.6 Investigation of inducers on RU-KM3$_L$ hydantoinase and carbamylase activity

RU-KM3$_L$ seed culture grown to early stationary phase as in section 3.3 was used to inoculate 100ml nutrient broth supplemented with 0.01, 0.1, or 1% hydantoin, 5,5-dihydrouracil, 5,5-dimethylhydantoin, or 5-thiouracil to give an initial optical density of 0.02. Induction was continued to early stationary phase, and cells were harvested by centrifugation. The cells were resuspended in 7.5ml 0.1M cold potassium phosphate buffer, pH8. Disruption of cells was effected by French pressing, and 1 ml portions of the extract were incubated with an equal volume of 100mM hydantoin

or MH for an hour at 50°C. The concentrations of the respective products and protein were determined as in section 3.1.4.

3.7 Optimization of the time for hydantoinase and carbamylase assays

A loopful of cells which had been previously cultured on basal medium plates at 25°C, was inoculated into 300ml nutrient broth supplemented with 1% hydantoin. Induction was continued to early stationary phase. Cells were harvested by centrifugation and suspended in 22.5ml cold 0.1M potassium phosphate buffer, pH8 prior to disruption by French Press. The crude extract was made up to 37.5ml with buffer and 1ml portions were incubated with equal volumes of 100mM MH at 50°C. Samples were taken at specific time intervals for determination of the concentrations *N*-carbamylalanine (NCA), alanine (ALA) and protein as in section 3.1.4.

3.8 The pH profile for activity of hydantoinase and carbamoylase enzymes in RU-KM3$_L$ crude extract

A loopful of cells which had been previously cultured at 25°C on basal medium plates was inoculated into 500ml nutrient broth supplemented with 1% hydantoin. Cells were harvested by centrifugation after induction to early stationary phase, and washed with 0.05M cold potassium phosphate buffer at pH7. Cells were resuspended in 37.5ml deionized water, and disrupted by French pressing. The extract was made up to 62.5ml with deionized water. 1ml protions of the extract were immediately incubated with an equal volume of 100mM MH in 0.1M citric acid/ trisodium citrate buffer (pH4-5), potassium phosphate buffer (pH6-8), Tris/ HCl buffer (pH7-9) or sodium hydrogen carbonate/ disodium carbonate buffer (pH9-11) for 3 hours at 50°C. Assays were carried out as in section 3.1.4 to determine the concentrations of NCA and ALA produced, and the concentration of protein.

3.9 The effect of the presence of metal ions on the hydantoinase and carbamylase activity of RU-KM3$_L$ crude extract

Cells were cultured on basal medium plates as before, inoculated into 800ml nutrient broth supplemented with 1% hydantoin, and induced to early stationary phase. Cells were harvested by centrifugation and resuspended in 60ml 0.05M cold Tris/ HCl buffer, pH9, then disrupted by French Press and the extract made up to 100ml with buffer. 1ml aliquots were assayed for hydantoinase and carbamylase activity for 3 hours at 50°C in the presence of 100mM MH as substrate. The remainder was dialyzed (SpectraPor® dialysis tubing, MWCO 6-8000) against 1L 0.05M Tris-HCl buffer containing 1mM EDTA, for 3 hours at 4°C, changing the buffer every hour. The dialyzed extract was assayed for MH conversion using 1ml aliquots, in the absence or presence of various metal ions at 1mM concentration. The sulphate salts of calcium, copper, sodium, magnesium, potassium, and the chloride salts of cobalt, manganese and nickel were used. Product and protein concentrations were determined as in section 3.1.4.

3.10 The effect of dialysis conditions on the effects of metal ions on RU-KM3$_L$ crude extract

Crude extract was prepared as described in section 3.9 and used as in the following sub-sections:

3.10.1 Dialysis of crude extract followed by assay in the presence of various metal ions

The crude extract was dialysed against 0.1M Tris-HCl buffer, pH9 for 3h at 4°C with changing of buffer every hour. 1ml portions of the dialysed extract were incubated in the presence of 50mM methylhydantoin and various metal ions. The concentrations products and protein were determined as described in section 3.1.4.

3.10.2 Incubation of crude extract followed by assay in the presence of various metal ions

The crude extract was incubated on a gel rocker at 4°C for 3h, after which 1ml portions of the extract were incubated for 1h at 50°C in the presence of 50mM methylhydantoin and various metal ions. The concentrations products and protein were determined as described in section 3.1.4.

3.10.3 Incubation of crude extract at 4°C in the presence of EDTA, followed by assay in the presence of various metal ions

Crude extract was incubated at 4°C on a gel rocker in the presence of 1mM EDTA (ethylenediaminetetraacetic acid) for 3 hours. The extract was then dialysed against 0.1M Tris-HCl buffer, pH9 for 3h at 4°C with changing of buffer every hour. 1ml portions of the dialysed extract were incubated in the presence of 50mM methylhydantoin and various metal ions. The concentrations products and protein were determined as described in section 3.1.4.

Results

3.2 Comparison of hydantoinase and carbamylase activities in mesophiles

3.2.1 Conversion of hydantoin by mesophilic strains, at 40°C

A comparison of the specific enzyme activities measured for the bacterial strains RU-KM3$_L$, RU-KM1, RU-KM3$_S$, RU-184$_S$, RU-OGB and RU-OR, in the conversion of hydantoin to NCG and GLY is shown in Figure 3.2.1

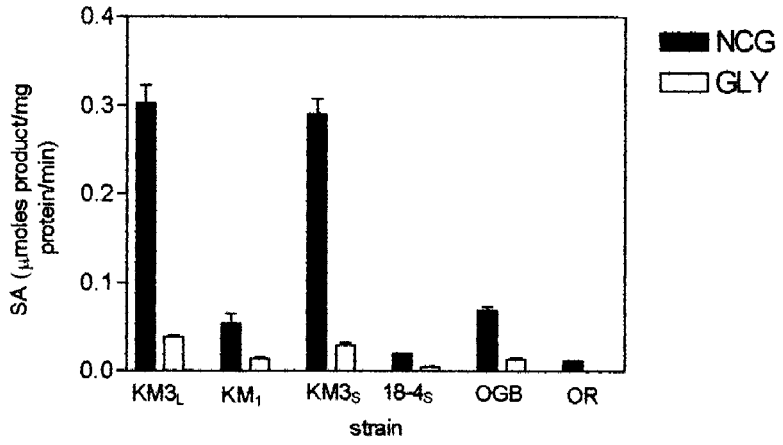


Figure 3.2.1 Specific activities of hydantoinase and carbamylase enzymes of mesophiles
Substrate conversion at 40 °C. NCG (*N*-carbamylglycine), GLY (glycine). The data represent the mean±SEM of triplicate determinations

3.2.2 Substrate conversion by mesophiles at elevated temperatures

The hydantoinase and carbamylase activities in previously-frozen extracts of RU-$KM3_L$, RU-KM1, RU-$KM3_S$, RU-184_S, RU-OGB and RU-OR were compared over the 40-55 °C temperature range Specific activity data for conversion of substrate by previously-frozen crude extracts of mesophiles at elevated temperatures are shown in Figure 3.2.2.

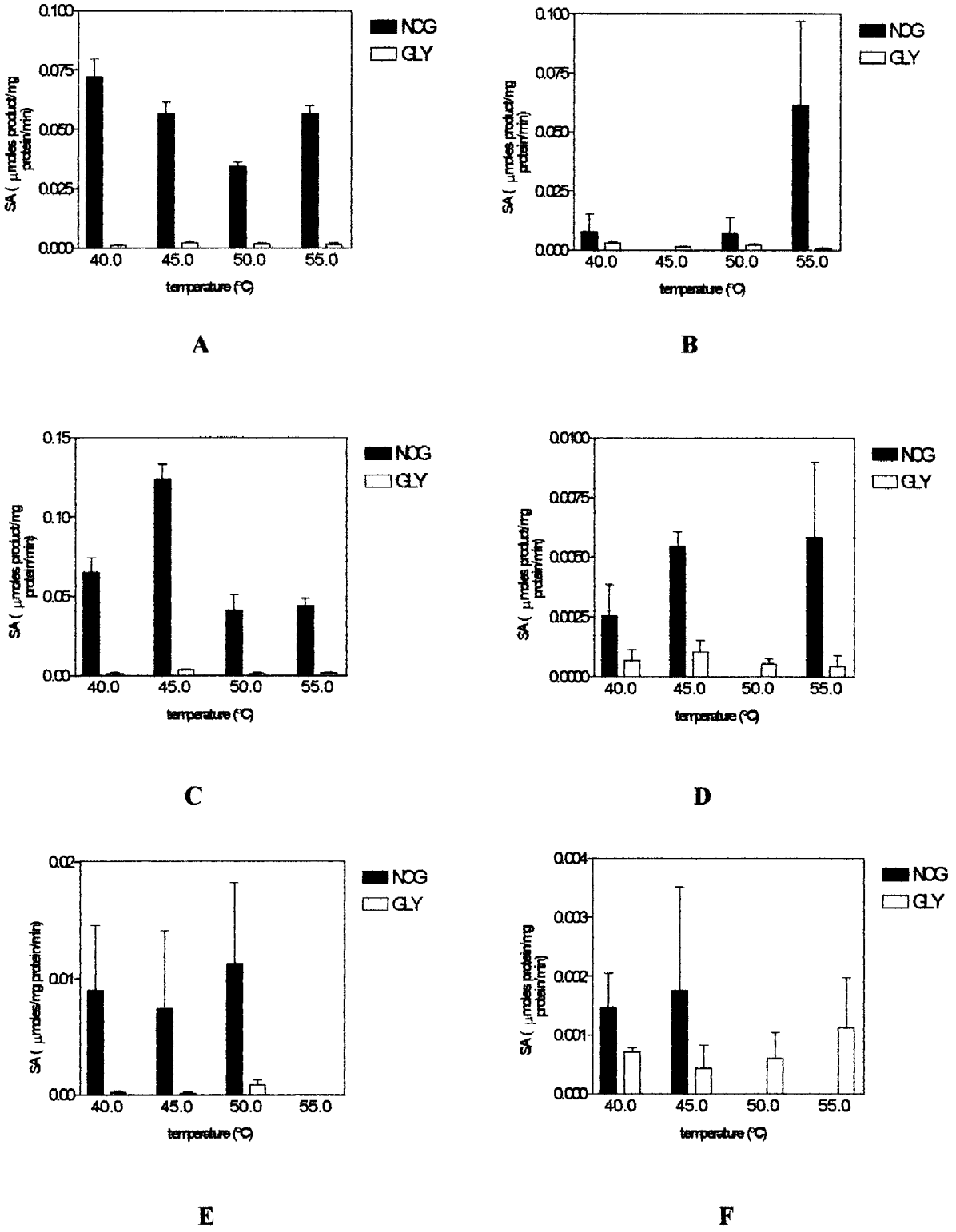
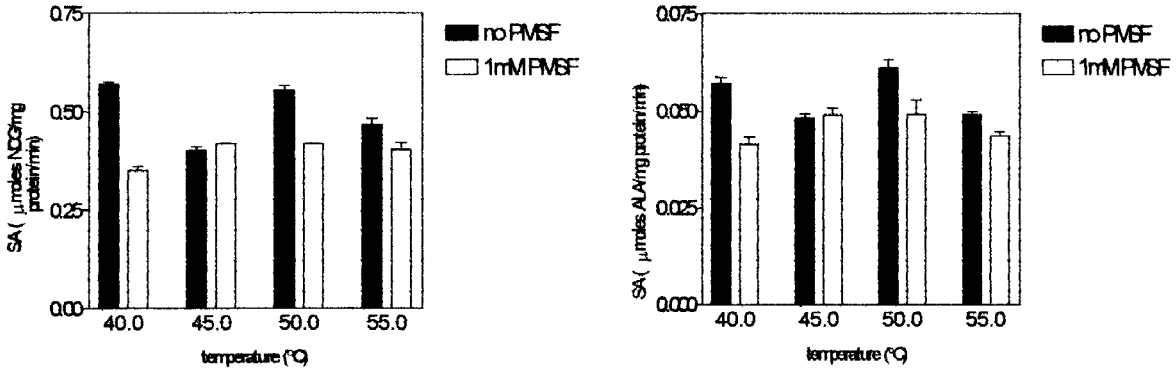


Figure 3.2.2 Conversion of hydantoin by mesophile cell extracts over a 40-55 °C temperature range (A) RU-KM3$_L$, (B) RU-KM1, (C) RU-KM3$_S$, (D) RU-184$_S$, (E) RU-OGB and (F) RU-OR. Extracts previously frozen at -40 °C. NCG (*N*-carbamylglycine), GLY (glycine). The data represent the mean±SEM of triplicate determinations

3.3 The effect of PMSF on enzyme activity of RU-KM3$_L$ crude extract

It was necessary to determine if the enzyme system was prone to inhibition by protease. The effect of incubation of crude extract in the presence or absence of PMSF is shown in Figure 3.3.



A B

Figure 3.3 The effect of PMSFon enzyme activity of RU-KM3$_L$ crude extract
(A) hydantoinase activity (B) carbamylase activity. PMSF at 1mM. NCG (*N*-carbamylglycine), GLY (glycine). The data represent the mean±SEM of triplicate determinations

3.4 Growth kinetics for RU-KM3$_L$

Growth kinetics would be necessary to determine the optimum time to harvest cells for maximum enzyme induction. This study was conducted in nutrient broth and in basal medium as a comparison. The results for growth kinetics of RU-KM3$_L$, and the enzyme activities monitored over the growth period are shown in Figure 3.4 (a) and Figure 3.4 (b) respectively.

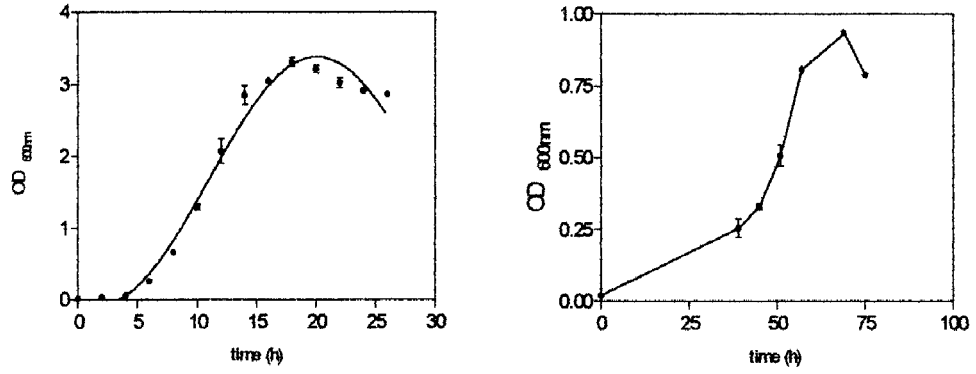


Figure 3.4a Growth kinetics for RU-KM3$_L$
(A) nutrient broth and (B) basal medium. The data represent the mean±SEM of duplicate determinations

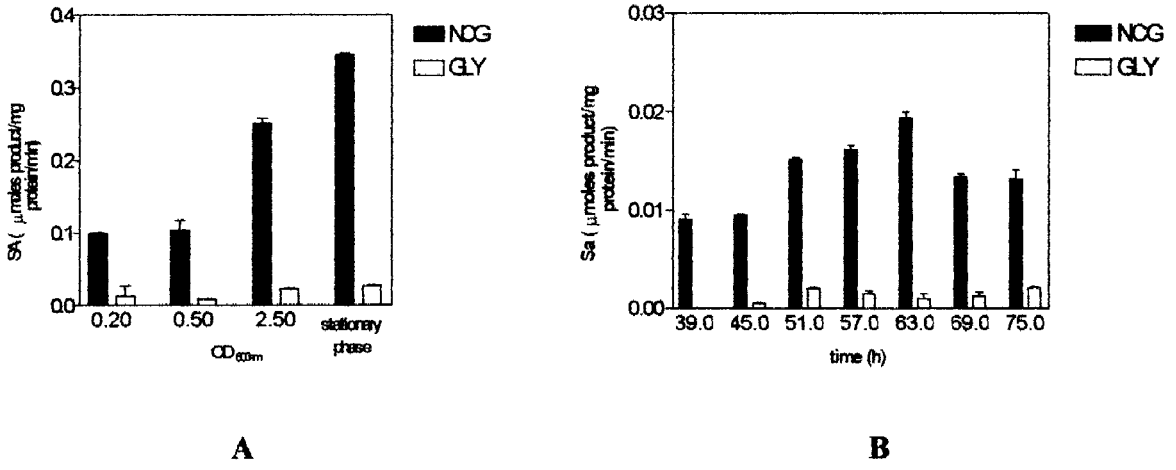


Figure 3.4b Enzyme activities in RU-KM3$_L$ crude extract
(A) nutrient broth and (B) basal medium. NCG (*N*-carbamylglycine), GLY (glycine). The data represent the mean±SEM of triplicate determinations

3.5 Substrate specificity of the crude extract of strain RU-KM3$_L$

Studies on the substrate specificity of RU-KM3$_L$ crude extract were conducted, and the result is illustrated in Figure 3.5.

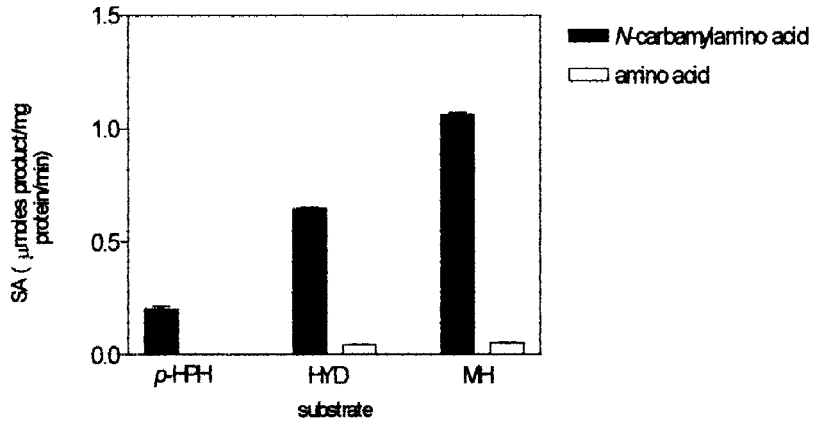


Figure 3.5 Specific activity for the crude extract of RU-KM3$_L$ using different substrates.
Substrates *p*-HPH (*p*-hydroxyphenylhydantoin), HYD (hydantoin), MH (5-methylhydantoin) with *N*-carbamylamino acids NCHPG (*N*-carbamylhydroxyphenylglycine), NCG (*N*-carbamylglycine) and NCA (*N*-carbamylglycine) respectively. Amino acids are HPG (hydroxyphenylglycine), GLY (glycine) and ALA (alanine) respectively. The data represent the mean±SEM of triplicate determinations

3.6 Investigation of inducers on RU-KM3$_L$ hydantoinase and carbamoylase activity

The effect of different inducers on the conversion of various substrates by RU-KM3$_L$ specificity is shown in Figure 3.6.

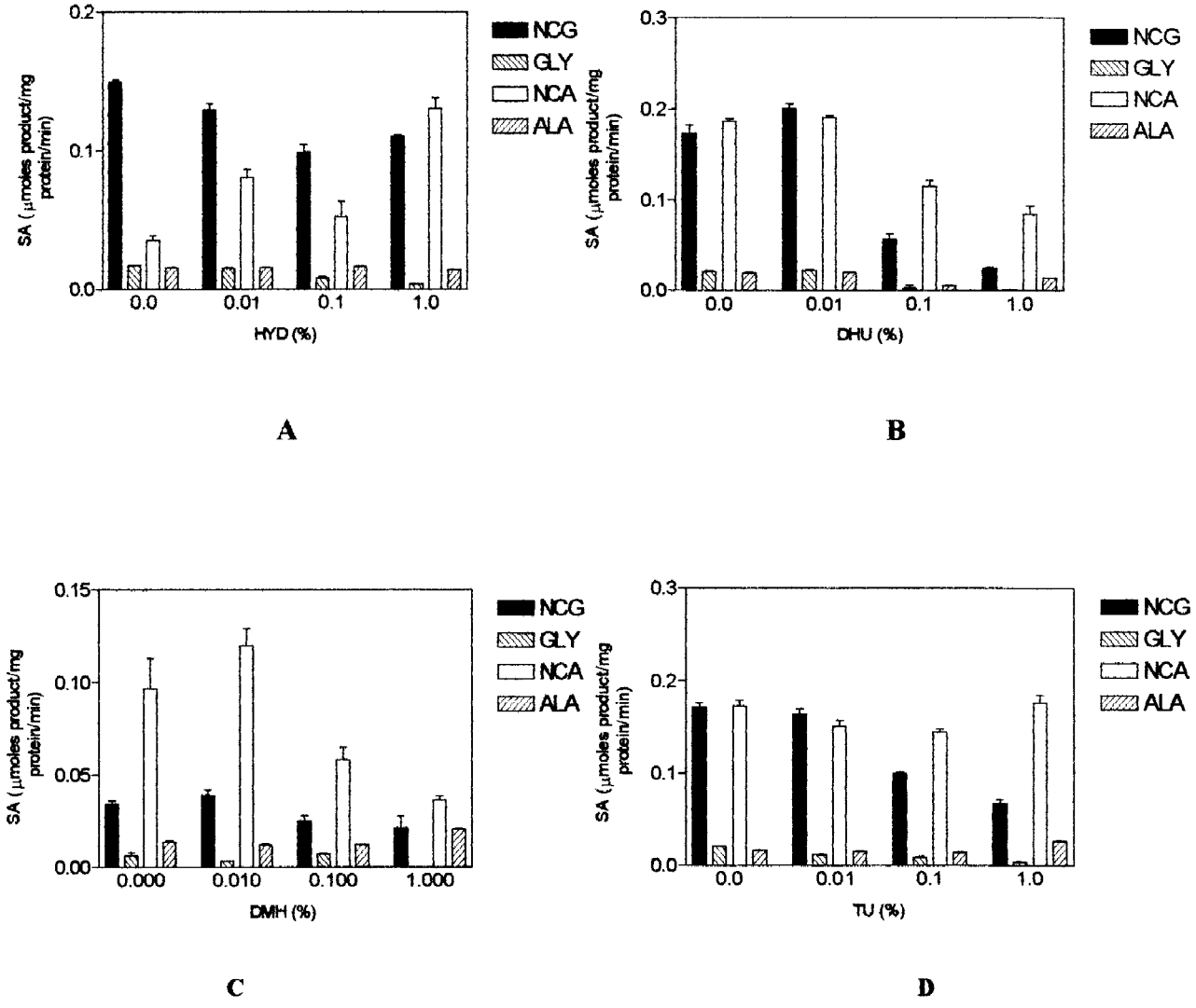


Figure 3.6 The effect of inducers on substrate conversion by the crude extract of RU-KM3$_L$.
Inducers (A) HYD (hydantoin), (B) DHU (5,5-dihydrouracil), (C) DMH (5,5-dimethylhydantoin), (D) TU (5-thiouracil). Substrates HYD (hydantoin), MH (5-methylhydantoin). Products NCG (*N*-carbamylglycine), NCA (*N*-carbamylalanine), GLY (glycine), ALA (alanine). The data represent the mean±SEM of triplicate determinations

3.7 Optimization of the time for hydantoinase and carbamylase assays

Optimization of the time for assay of the hydantoinase and carbamylase enzymes in crude extract was conducted, and MH was used because it was previously shown to be the preferred substrate (result of section 3.5).The results used to determine the optimal time for assay of conversion of MH by crude extract are shown in Figure 3.7.

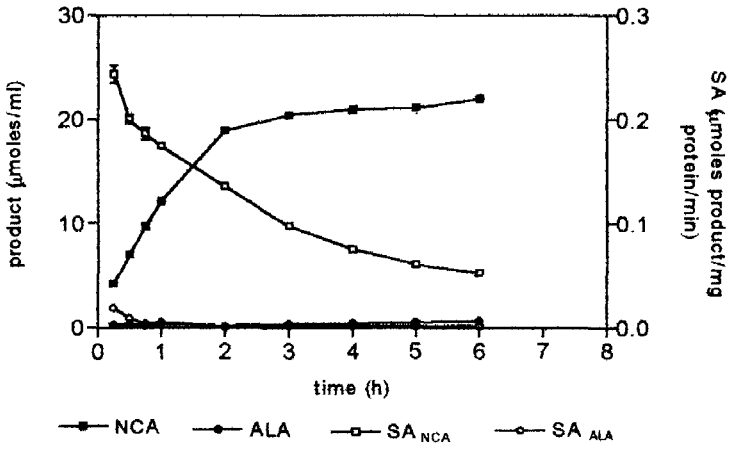


Figure 3.7 Conversion of MH by RU-KM3$_L$ crude extract over a 6h period.
NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean±SEM of triplicate determinations.

3.8 The pH profile for activity of hydantoinase and carbamylase enzymes in RU-KM3$_L$ crude extract

The pH profile for the hydantoinase and the carbamylase enzymes were determined using different buffer systems, with a view to using a buffer system that would not compromise enzyme activity, nor promote precipitation of metal ions in the ensuing metal ion studies. The pH profile of both enzymes of the crude extract of RU-KM3$_L$ is shown in Figure 3.8.

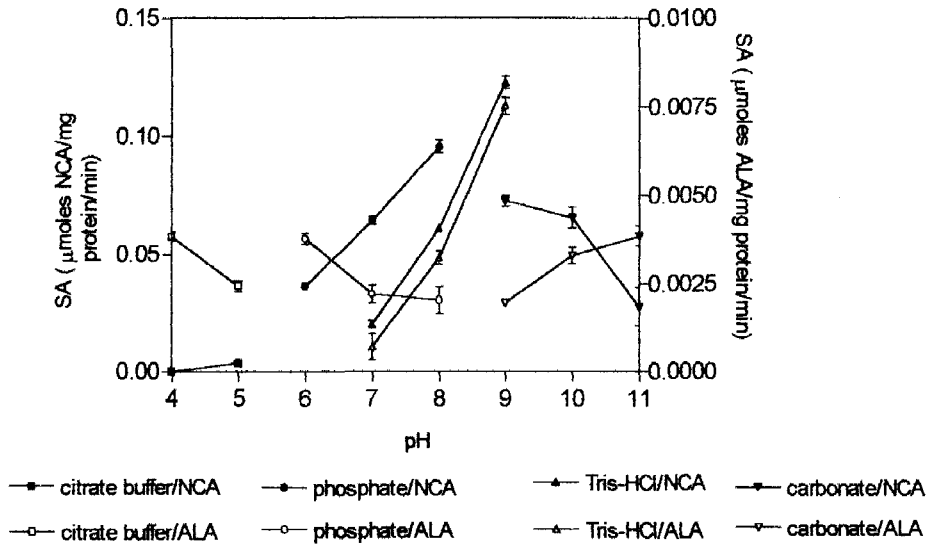


Figure 3.8 pH profiles for the hydantoinase and carbamylase enzymes of RU-KM3$_L$ crude extract. NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean±SEM of triplicate determinations

3.9 The effect of the presence of metal ions on the hydantoinase and carbamylase activity of RU-KM3$_L$ crude extract

The selection of a suitable buffer system, namely Tris-HCl, with respect to enzyme activity allowed for the study of the effects of metal ions on hydantoinase and carbamylase activities in the crude extract of RU-KM3$_L$, and the results are shown in Figure 3.9

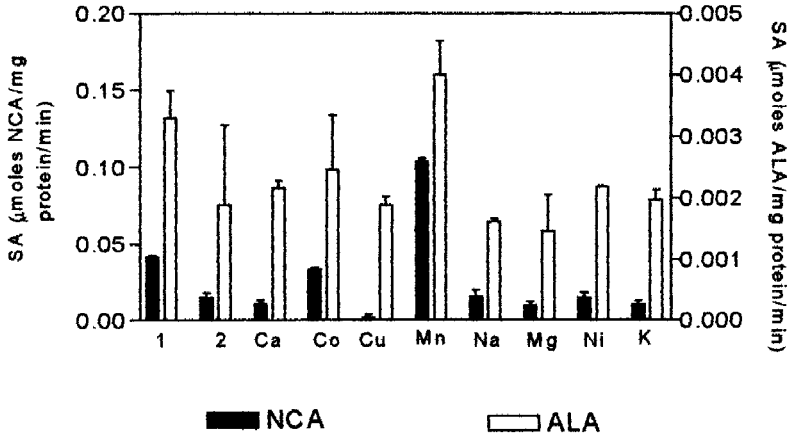


Figure 3.9 The effect of the presence of metal ions on hydantoinase and carbamylase enzyme activities in RU-KM3$_L$ crude extract

(1) before dialysis (2) after dialysis and in the absence of metal ion. The data represent the mean±SEM of triplicate determinations

3.10 The effect of dialysis conditions on the effects of metal ions on RU-KM3$_L$ crude extract

In addition to the establishing the effect of dialysis conditions on hydantoinase and carbamylase activities in the presence of metal ions, the possibility of competition for binding sites between Mn^{2+}, Co^{2+} and Cu^{2+} ions was investigated, and the result is shown in Figure 3.10.

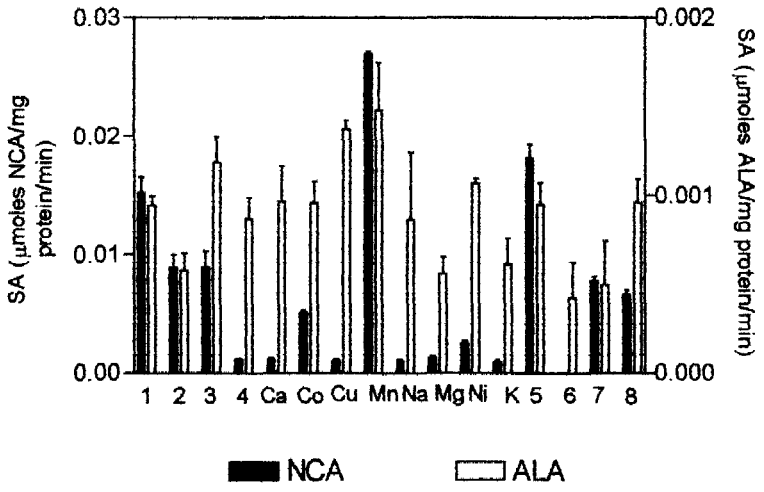


Figure 3.10 The effect of dialysis conditions on hydantoinase and carbamylase activities of RU-KM3$_L$ crude extract in the presence of metal ions
(1) before treatment (2) after dialysis at 4 °C (3) after incubation at 4 °C (4) after dialysis and incubation at 4 °C, and in the absence of metal ion (5) Co and Mn (6) Cu and Co (7) Cu and Mn (8) Co, Cu and Mn ions. The data represent the mean±SEM of triplicate determinations

Discussion

The specific activity of the hydantoinase enzyme of fresh extract of RU-KM3$_L$ was high (about 0.3U/mg) and comparable with that of the strain RU-KM3$_S$ (Figure 3.2.1). Figure 3.2.2A shows that the specific activities of the hydantoinase enzyme and the carbamylase enzyme in previously-frozen RU-KM3$_L$ extract did not change significantly over the entire temperature range.Both enzyme activities in RU-KM3$_L$ were relatively high at 55 °C. RU-KM3$_L$ was selected for further studies based on these observations.

Comparison of Figures 3.2.1 and 3.2.2 shows that a considerable amount of activity was lost in extracts of all strains, probably as a consequence of the freezing and thawing. It was therefore decided

to use fresh extract of RU-KM3$_L$ for subsequent biocatalysis studies.

PMSF was added at 1mM concentration in the previous experiments (section 3.2) as a cautionary measure against degradation by protease. Owing to the instability of PMSF in aqueous solution (Bollag *et al.*, 1996) and thus the need to constantly treat the extract with the protease inhibitor, it was necessary to determine whether the enzyme system was prone to inhibition by proteases. The result in Figure 3.3 suggests that 1mM PMSF did not improve the conversion of hydantoins by inhibition of protease. The lower activities observed in the presence of PMSF may be attributed to inhibition of the enzymes by the PMSF, since these enzymes function by a hydrolysis mechanism which may be similar to that of proteases. This lowering of activity was more marked at the PMSF concentration used, namely, 1mM as opposed to 0.1mM reported in the literature (Runser and Meyer, 1993; Lee *et al.*, 1994), and the optimization of PMSF concentration over the 0.1 to 1mM concentration range (Bollag *et al.*, 1996) would have been beneficial. Since the PMSF effect is neither significantly advantageous nor disadvantageous, it was not added in preparing extracts subsequently. The relatively high activity of the hydantoinase enzyme at 50°C justified subsequent assays at that temperature.

The culture conditions for growth of RU-KM3$_L$ cells were studied, and the growth kinetics of RU-KM3$_L$ were compared in nutrient broth and basal medium. Nutrient broth supplemented with 1% hydantoin was found to be suitable as an induction medium since much higher hydantoinase enzyme specific activity (0.35U/mg) was observed in this case as compared to activities measured after induction in basal medium (Figure 3.4b). Maximum optical density was observed after 18 hours

induction in nutrient broth (Figure 3.4a), although enzyme activities were observed in extracts prepared after 26 hours induction (Figure 3.4b) in the same medium. It was for this reason that subsequent harvesting was carried out at early stationary phase (maximum optical density was observed after 16-18 hours induction); the optical density would reach its maximum when the highest cell mass was reached, so that enzyme stability and activity are not compromised.

Experiments on the time required for culture of RU-KM3$_L$ cells in basal medium showed that 69 hours would be optimal with respect to obtaining maximum optical density (Figure 3.4a), and 63 hours was optimal for obtaining the highest hydantoinase activity in the cell extracts (Figure 3.4b). Conversion of hydantoin in extracts obtained from cells cultured in basal medium was generally low, and therefore culturing of cells in inducer-supplemented nutrient broth was the method of choice for subsequent experiments.

Studies were conducted on the substrate selectivity of the hydantoinase and carbamylase enzymes in crude extract of RU-KM3$_L$. It is clear from Figure 3.5 that 5-methylhydantoin was the preferred substrate, followed by hydantoin and then, *p*-hydroxyphenylhydantoin. Poor utilization of *p*-HPH may be attributed to its poor solubility in aqueous medium, and hence biocatalytic assays with *p*-HPH as substrate in aqueous medium were discontinued.

Inducer studies were conducted to determine the best inducer and the effects of the inducer on substrate specificity. Results shown in Figure 3.6 suggested that lowered activity of the hydantoinase enzyme could be concentration-dependent where DHU (Figure 3.6B) DMH (Figure 3.6C) and TU

(Figure3.6D) were used as the inducers. Both HYD and MH conversion were decreased with increase in concentration of these inducers except when TU was the inducer; in this case only HYD conversion was decreased (Figure3.6D). The use of HYD as inducer seemed to increase the hydantoinase activity when MH was the substrate (Figure3.6A). Reduced activity of the carbamylase enzyme was concentration dependent for all inducers in the presence of HYD as substrate (Figure 3.6A). No significant effect on the carbamylase enzyme was observed when MH was used as the substrate for any of the inducers. At higher concentrations (0.1-1%) of the inducers HYD (Figure 3.6A), DHU (Figure 3.6B) and TU (Figure3.6D), MH was observed to be the preferred substrate. This was also observed over the entire concentration range for DMH as inducer. Preference for 5-methylhydantoin over hydantoin, when the inducer concentration was increased, suggests that the substrate specificity may be controlled by altering concentration of the inducers hydantoin, 5,5-dihydrouracil and 5-thiouracil. It is proposed that higher concentrations of these inducers may promote a change in the enzyme conformation so as to switch substrate preference from hydantoin to 5-methylhydantoin.

It may be proposed that RU-KM3$_L$ hydantoinase activity need not be induced, i.e., that the enzymes are constitutively expressed since the activity in the absence and in the presence of 0.01% DHU, DMH or TU as inducer was similar (Figures 3.6B, C and D).The constitutive expression of hydantoinase enzymes has been reported for other organisms (Kim and Kim, 1993; Morin *et al.*, 1986b). However, 0.01% inducer may not be a significant amount to exert an effect (whether repression or activation) different from that of the control. It is noted that cells used to inoculate nutrient broth supplemented with the different inducers were cultured on basal medium plates and then in basal medium broth for a seed culture. Both media were supplemented with 1% hydantoin.

Therefore cells assayed in the absence of inducer may already have been induced, hence the high activity observed. HYD used as inducer (Figure 3.6A) was observed to lead to induction of both enzymes, even at 1% concentration, when MH was the substrate, and hence this inducer was used at this concentration for subsequent experiments. An advantage of this would be that this concentration of the inducer would not be depleted over the induction period.

The optimal time for assay of conversion of substrate was determined. Three hours as the assay time appeared to be optimal (Figure 3.7) since after that time, NCA production did not develop further; a plateauing effect was observed, and may be ascribed to product inhibition. However specific activity for NCA production decreased over the entire time period studied, and was due to the increasing time factor used to calculate specific activity detected with time. The rate of the reaction would have to be determined over a linear portion of the curve in kinetics studies. The rates of conversion by the carbamylase enzyme were observed to be low, and conclusions could not be made, except that the trends of activity with time were similar to those of the hydantoinase enzyme.

The pH profiles for the enzymes in different buffer systems was established. The use of Tris-HCl buffer at pH 9 appeared to be optimal for both enzymes, as shown in Figure 3.8. The activity of the enzyme system at high pH is similar to that reported in the literature for the hydantoinase enzyme (Olivieri *et al.*, 1981; Xu and West, 1994; Durham and Weber, 1995; Gokhale *et al.*, 1996) and for the carbamylase enzyme (Olivieri *et al.*, 1981; Ogawa *et al.*, 1993; Ogawa *et al.*, 1995b; Louwrier and Knowles, 1996). Different buffer effects were observed for the two enzymes. The use of citrate and potassium phosphate buffers resulted in an increase of hydantoinase activity with pH. The

opposite effect was observed for the carbamylase enzyme using the same buffers. Both enzyme activities increased with the pH of the Tris-HCl buffer. In the presence of sodium carbonate buffer, hydantoinase enzyme activity decreased with pH, while an increase in carbamylase activity was observed. The relatively high activity of both enzymes in Tris-HCl buffer over potassium phosphate buffer was an advantage in that the use of the former buffer would eliminate interference by K^+ ions in metal ion studies. In addition, metal ions would readily precipitate out in the presence of potassium phosphate buffer, pH 8, during assays for activity of the hydantoinase enzyme in the presence of metal ions. Therefore Tris-HCl buffer was chosen for the study of hydantoinase and carbamylase enzyme activities in the presence of various metal ions. The observed activity at high pH could be exploited for the chemical racemization of substrate, leading to the complete conversion of substrate to products.

The results shown in Figure 3.9 indicate that the hydantoinase activity decreased about 3-fold after dialysis. Mn^{2+} ions increased the activity of the dialyzed extract about 7-fold. Co^{2+} ions appeared only to preserve hydantoinase activity rather than activate or inhibit it. The hydantoinase enzyme may be readily activated by Mn^{2+} ions, and slightly activated by Co^{2+} ions. Cu^{2+} ions were found to be potent inhibitors of hydantoinase enzyme activity. The metal ion effects observed were similar to those reported in the literature (Morin *et al.*, 1986b, Syldatk *et al.*, 1987, Xu and West, 1994, Mukohara *et al*, 1994, Lee *et al.*, 1995, Kim *et al.*, 1997b) for the hydantoinase enzyme. The metal ions Mn^{2+}, Co^{2+} and Ni^{2+} activated the carbamylase enzyme the most (Figure 3.9) and the effects on the carbamylase enzyme were similar to those reported in the literature by Ishikawa *et al*, 1994, Ogawa *et al.*, 1995b, and Ishikawa *et al.*, 1996.

Experiments to determine the nature of the Cu^{2+} effect observed in Figure 3.9 on the hydantoinase enzyme were designed. In view of the observation that Mn^{2+} and Co^{2+} ions were the only two of the eight metal ions shown to activate both the hydantoinase and the carbamylase enzymes, it was considered possible that the dialysis steps influenced enzyme activities in the presence of metal ions other than Mn^{2+}, Co^{2+}, and Ni^{2+}. Dialysis conditions were investigated for their effect on enzyme activity in the presence of metal ions. In addition, the possibility of competition for binding sites by Mn^{2+}, Co^{2+} and Cu^{2+} ions was explored. The data in Figure 3.10 indicates that dialysis or incubation at 4°C, resulted in a loss of activity. However, dialysis of the extract followed by incubation at 4°C resulted in a greater loss of enzyme activity. Mn^{2+} and Co^{2+} ions stimulated the hydantoinase activity about 23.7-fold and 4.5-fold respectively after both treatments, and the stimulation of activity by Mn^{2+} ions was clearly reproducible (compare Figure 3.9). There was still no activation of the hydantoinase enzyme by Ca^{2+}, Na^{+}, Mg^{2+}, K^{+} ions. These results suggest that the dialysis conditions used did not inhibit enzyme activity in such a way as to impair the ability of these metal ions to activate hydantoinase activity.

Cobalt and manganese ions, co-incubated, did not activate hydantoinase activity more than manganese alone. This suggested a competition effect by both ions for the same site, where the manganese ions were possibly preferred over the cobalt ions. Copper and cobalt ions together inhibited the hydantoinase enzyme completely, suggesting toxicity by copper ions as cobalt ions were observed to be poor activators of activity. This was also suggested by the co-incubation of copper and manganese ions: the activating effect by manganese ions was decreased to a third of the effect by manganese ions alone. The activating effect of manganese is less in the presence of copper and

cobalt, owing possibly to the combined competition effects of copper and cobalt ions. It would be interesting to determine if the inhibitory effect by copper ions is reversible or not, after binding and displacement. This may indicate which metal ions are structurally or functionally involved, thus distinguishing clearly between metallodependence and enzyme activation. It would be of interest to determine if activation by metal ions is pH-dependent. This may identify a better activator than manganese at a different pH.

Conclusion

The reaction conditions for the hydantoinase and carbamylase activities of RU-KM3$_L$ crude extract were optimised with respect to induction conditions, substrate specificity, optimal assay time for resting cell reactions, pH conditions, and the presence of metal ions. These optimisation experiments were conducted for a comparison of enzyme activity in aqueous systems with non-aqueous systems.

CHAPTER 4: RELEASE OF MEMBRANE-ASSOCIATED ENZYME

With the reaction conditions for the activity of the hydantoinase and carbamoylase enzymes in the crude extract RU-KM3$_L$ optimized, it was suggested that the enzymes, more especially the hydantoinase enzyme, be at least partially purified for use in biocatalytic asssays in non-aqueous sytems. This would require the localization of the enzyme system within the cell so that specific purification methods may be applied.

Materials and method

4.1 Chemicals

Hydantoin, *N*-carbamylglycine (NCG), *N*-carbamyl-D,L-alanine (NCA), glycine (GLY) and D,L-alanine (ALA) were purchased from Sigma Chemicals. 5-methylhydantoin (MH) was synthesized by the Bucherer-Bergs method (section 3, Appendix). All other reagents were of analytical grade.

4.2 Localization of hydantoinase and carbamylase enzyme activities

4.2.1 The effect of cell debris removal after cell disruption

A loopful of cells cultured on basal medium plates at 25°C was inoculated into 400ml nutrient broth supplemented with 1% hydantoin. Induction of cells at 25°C was continued to early stationary phase. Cells were harvested and resuspended in 30ml 0.1M cold potassium phosphate buffer, pH8. The cells were disrupted by French Press and the extract was made up to 50ml with buffer. 1ml aliquots of the extract were incubated with an equal volume of 100mM hydantoin for 3 hours at 50°C. The

remainder of the extract was clarified by microcentrifugation (1200xg for 3 minutes) and the supernatant was assayed in 1 ml aliquots for hydantoinase and carbamylase activity as for the cell debris-containing fraction. Product and protein concentrations were determined as in section 3.1.4.

4.2.2 Ammonium sulfate fractionation of crude extract

Crude extract was obtained as described in section 4.1.1 from 200ml induction medium. Ammonium sulfate was slowly added to the crude extract (25ml) to 20% saturation at 0°C. The extract was centrifuged at 15000rpm for 10 minutes at 0°C to afford a pellet that was resuspended in 5ml buffer and dialyzed (Slide-A-Lyzer™ 10K dialysis cassettes, MWCO 10000) for 3 hours against 1L 50% buffer, with buffer changes every hour. The dialyzed extract was incubated in 1ml aliquots at 50°C in the presence of 50mM MH (5-methylhydantoin) for 3 hours and assayed for enzyme activity. Ammonium sulfate was added to the supernatant to 40% saturation. The mixture was centrifuged and the pellet was dialyzed and assayed as for the 20% ammonium sulfate fraction. Ammonium sulfate was added to the supernatant of the 40% fraction to 60 % saturation, and the procedure was repeated to 80% ammonium sulfate saturation. The supernatant of the final 80% ammonium sulfate fraction was not assayed.

4.3 Release of membrane-associated enzyme

4.3.1 The effects of detergents on membrane-associated hydantoinase and carbamylase enzymes

A loopful of cells cultured on basal medium plates at 25°C was inoculated into 200ml nutrient broth supplemented with 1% hydantoin. Induction was continued to early stationary phase. The cells were harvested by centrifugation and resuspended in 15ml 0.1M cold potassium phosphate buffer, pH8.

Cells were disrupted by French Press, and the extract was made to 25ml with buffer. 5ml portions of the extract were assayed for hydantoin-hydrolyzing activity in the presence of 50mM MH at 50°C for 3 hours. The remainder of the extract (20ml) was clarified by centrifugation at 9000rpm for 30 minutes at 4°C. The supernatant was assayed for enzyme activity, and the pellet was resuspended in 40ml buffer. 10 ml portions of the extract were incubated for one hour on ice in the absence or presence of an equal volume of detergent (polyoxyethylene ether W-1 or sodium deoxycholate or Tween 20 or Triton X-114) for a final detergent concentration of 0.01 or 0.1 or 1%. The remainder of the extract was clarified by centrifugation, and the supernatant was assayed for enzyme activity. Where Triton X-114 was used, the viscous layer was assayed for the presence of both the hydantoinase and the carbamylase enzyme activities. Product and protein concentrations were determined as in section 3.1.4.

4.3.2 The effects of toluene on membrane-associated hydantoinase and carbamylase enzymes

A loopful of cells was inoculated into 800ml nutrient broth supplemented with 1% hydantoin and induced to early stationary phase. The cells were harvested by centrifugation, resuspended in 60ml 0.1M cold potassium phosphate buffer, pH8 and disrupted by French Press. The extract was made up to 100ml, and 1ml portions were assayed at 50°C for 3 hours with 50mM MH as substrate. 5ml portions of the extract were incubated at 4°C (with mixing at 15-minute intervals) for 30 minutes, 1, 2, and 4 hours in the absence or presence of an equal volume of toluene at 0.2, 2, or 10% (v/v) (for final toluene concentrations of 0.1, 1 or 5% (v/v)). After the incubation times, the toluene-extract fractions were centrifuged at 15000rpm for 15 minutes at 4°C. The supernatant was assayed for enzyme activity, and the pellet was resuspended in 5ml buffer and assayed in the same manner. NCA,

ALA and protein concentrations were determined as in section 3.1.4.

4.3.3 The effect of sonication on membrane-associated hydantoinase and carbamylase enzymes

Nutrient broth (300ml) supplemented with 1% hydantoin was inoculated with cells cultured on basal medium plates. Induction was carried out to early stationary phase, and the cells were resuspended in 22.5ml 0.1M cold potassium phosphate buffer, pH8, and were disrupted by French Press. The extract was made to 37.5ml with buffer and sonicated in 5ml portions at 30nm strength (just below frothing strength) in 10-second bursts for up to 20 minutes at 0°C, and centrifuged at 15000rpm for 15 minutes at 0°C. Both the supernatant and pellet resuspended in 5ml buffer were assayed at 50°C with 50mM MH as substrate for 3 hours. NCA, ALA and protein concentrations were determined as in section 3.1.4.

Specific activity (SA) was expressed as units per milligram of protein, where one unit (U) was defined as the amount of enzyme required to produce 1μmol of product in one minute under the specified conditions. The data is presented as specific activity, unless otherwise stated.

Results

4.2 Localization of hydantoinase and carbamylase enzyme activities

4.2.1 The effect of cell debris removal after cell disruption

Hydantoinase and carbamylase enzyme activities were determined in crude extract with cell debris,

and in fractions where cell debris was removed. Enzyme activity in cell debris-containing fractions would suggest association of activity with cell structures such as membranes. The result for the effect of cell debris removal on hydantoinase and carbamylase activities in crude extract of RU-KM3$_L$ is shown in Figure 4.2.1.

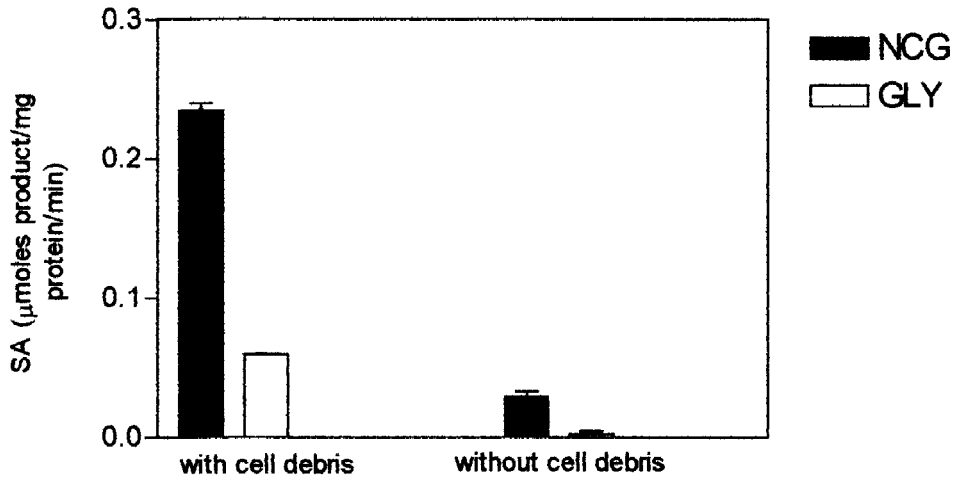


Figure 4.2.1 The effect of removal of cell debris on enzyme activity in RU-KM3$_L$ crude extract
The data represent the mean ±SEM of triplicate determinations.

4.2.2 Ammonium sulfate fractionation of crude extract

Ammonium sulfate fractionation was conducted to provide further evidence for the association of hydantoinase and carbamylase enzymes with the cell membrane, and the result is shown in Table 4.2.2.

Table 4.2.2 Ammonium sulfate treatment of RU-KM3$_L$ crude enzyme extract

ammonium sulphate	specific activity $_{NCA}$	specific activity $_{ALA}$
none	0.17 (±0.0022)	0.03 (±0.0013)
20%	0.08 (±0.0005)	0.013 (±0.0011)
40%	0.06 (±0.0)	0.25 (±0.0028)
60%	0.02 (±0.0107)	0.05 (±0.0011)
80%	0.06 (±0.0132)	0.18 (±0.0019)

NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean ±SEM of triplicate determinations.

4.3 Release of membrane-associated enzyme

As the data from the cell debris removal and ammonium sulfate fractionation experiments suggested that the hydantoinase enzyme in particular was membrane-associated, several techniques were used in an attempt to release enzyme from the membrane, and included the use of detergents, organic solvents such as toluene, and sonication.

4.3.1 The effects of detergents on membrane-associated hydantoinase and carbamylase enzymes

The effects of detergents on the release of membrane-associated hydantoinase and carbamylase enzymes are shown in Tables 4.3.1a and b.

Table 4.3.1a The effects of detergents on membrane-associated hydantoinase enzyme

deterg	Specific activity ($U.mg^{-1}$)									
	extract	supn	extract in presence of detergent				supernatant of detergent-treated extract			
	detergent concentration (%)									
			0	**0.01**	**0.1**	**1**	**0**	**0.01**	**0.1**	**1**
W-1	0.88	0.08	0.22	0.19	0.34	2.52	0.1	0.19	0.37	0
	(0.03)	(0.01)	(0.02)	(0.01)	(0.04)	(0.05)	(0.05)	(0.09)	(0.03)	
NaDC	0.52	0.17	0.42	0.33	0.23	0.85	0.33	0.14	0	0
	(0.02)	(0.004)	(0.004)	(0.03)	(0.02)	(0.12)	(0.02)	(0.08)		
TW20	1.01	0.034	0.35	0.4	0.32	0.36	0.09	0.12	0.03	0
	(0.02)	(0.02)	(0.02)	(0.01)	(0.02)	(0.02)	(0.05)	(0.01)	(0.03)	
TX114	0.32	0.06	0.19	0.13	0.19	0	1.78	0.2	0.1	0
	(0.01)	(0.01)	(0.01)	(0.02)	(0.01)		(0.34)	(0.06)	(0.02)	
										1.58
										(0.15)

deterg (detergent), supn (supernatant of clarified extract), W-1 (polyoxyethylene ether W-1), NaDC (sodium deoxycholate), (TW20) Tween 20, TX114 (Triton X-114). Viscous Triton X-114 layer. The data represent the mean ±SEM (SEM in parentheses) of triplicate determinations.

Table 4.3.1b The effects of detergents on membrane-associated carbamylase enzyme

deterg	Specific activity ($U.mg^{-1}$)									
	extract	supn	extract in presence of detergent				supernatant of detergent-treated extract			
			detergent concentration (%)							
			0	**0.01**	**0.1**	**1**	**0**	**0.01**	**0.1**	**1**
W-1	0.07	0.09	0.09	0.09	0.15	1.14	0.4	0.68	0.79	0
	(0.002)	(0.005)	(0.003)	(0.004)	(0.005)	(0.06)	(0.01)	(0.03)	(0.02)	
NaDC	0.04	0.07	0.05	0.05	0.05	0.39	0.52	0.45	0.07	0.05
	(0.001)	(0.001)	(0.001)	(0.001)	(0.001)	(0.004)	(0.01)	(0.01)	(0.002	(0.001)
TW20	0.12	0.14	0.07	0.08	0.07	0.12	0.8	0.89	0.9	0
	(0.005	(0.01)	(0.001)	(0.001)	(0.002)	(0.01)	(0.02)	(0.04)	(0.02)	
TX114	0.03	0.04	0.06	0.07	0.05	0	3.42	0.39	0.12	0
	(0.001)	(0.003)	(0.001)	(0.01)	(0.001)		(0.08)	(0.004)	(0.003)	
										0.13
										(0.01)

deterg (detergent), supn (supernatant of clarified extract), W-1 (polyoxyethylene ether W-1), NaDC (sodium deoxycholate), (TW20) Tween 20, TX114 (Triton X-114). Viscous Triton X-114 layer. The data represent the mean ±SEM (SEM in parentheses) of triplicate determinations.

4.3.2 The effects of toluene on membrane-associated hydantoinase and carbamylase enzymes

Tables 4.3.2a and b show the result of toluene treatment on membrane-associated hydantoinase and carbamylase enzymes respectively.

Table 4.3.2a The effects of toluene on membrane-associated hydantoinase enzyme

time (h)	NCA(μmoles/ml)							
	pellet				supernatant			
	toluene concentration (%)							
	0	**0.1**	**1**	**5**	**0**	**0.1**	**1**	**5**
0.5	17.43 (0.47)	16.99 (0.45)	14.0 (1.96)	13.32 (2.83)	0.37 (0.08)	0	0.05 (0.03)	0.86 (0.08)
1	15.28 (0.26)	15.28 (0.29)	15.34 (0.09)	3.59 (0.08)	0.25 (0.11)	0	0.19 (0.03)	0.14 (0.05)
2	15.96 (0.58)	18.16 (0.63)	9.01 (0.36)	5.54 (0.14)	0.51 (0.08)	0.19 (0.11)	0.1 (0.03)	0.51 (0.26)
4	15.14 (0.29)	14.36 (0.32)	2.33 (0.06)	2.77 (0.24)	0.35 (0.06)	0	0.29 (0.16)	0.34 (0.02)

NCA (*N*-carbamyl alanine). The data represent the mean ±SEM (SEM in parentheses) of triplicate determinations.

Table 4.3.2b The effects of toluene on membrane-associated carbamylase enzyme

time (h)	ALA (μmoles/ml)							
	pellet				supernatant			
	toluene concentration (%)							
	0	**0.1**	**1**	**5**	**0**	**0.1**	**1**	**5**
0.5	0.51 (0.11)	0.89 (0.12)	0.38 (0.02)	0.59 (0.09)	0.49 (0.02)	0.58 (0.03)	0.57 (0.08)	0.44 (0.02)
1	0.48 (0.01)	0.48 (0.01)	0.52 (0.01)	0.31 (0.02)	0.29 (0.01)	0.33 (0.01)	0.27 (0.01)	0.3 (0.01)
2	0.33 (0.05)	0.49 (0.02)	0.28 (0.01)	0.39 (0.003)	0.27 (0.01)	0.26 (0.01)	0.26 (0.02)	0.23 (0.01)
4	0.28 (0.03)	0.38 (0.01)	0.09 (0.02)	0.1 (0.06)	0.21 (0.02)	0.21 (0.01)	0.23 (0.02)	0.18 (0.01)

ALA (alanine). The data represent the mean ±SEM (SEM in parentheses) of triplicate determinations.

4.3.3 The effect of sonication on membrane-associated hydantoinase and carbamylase enzymes

Previous attempts (the use of detergents and toluene) to release hydantoinase and carbamylase enzymes from the membrane suggested that the enzymes were tightly associated with the membrane. Therefore a mechanical means to release enzymes from the membrane, namely sonication, was considered. Changes in protein distribution between membrane and supernatant fractions during sonication were studied.The data for the sonication experiment are shown in Figures 4.3.3a and b.

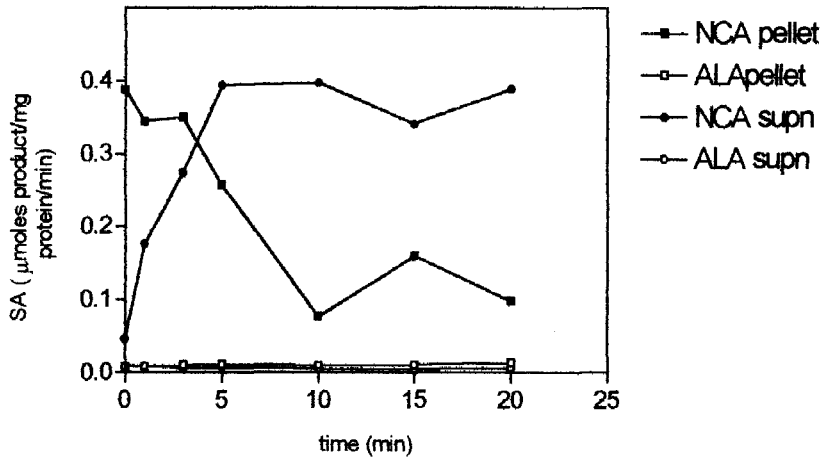


Figure 4.3.3a Release of membrane-associated enzyme by sonication
NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean ±SEM of triplicate determinations.

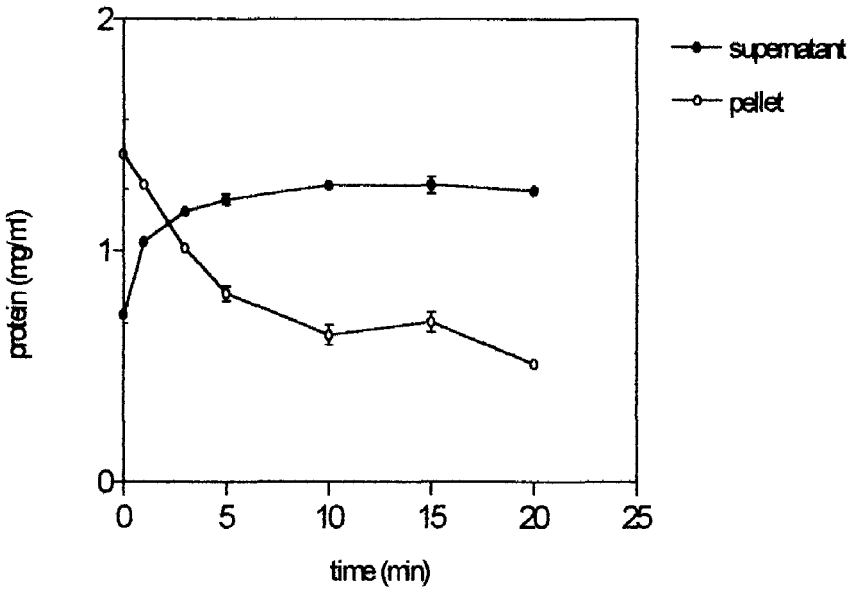


Figure 4.3.3b Protein distribution between the cell debris containing fraction and supernatant during sonication.
The data represent the mean ±SEM of duplicate determinations.

Discussion

The data in Figure 4.2.1 suggests that enzyme activity was confined to the cell debris-containing fraction and could indicate association of hydantoinase and carbamylase activities with the cell membrane. Further evidence for this result using ammonium sulfate fractionation was considered. It was speculated that if activity is associated with the early ammonium sulfate fractions, then it is likely that activity is indeed associated with the membrane: membrane proteins and lipids are detected in early ammonium sulfate cuts. The ammonium sulfate fractionation data in Table 4.2.2 suggest that activity, in particular the hydantoinase activity, was membrane-associated because most of the hydantoinase activity was shown to be confined to the 0-20% ammonium sulfate fraction. These results are different from that reported in the literature: clarification of crude extract afforded an active supernatant fraction. The results for specific activity of the carbamylase enzyme at each ammonium sulfate fractionation may be suspect owing to $(NH_4)_2SO_4$ interference in the Ninhydrin assay for amino acids, especially at the higher $(NH_4)_2SO_4$ concentrations. A means to circumvent this would be to dialyse the ammonium sulfate-treated fraction for longer than one hour prior to assay. However, this approach causes risk of losing carbamylase activity due to its instability.

The release of membrane-associated enzyme using detergents, organic solvents or mechanical means was considered. Table 4.3.1a shows that the result that higher hydantoinase activity occurred in the presence of cell debris, as opposed to in clarified extract, was reproducible. Clarification of the crude extract and assay of the supernatant fraction showed that negligible amounts of hydantoinase activity could be detected. The presence of polyoxyethylene ether W-1 in the crude extract appeared to

stimulate hydantoinase activity with increase in detergent concentration. A lowering effect was observed for sodium deoxycholate, while Tween 20 and Triton X-114 seemed to have preserved hydantoinase activity. For all the detergents tested, the activity of the hydantoinase enzyme in untreated extract was higher than that in the treated extract. This is ascribed to activity loss during pellet reconstitution in buffer and incubation in the presence of detergent. Centrifugation of the detergent-treated extract and assay of the supernatant for hydantoinase activity showed even lower activity in that fraction. The absence of activity observed at 1% detergent in the supernatant is ascribed to detergent interference in the colorimetric assay for *N*-carbamyl amino acids as well as the Bradford assay for protein. The high specific activity in the supernatant in the absence of Triton X-114 is ascribed to low protein detection. Phase separation afforded a viscous detergent phase in which hydantoinase activity was low. In comparing activity before and after clarification, it appeared that the detergents did not effect enzyme release, or that the enzyme released was inactive.The supernatant of the clarified extract was shown to have more carbamylase activity than the cell debris-containing crude extract (Table 4.3.1b).This suggested that the hydantoinase and carbamylase enzymes may be located in different parts of the cell, or that the association of the hydantoinase enzyme with the membrane was tighter than that of the carbamylase enzyme. However, more research would have to be done to confirm these findings.

The crude extract was treated with detergent to determine if this would release the enzyme into the medium. The supernatant was assayed to establish whether released enzyme had been solubilized. Polyoxyethylene ether W-1 at 1% appeared to increase both specific activities in unclarified extract (Tables 4.3.1a and b). This was more marked for the carbamylase enzyme in the supernatant of the

detergent-treated extract (Table 4.3.1b).

Sodium deoxycholate appeared to have a concentration-dependent lowering effect on the hydantoinase enzyme specific activity in unclarified extract (Table 4.3.1a). A significant increase in carbamylase activity was observed in the presence of 1% sodium deoxycholate in unclarified extract (Table 4.3.1b). However, upon clarification of the extract, the lowering effect appeared to be dependent upon the concentration of the detergent for both the hydantoinase and carbamylase enzymes, as before.

The presence of Tween 20 did not have any effect on either enzyme before clarification, but appeared to activate the carbamylase enzyme in clarified extract (Table 4.3.1b).

The presence of Triton X-114 did not have an effect on the enzyme systems in unclarified extract, but appeared to lower the activity increasingly with increase in concentration of detergent in the supernatant fraction (Tables 4.3.1a and b). Phase separation afforded two layers, one of which was the viscous detergent layer, and was assayed in case either enzyme was amphiphilic and could be detected in it. No appreciable activity was observed. It is proposed that since the non-ionic detergent Tween 20 was not observed to affect the activity of either enzyme, little or no hydrophobic interaction occurs between this detergent and enzyme, suggesting that the enzymes are hydrophilic. This is possibly confirmed by considering activity in the supernatant of Triton X-114-treated extract. The amount of protein in the hydrophilic phase during phase separation was proportional to detergent concentration. The specific activity in the supernatant thus appeared to diminish with detergent

concentration. Assay of the detergent phase showed high *specific* activity owing to the minimal amount of protein present.

Considerable interference in the assays, especially the Ninhydrin assay for amino acids, by the detergents, was observed. This was particularly marked where polyoxyethylene ether W-1, Tween 20 and Triton X-114 were used at the higher concentrations. In addition, it was foreseen that detergent removal would be difficult. Detergent studies were therefore discontinued, and an alternative approach, namely the use of organic solvents to release membrane-associated enzyme, was considered.

It was suggested that toluene treatment of crude extract be used to release enzyme from the membrane. Toluene-treated extract was clarified, and both resuspended pellet and supernatant were assayed for residual activity and solubilised activity respectively. It was observed that most of the hydantoinase activity was still confined to the membrane (pellet) fraction after treatment with toluene (Table 4.3.2a). Prolonged incubation with increasing toluene concentrations possibly resulted in decrease in specific activity. It is suggested that toluene had an inhibitory effect on hydantoinase activity at higher concentrations. This is more pronounced as incubation time increased, and may be explained by excessive partitioning of toluene into the membrane to disrupt membrane functions (Sikkema *et al.*, 1994 cited in Weber *et al.*, 1994). Loss of enzyme stability is unlikely as a cause of the lowered hydantoinase activity at high toluene concentrations since in the absence of toluene, activity was not observed to significantly decrease with time. The supernatant of toluene-treated crude extract showed negligibly low enzyme activity and confirmed that most activity is still

associated with the cell membrane. The same trends in toluene effects were observed for the carbamylase enzyme as for the hydantoinase enzyme (Table 4.3.2b), i.e., association with the membrane, and a decrease in activity with increasing incubation time in the presence of toluene.

As the toluene and detergent treatments were shown to be ineffective with respect to enzyme release from the membrane, a mechanical means to release enzyme was suggested (D. Cowan, personal communication). In addition, previous attempts to release enzymes from the membrane seemed to indicate that the enzymes were either integral proteins, or were associated with the membrane by other means in addition to ionic and hydrogen bonds. Thus sonication was investigated as a means to release enzyme from the cell membrane. Sonication effected the release of the hydantoinase enzyme from the membrane (Figure 4.3.3a). Sonication of the crude extract for 10 minutes was sufficient to release all of the hydantoinase enzyme. It is of significance that all of the hydantoinase activity was retained in the supernatant after sonication. When sonication was applied to the extract at optimum pH 9-10, the activity was lost. It is proposed that the high alkaline conditions are unfavourable for released enzyme. The carbamylase activity was very low, so drawing of conclusions was difficult.

It is suggested that the hydantoinase and carbamylase enzyme might be membrane-associated, but direct evidence would have to be provided to confirm this. This would involve co-precipitation of the hydantoinase and carbamylase enzymes with known membrane-bound enzymes (possibly incorporating a membrane marker), or the use of sucrose gradient density centrifugation to isolate membrane fractions and demonstrate enzyme activity in these fractions.

Protein determination (Figure 4.3.3b) in all the samples showed that the protein concentration decreased in the pellet fractions, and increased in the supernatant fractions, which corresponded with the data in Figure 4.3.3a. It would be of interest to detect hydantoinase activity of RU-KM3$_L$ using activity gels, as has already been reported by Kim *et al.*(1997a) for *Agrobacterium* sp. I-671, *B. stearothermophilus* SD-1, and *Bacillus thermocatenulatus* GH2 strains. Gel electrophoresis under non-denaturing conditions and incorporation of phenol red to detect hydantoinase and estimate molecular weight was applied without further treatment of the clarified crude extract (Kim *et al.*, 1997a). This approach could be modified by using the supernatant of sonicated RU-KM3$_L$ crude extract. Supernatant could have been treated further (size exclusion chromatography or heat treatment) to afford a fraction with less cell debris and contaminating protein. These treatments would, however, incur the risk of loss of enzyme activity.

Other approaches could have been employed to obtain membrane-bound enzyme in a partially-purified form if time had allowed. These include EDTA/ lysozyme treatment of whole cells to yield protoplasts. The protoplasts could be subjected to fractional centrifugation to afford membranes (Burrous and Wood, 1962). Detergents may still be required to release enzyme from the isolated membranes. In order to circumvent the use of detergents, enzyme could be released from membranes by n-butanol extraction: n-butanol is highly lipophilic and is inserted into the membrane giving a detergent-like phospholipid-displacing effect (Gondolf *et al.*, 1990). Lipid interference in protein assays may be eliminated by use of a modification of the Amido Black 10B protein assay (Kaplan and Pedersen, 1985).

The stability of the crude extract could be retained by freezing in liquid nitrogen followed by rapid, gentle crushing into a fine powder by pestle-and mortar and suspension in ice-cold buffer for sonication.

An alternative mechanical approach would be repeated freezing and thawing to release membrane-bound enzyme, although there is the risk of enzyme inhibition (as reported for frozen and thawed extracts). Fouling of the thawed extract could be prevented by treatment with polyethyleneimine to remove nucleic acids (Kirk and Cowan, 1995). Incorporated into the treatment of the crude extract could be rapid ("flash") heating for separation of desired activity from precipitated extract particulates, insoluble cell debris, and other heat-labile proteins. This type of heating rapidly denatures proteolytic enzymes. The thermostability of the hydantoinase enzyme would be exploited, provided the upper limit of temperature tolerance is known. The time taken to reach the upper temperature limit should be taken into account since proteases may still be active at lower temperatures, and the upper temperatures of enzyme activity may be incorrectly interpreted.

These techniques, although attractive, were not investigated, owing to the limited time and scope of the present study.

Conclusion

Although the release of enzyme by sonication was reproducible, variations in sonication efficiency and lack of enzyme stability as observed in some of the subsequent experiments proved this technique to be unsuitable in view of the targeted objective, i.e., the characterization of enzyme activity in

organic solvents. It was thus considered adequate to pursue organic solvent studies using crude extract.

CHAPTER 5: OPTIMIZATION OF LYOPHILIZATION CONDITIONS FOR CRUDE EXTRACT OF RU-KM3$_L$

Prior to determining the effects of organic solvents on the hydantoinase enzyme system, it was necessary to obtain the crude extract in active lyophilised form at optimum pH, with a view to adding crude enzyme extract in powder form to water-immiscible organic solvents in the presence of substrate.

Materials and method

5.1 Chemicals

N-carbamyl-D,L-alanine (NCA) and D,L-alanine (ALA) were purchased from Sigma Chemicals. 5-methylhydantoin (MH) was synthesized by the Bucherer-Bergs method (section 3, Appendix). All other reagents were of analytical grade.

5.2 Optimum pH for lyophilisation

Nutrient broth (400ml) supplemented with 1% hydantoin was inoculated with a loopful of cells cultured on basal medium plates at 25°C. Induction was continued to early stationary phase after which 40ml aliqouts cells were harvested by centrifugation and resuspended in 3ml 0.1M cold potassium phosphate buffer at pH3-12. Cells were disrupted by French Press and made up to 5ml with buffer of corresponding pH. Incubation of 1ml portions of extract was conducted for 3 hours in the presence of 50mM MH (5-methylhydantoin) at 50°C. Product and protein concentrations were

determined as in section 3.1.4.

5.3 Lyophilisation of crude extract

A loopful of cells cultured on basal medium plates was inoculated into 100ml nutrient broth supplemented with 1% hydantoin. Induction was continued to early stationary phase. The cells were harvested by centrifugation, and resuspended in 7.5ml 0.1M cold potassium phosphate buffer, pH10. Cells were disrupted by French Press, and the extract was made up to 12.5ml with buffer. Incubation of the extract was conducted with 50mM MH at 50°C for 3 hours. 5ml aliquots of the extract were lyophilised for 6-10 hours, resuspended in the same volume of buffer and assayed for hydantoinase and carbamylase activities. Product and protein concentrations were determined as in section 3.1.4.

5.4 Strategies for obtaining active lyophilised crude extract

5.4.1 Lyophilisation of concentrated crude extract pellet

Crude enzyme extract was obtained as described in section 5.3 from 100ml induction culture. The extract was centrifuged at 15000rpm for 15 minutes at 4°C. The pellet was resuspended in 1ml buffer (which afforded a volume in excess of 1ml), and was lyophilized for 3 hours prior to resuspension in 6.25ml buffer and assaying of 1ml aliquots at 50°C in the presence of 50mM MH for 3 hours. Buffer (5.25ml) was added to the remainder of the resuspended pellet from centrifugation and assayed as for the lyophilised fraction. Product and protein concentration were determined as in section 3.1.4.

5.4.2 Dialysis of crude extract before lyophilisation

Cells were cultured and the crude extract was obtained as described in section 5.3. 1ml aliquots were

assayed for 3 hours at 50°C with 50mM hydantoin as substrate. A second fraction was dialysed (Slide-A-Lyzer™ 10K dialysis cassettes, MWCO 10000) against distilled water at 4°C for 2 hours, with changes every 30 minutes.The dialysed fraction was assayed in 1ml fractions for enzyme activity. 5ml aliquots of the remainder of the dialysed fraction were lyophilised for 6-10 hours, resuspended in the same volume of buffer, and assayed in 1ml aliquots. Protein and product determinations were conducted as in section 3.1.4.

5.4.3 Lyophilisation of crude extract in the presence of lower ionic strength and pH buffers

Nutrient broth (100ml) supplemented with 1% hydantoin was inoculated with cells cultured on basal medium plates and cells were induced to early stationary phase. Harvesting of cells was carried out by centrifugation and the pellet was resuspended in 7.5ml 0.01 or 0.05M cold potassium phosphate buffer at pH 7, 8, 9 or 10. Cells were disrupted by French Press, and the extract was made up to12.5ml with the appropriate buffer. 1ml portions of the extract were assayed in the presence of 50mM MH at 50°C for 3 hours. 5ml portions of the remainder of the extract were lyophilised for 6-10 hours, resuspended in the same volume of the corresponding buffer, and assayed in 1ml portions. Product and protein concentration determinations were conducted as in section 3.1.4.

5.4.4 Lyophilisation of crude extract in the presence of different buffer systems

The inoculation of 100ml nutrient broth and induction of cells were conducted as described in section 5.4.3. Harvested cells were resuspended in 7.5ml 0.05M cold Glycine-NaOH or Tris-HCl buffer, pH10. Cells were disrupted by French Press and the extract was made up to 12.5ml with the corresponding buffer. 1ml portions were assayed at 50°C with 50mM MH as substrate for 3 hours.

5ml portions of the remaining extract were lyophilized for 6-10 hours and resuspended in the same volume of the corresponding buffer. Assaying of the lyophilised crude extract was carried out using 1ml portions. Product and protein concentrations were determined as in section 3.1.4.

5.4.5 Lyophilisation of RU-KM3$_L$ whole cells

A loopful of cells cultured on basal medium plates was inoculated into 100ml nutrient broth supplemented with 1% hydantoin, and this was followed by induction of cells to early stationary phase. The cells were harvested by centrifugation, and resuspended in 7.5ml 0.1M cold potassium phosphate buffer, pH8 for 100mg cells per reaction. The incubation of whole cells was conducted at 50°C for 6 hours in the presence of 50mM MH. Determinations of product and protein concentration were conducted as described in section 3.1.4.

5.4.6 The incorporation of additives during the lyophilisation of crude extract

Nutrient broth (400ml) supplemented with 1% hydantoin was inoculated with a loopful of cells cultured on basal medium plates. Induction of cells was continued to stationary phase, and this was followed by harvesting of cells by centrifugation. The cells were resuspended in 30ml 0.05M potassium phosphate buffer and disrupted by French Press. The extract was made to 50ml with buffer, and assayed for conversion of 50mM MH in 1ml portions at 50°C in the presence of 1 or 10 or 30 or 50% (w/v) sucrose. The remainder of the extract was lyophilised in 5ml aliquots in the presence of an equal volume of 1, 10, 30 or 50% (w/v) final concentration of sucrose for 6-10 hours. Lyophilised extract was resuspended in 5ml buffer, and assayed using 1ml portions, for enzyme activity. Product and protein concentrations were determined as in section 3.1.4.

5.4.7 The effect of sucrose as an additive on the thermostability of lyophilised crude extract

Crude extract was prepared as in section 5.4.6. 1ml portions were assayed at 40, 50 or 60°C in the presence of 50mM MH and 50% (w/v) sucrose for 3 hours. The remainder of the extract was lyophilised for 6-10 hours in 5ml aliquots in the presence of 50% (w/v) sucrose. Resuspension of lyophilised extract was conducted in 5ml buffer, and assaying was conducted under the same conditions used for the unlyophilised extract. Product and protein concentrations were determined as in section 3.1.4.

Specific activity (SA) was expressed as units per milligram of protein, where one unit (U) was defined as the amount of enzyme required to produce 1μmol of product in one minute under the specified conditions. The data is presented as specific activity, unless otherwise stated.

Results

5.2 Optimum pH for lyophilisation

Enzymes assume an ionisation state optimal for catalysis in aqeous media, and this ionisation state may be retained in non-aqueous media if the enzyme is lyophilised from buffer of optimal pH (Zaks and Klibanov, 1985 cited in Deetz and Rozzell, 1988). In order to determine the optimal pH for lyophilisation, the pH profile of RU-KM3$_L$ crude extract in potassium phosphate buffer was determined, and the result is shown in Figure 5.2.

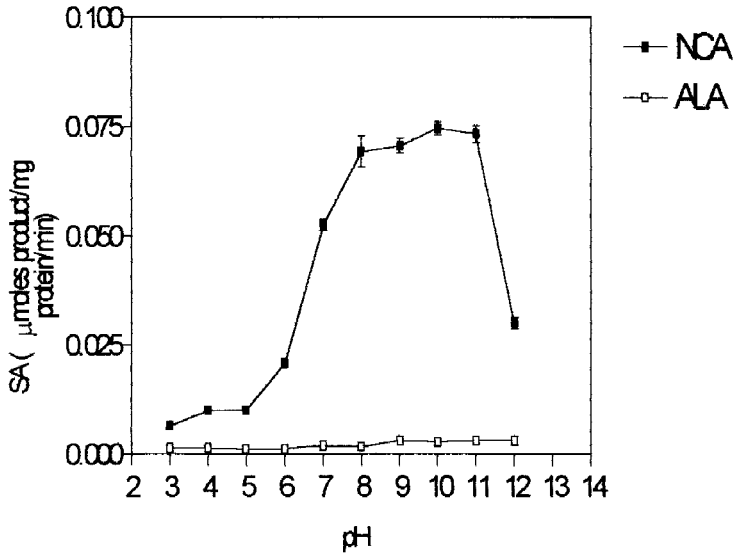


Figure 5.2 The pH profile for RU-KM3$_L$ crude extract in potassium phosphate buffer.
NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean ±SEM of triplicate determinations.

5.3 Lyophilisation of crude extract

The lyophilisation of crude extract from aqueous medium at pH10 was conducted with the aim of obtaining crude extract in powder form for use in organic solvent studies, and the result is shown in Table 5.3

Table 5.3 The effect of lyophilisation on enzyme activity in the crude extract of RU-KM3$_L$

	before lyophilisation (U.mg^{-1})	after lyophilisation (U.mg^{-1})
NCA	0.099 (±0.001)	0.007 (±0.001)
ALA	0.003 (±0.0001)	0.002 (±0.0001)

NCA (*N*-carbamylalanine), ALA (alanine).The data represent the mean ±SEM of triplicate determinations.

5.4 Strategies for obtaining active lyophilised crude extract

Lyophilisation of crude extract was shown to be detrimental under the conditions used in section 5.3, and therefore methods of preserving enzyme activity during lyophilisation were investigated.

5.4.1 Lyophilisation of concentrated crude extract pellet

It was thought that lyophilisation of concentrated crude extract pellet would render the hydantoinase and carbamylase enzymes more stable, and this would substantially decrease loss in enzyme activity. The effect of lyophilisation of concentrated crude extract is shown in Table 5.4.1.

Table 5.4.1 Lyophilisation of concentrated crude extract pellet

	before lyophilisation ($U.mg^{-1}$)	after lyophilisation ($U.mg^{-1}$)
NCA	0.111(±0.005)	0.016 (±0.0004)
ALA	0.005 (±0.0004)	0.004 ($\pm 1.15 \times 10^{-6}$)

NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean ±SEM of triplicate determinations.

5.4.2 Dialysis of crude extract before lyophilisation

It was suspected that a compound inhibitory to enzyme activity was possibly concentrated and/or activated upon lyophilisation of the crude extract. The crude extract was dialysed before lyophilisation to remove the inhibitory compound if present, the result of which is shown in Table 5.4.2.

Table 5.4.2 Dialysis of crude extract prior to lyophilisation

	before lyophilisation		after lyophilisation
	before dialysis	after dialysis	after dialysis
NCA	0.068 (±0.003)	0.065 (±0.001)	0.009 (±0.0002)
ALA	0.0012 (±0.001)	0.0028 (±0.0002)	0.0015 (±0.0001)

NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean ±SEM of triplicate determinations.

5.4.3 Lyophilisation of crude extract in the presence of lower ionic strength and pH buffers

It was suggested that changes in pH and increase in ionic strength that accompany lyophilisation could easily be detrimental to enzyme activity (D. Cowan, personal communication) and therefore the effect of lypohilisation from different concentrations and pH of potassium phosphate buffer on enzyme activity were investigated. The result is shown in Tables 5.4.3a and b.

Table 5.4.3a Lyophilsation using 0.01M potassium phosphate buffer, pH 7-10.

pH	before lyophilisation ($U.mg^{-1}$)		after lyophilisation ($U.mg^{-1}$)	
	NCA	ALA	NCA	ALA
7	0.026 (±0.0)	0.001 (±0.0003)	0.018 (±0.004)	0.001 (±0.0001)
8	0.024 (±0.003)	0.002 (±0.0001)	0.011 (±0.001)	0.001 (±0.0001)
9	0.04 (±0.002)	0.002 (±0.0002)	0.011 (±0.0001)	0.001 (±0.0001)
10	0.035 (±0.001)	0.002 (±0.0001)	0.02 (±0.0002)	0.001 (±0.00002)

Lyophilisation using 0.01M potassium phosphate buffer, pH 7-10
NCA (*N*-carbamylalanine), ALA (alanine).The data represent the mean ±SEM of triplicate determinations.

Table 5.4.3b Lyophilisation using 0.05M potassium phosphate buffer, pH 7-10.

pH	before lyophilisation ($U.mg^{-1}$)		after lyophilisation ($U.mg^{-1}$)	
	NCA	ALA	NCA	ALA
7	0.06 (±0.001)	0.001 (±0.0002)	0.007 (±0.0002)	0.001 (±0.0002)
8	0.071 (±0.001)	0.002 (±0.0001)	0.011 (±0.001)	0.001 (±0.0002)
9	0.088 (±0.001)	0.003 (±0.00004)	0.004 (±0.0003)	0.002 (±0.0001)
10	0.098 (±0.001)	0.002 (±0.0003)	0.008 (±0.0001)	0.001(±0.0001)

Lyophilisation using 0.05M potassium phosphate buffer, pH 7-10
NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean ±SEM of triplicate determinations.

5.4.4 Lyophilisation of crude extract in the presence of different buffer systems

It was proposed that phosphate salts may render the hydantoinase and carbamylase enzymes inactive during lyophilisation. Buffers other than potassium phosphate were studied for their effect on enzyme activity during lyophilisation. Glycine-NaOH and Tris-HCl were chosen as they are suitable buffers for use at high pH. The results for Glycine-NaOH and Tris-HCl as alternatives to potassium

phosphate buffer are shown in Tables 5.4.4a and b.

Table 5.4.4a Lyophilisation of crude extract in the presence of Glycine-NaOH buffer

	before lyophilisation ($U.mg^{-1}$)	after lyophilisation ($U.mg^{-1}$)
NCA	0.012 (±0.0004)	0.002 (±0.0002)

0.05M Glycine-NaOH, pH10 as buffer. NCA (*N*-carbamylalanine). ALA (alanine) not determined due to interference in the Ninhydrin assay for amino acids. The data represent the mean ±SEM of triplicate determinations.

Table 5.4.4b Lyophilisation of crude extract in the presence of Tris-HCl buffer

	before lyophilisation ($U.mg^{-1}$)	after lyophilisation ($U.mg^{-1}$)
NCA	0.077 (±0.011)	0.027 (±0.001)
ALA	0.022 (±0.017)	0.003 (±0.0002)

0.05MTris-HCl, pH10 as buffer. NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean ±SEM of triplicate determinations.

5.4.5 Lyophilisation of RU-KM3$_L$ whole cells

Lyophilisation of whole cells was suggested as an alternative to lyophilisation of crude extract. Substrate and product transport, and hence enzyme activity may be retained as membranes would be intact, and intracellular pH would remain unaffected (D. Cowan, personal communication). The result of lyophilisation of whole cells is shown in Table 5.4.5.

Table 5.4.5 Lyophilisation of RU-KM3$_L$ whole cells

	before lyophilisation (µmoles/ml)	after lyophilisation (µmoles/ml)
NCA	19.43 (±0.29)	0.37 (±0.02)
ALA	2.05 (±0.22)	0.24 (±0.09)

Resting cell reaction conducted at 100mg cells per reaction.
Buffer conditions were modelled on those reported by Morin *et al* (1986b) for a *Pseudomonas putida* DSM 84 strain. NCA (*N*-carbamylalanine), ALA (alanine), µmoles/ml. The data represent the mean ±SEM of triplicate determinations.

5.4.6 The incorporation of additives during the lyophilisation of crude extract

The addition of enzyme-compatible solutes such as polyols, trehalose and ion exchange resins in the aqueous medium prior to lyophilisation was suggested as a means of preserving enzyme activity during lyophilisation (D. Cowan, personal communication). Initially, sucrose was used as an additive, as it had been used for the preservation of alcohol oxidase from *Pichia pastoris* (EC 1.1.3.13) (Zaks and Klibanov, 1988). In addition, the successful use of sugars as lyoprotectants had been reported (Dabulis and Klibanov, 1993 cited in Klibanov, 1997). The effect of sucrose on crude enzyme activity before and after lyophilisation are shown in Tables 5.4.6a and b respectively.

Table 5.4.6a The effect of sucrose on crude enzyme activity before lyophilisation

	Specific activity ($U.mg^{-1}$)				
	sucrose (%)				
	0	**1**	**10**	**30**	**50**
NCA	0.044	0.038	0.037	0.033	0.03
	(±0.001)	(±0.001)	(±0.005)	(±0.001)	(±0.001)
ALA	0.0019	0.0021	0.0016	0.0015	0.0011
	(±0.0003)	(±0.0001)	(±0.0004)	(±0.0004)	(±0.0002)

NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean ±SEM (SEM in parentheses) of triplicate determinations.

Table 5.4.6b The effect of sucrose on crude enzyme activity after lyophilisation

	Specific activity ($U.mg^{-1}$)				
	sucrose(%)				
	0	**1**	**10**	**30**	**50**
NCA	0.011	0.028	0.052	0.062	0.082
	(±0.0004)	(±0.002)	(±0.001)	(±0.002)	(±0.002)
ALA	0.001	0.0013	0.009	0.003	0.0033
	(±0.0001)	(±0.0001)	(±0.007)	(±0.0001)	(±0.0003)

NCA (*N*-carbamylalanine), ALA (alanine). The data represent the mean (±SEM) (SEM in parentheses) of experiments conducted in triplicate.

5.4.7 The effect of sucrose as an additive on the thermostability of lyophilised extract

The incorporation of additives during lyophilisation has been shown to decrease the thermoinactivation of enzymes (Triantafyllou *et al.*, 1997). It was proposed that sucrose may have the same effect. The effect of sucrose on thermostability of crude extract before and after lyophilisation is shown in Tables 5.4.7a and b respectively.

Table 5.4.7a The effect of sucrose on the thermostability of crude extract before lyophilisation

	Specific activity ($U.mg^{-1}$) **no sucrose** temperature (°C)			Specific activity ($U.mg^{-1}$) **sucrose (50%)** temperature (°C)		
	40	**50**	**60**	**40**	**50**	**60**
NCA	0.062	0.074	0.073	0.052	0.051	0.054
	(±0.00058)	(±0.00112)	(±0.00069)	(±0.00132)	(±0.00101)	(±0.00051)
ALA	0.0036	0.0031	0.0031	0.0026	0.0015	0.0016
	(±0.000029)	(±0.00004)	(±0.000156)	(±0.000217)	(±0.000157)	(±0.000246)

NCA (*N*-carbamylalanine), ALA (alanine). Table entries are mean specific activities (±SEM) (SEM in parentheses) of experiments conducted in triplicate.

Table 5.4.7b The effect of sucrose on the thermostability of crude extract after lyophilisation

	Specific activity ($U.mg^{-1}$) **no sucrose** temperature (°C)			Specific activity ($U.mg^{-1}$) **sucrose (50%)** temperature (°C)		
	40	**50**	**60**	**40**	**50**	**60**
NCA	0.03	0.022	0.014	0.082	0.089	0.076
	(±0.00065)	(±0.00046)	(±0.00049)	(±0.00267)	(±0.00160)	(±0.00113)
ALA	0.0016	0.0015	0.0013	0.0029	0.0036	0.0030
	(±0.000135)	(±0.000022)	(±0.000073)	(±0.000489)	(±0.000502)	(±0.000181)

NCA (*N*-carbamylalanine), ALA (alanine). Table entries are mean specific activities (±SEM) (SEM in parentheses) of experiments conducted in triplicate.

Discussion

The pH profile for the crude extract was determined using a potassium phosphate buffer system as it was of concern that unpredictable buffer effects by other buffers would occur in the organic solvent studies. As suggested in Figure 5.2, pH 9-11 was the range for activity of the hydantoinase enzyme, with pH10 as the optimum.This result was comparable with the optimal pH of 9 in Tris-HCl buffer reported in this study (section 3.8). Similarity to pH optima reported in the literature was observed (Runser and Ohleyer, 1990, Durham and Weber, 1995). The data suggest that the carbamylase enzyme activity was in the pH range 9-12, although it did not differ significantly from other pH points. The observed activity of the hydantoinase enzyme at high temperature and in alkaline conditions may be exploited to effect spontaneous racemisation of substrates, and hence complete

enzymic conversion of substrate to products.

Lyophilisation of crude extract in aqueous medium at pH10 was attempted. Lyophilisation under these conditions had a detrimental effect on the activity of the hydantoinase enzyme (Table 5.3). The specific activity was decreased to about a tenth of the original activity by the lyophilisation conditions used, and the activity of the carbamylase activity was too low to justify comment. A number of alternative approaches to obtain active lyophilised crude extract were taken.

Concentration of crude extract prior to lyophilisation did not stabilize the enzymes so that activity could be preserved during lyophilisation (Table 5.4.1). The hydantoinase specific activity after lyophilisation was observed to be 14% of the original activity. The activity of the carbamylase enzyme was low before and after lyophilisation, and this was possibly due to enzyme instability.

It was suggested that an enzyme-inhibitory compound may be concentrated and/or activated during the lyophilisation process, and that dialysis of the extract before lyophilisation could remove the inhibitory compound. As shown in Table 5.4.2, dialysis does not protect the hydantoinase enzyme from inactivation during lyophilization. This implies that a possible inhibitor was not present, or was not removed by dialysis.

Changes in pH and increases in ionic strength that accompany lyophilisation could render enzymes unstable (D. Cowan, personal communication). Therefore lyophilisation under conditions of lower ionic strength (10 and 50mM) over pH 7-10 range was explored. Irrespective of the ionic strength,

significant loss of enzyme activity occurred during lyophilisation, and did not show a trend over the pH range in question (Tables 5.4.3a and b). However, maximum hydantoinase activity at pH10 was reproducible, and was observed using 50mM potassium phosphate buffer (Table 5.4.3b). Much lower hydantoinase activity was observed using 10mM potassium phosphate buffer before and after lyophilisation (Table 5.4.3a). Carbamylase activity was too low to justify comment. Thus use of the buffer at 50mM ionic strength was selected for subsequent experiments, as this buffer ionic strength was found to be suitable for enzyme activity.

Possible inactivation by phosphate salts was suspected, and hence lyophilisation experiments were carried out using Glycine-NaOH and Tris-HCl as buffers. Glycine-NaOH (Table 5.4.4a) did not protect the hydantoinase enzyme from inactivation during lyophilisation, and activity was observed to be low before lyophilisation. A negative feedback effect by the glycine for an accumulation of *N*-carbamyl alanine which would in turn inhibit the hydantoinase enzyme is possible. The carbamylase enzyme activity was not assayed due to interference by glycine in the Ninhydrin assay for amino acids. Tris-HCl buffer did not protect either of the enzymes from inactivation during lyophilisation (Table 5.4.4b). The use of 50mM potassium phosphate buffer at pH10 was re-considered.

It was suggested that lyophilisation of whole cells would be an alternative to lyophilisation of crude extract, but the amounts of *N*-carbamyl alanine and alanine produced by lyophilised whole cells were low (Table 5.4.5).

Addition of sucrose as an additive prior to lyophilisation was observed to retain enzyme activity.

However, an inhibitory effect on both the hydantoinase and carbamylase enzymes was observed in the presence of increasing sucrose concentration, before lyophilisation (Table 5.4.6a). This relationship was reversed upon the assay of extract which had been lyophilised in the presence of increasing amounts of sucrose. Therefore sucrose was used as an additive to preserve enzyme activity during lyophilisation in subsequent experiments.

The lyoprotective effect by sucrose was similar to that reported for salts such as KCl (Khmelnitsky *et al.*, 1994). KCl was suggested to maintain native enzyme structure during lyophilisation of α-chymotrypsin and subtilisin Carlsberg, and it is proposed, here, that sucrose had the same effect. Triantafyllou *et al.* (1997) proposed that the stability conferred by additives during lyophilisation is due to an involvement by the additive in the distribution of water around protein. Griebenow and Klibanov (1997) suggested that it is the secondary structure of proteins which is preserved during lyophilization in the presence of salts such as KCl, and this may be responsible for the increased activity after lyophilisation observed here.

Additives incorporated during lyophilisation have been shown to decrease the thermoinactivation of enzymes (Triantafyllou *et al.*, 1997). It was proposed that sucrose may have the same effect on the hydantoinase enzyme, and this was investigated in section 5.4.7. As suggested by the results shown in Table 5.4.7a, the optimum temperature for hydantoinase activity before lyophilisation was observed to be 60°C in the presence of sucrose. This optimum shifted to 50°C after lyophilisation (Table 5.3.7b). Sucrose was therefore not observed to offer an increase in the thermostability of the enzymes of the lyophilised crude extract. However, the stabilizing effect by sucrose at 50% (w/v) on

hydantoinase enzyme activity during lyophilisation was reproducible.

Conclusion

The optimal conditions for obtaining the crude extract in lyophilised form for use in water-immiscible organic solvent studies may be summarized as follows:

a) 0.05M potassium phosphate buffer, pH10

b) 50% (w/v) sucrose as lyophilisation additive

c) assay of lyophilised sucrose-containing extract at 50°C.

CHAPTER 6: ORGANIC SOLVENT STUDIES

Reaction conditions for producing lyophilized crude extract had been optimized, and therefore RU-$KM3_L$ could be cultured on a large scale to afford a suitable batch of lyophilized, active crude enzyme extract for use in water-immiscible organic solvent studies. These studies would entail characterizing the hydantoinase and carbamylase activities with respect to substrate specificity, reversal of the hydantoinase reaction, partition of substrate and/or product into hydrophobic organic solvents, and the possibility of peptide synthesis. However studies on enzyme activity in water-miscible organic solvents were conducted on unlyophilized ("wet") extract first, considering the effects of different concentrations of water-miscible solvents, and whether the solubility of substrates such as *p*-hydroxyphenylhydantoin, which are poorly soluble in aqueous media, could be enhanced in the presence of these solvents.

Materials and method

6.1 Chemicals

Hydantoin, 5,5-dimethylhydantoin (DMH), 5-benzylhydantoin (BH), *N*-carbamylglycine (NCG), *N*-carbamyl-D,L-alanine (NCA), *N*-carbamylphenylalanine (NCpheALA), glycine (GLY), D,L-alanine (ALA) and phenylalanine (PHE) were purchased from Sigma Chemicals. 5-methylhydantoin (MH) was synthesized by the Bucherer-Bergs method (section 3, Appendix). All other reagents were of analytical grade.

6.2 Water-miscible organic solvent studies

6.2.1 The effect of water-miscible organic solvents at 1-40% (v/v) concentration

Nutrient broth (700ml) supplemented with 1% hydantoin was inoculated with cells cultured on basal medium plates. Induction of cells was continued to early stationary phase prior to harvesting of cells by centrifugation. Cell resuspension was conducted in 52.5ml 0.05M cold potassium phosphate buffer, pH10. Cells were disrupted by French Press, and the extract was made to 87.5ml with buffer. Incubation of 1ml aliquots of extract was conducted at 50°C for 3 hours in the presence of 50mM MH (5-methylhydantoin) and organic solvents methanol, acetonitrile, acetone, ethanol, or 2-propanol at 1, 10 or 40% (v/v) in capped vials. Products and protein concentrations were determined as in section 3.1.4.

6.2.2 The effect of water-miscible organic solvents at 1-10% (v/v) concentration

The crude extract was prepared and assayed as in 6.2.1, with the exception that the solvents used were at 1, 5 or 10% (v/v) concentration.

6.3 Water-immiscible organic solvent studies

6.3.1 Substrate and product solubilities in water-immiscible organic solvents

Solvents TE (triethylamine), HXL (1-hexanol), CHL (chloroform), TOL (toluene), PEN (n-pentane), HEX (hexane), HEP (n-heptane) and OCT (n-octane), chosen on the basis of increasing log P value (hydrophobicity), were added to substrates HYD (hydantoin), MH (5-methylhydantoin), DMH (5,5-

dimethylhydantoin), BH (5-benzylhydantoin) and products NCG (*N*-carbamylglycine), NCA (*N*-carbamyl alanine), GLY (glycine) and ALA (D,L-alanine) in powder form for 50mM concentration in 1ml solvent (or 20mM for GLY and ALA). Mixing of test tube contents was achieved by shaking by hand at room temperature. 1ml 0.05M potassium phosphate buffer was added to test tubes with solvent and NCG, or NCA for mixing by hand. 0.5ml acidic 10% *p*-dimethylaminobenzaldehyde solution was added to the tubes containing the *N*-carbamylamino acids to determine which phase (aqueous or organic) they had partitioned into.

6.3.2 The effect of water-immiscible organic solvents on substrate specificity of lyophilised RU-KM3$_L$ crude extract

Lyophilized crude extract was suspended in 1ml 50mM HYD or MH or DMH or BH at 1g enzyme powder/ml substrate. An equal volume of water-immiscible solvent was added, and incubation was conducted with shaking at 50°C for 3 hours in capped vials. After incubation, vial contents were allowed to settle at room temperature, and the supernatant of samples taken from the aqueous phase was analyzed for product formation. Controls in the absence of solvent were run in the 0 to 50mM or 20mM *N*-carbamylamino acid or amino acid range respectively and were used to construct a calibration curve against which the solvent-containing reaction mixtures were read. Product and protein concentrations were determined as described in section in 3.1.4.

6.3.3 Reversal of the hydantoinase reaction by water-immiscible organic solvents

Lyophilized crude extract was suspended in 20mM D,L-alanine or 50mM NCA at 1g/ml. 1ml of organic solvent was added to an equal volume of extract and substrate, and the reaction mixture was

incubated with shaking for 3 hours at 50°C in capped vials. After incubation, reaction mixtures were allowed to settle at room temperature, and samples for analysis were taken from the aqueous layer. Controls were run simultaneously, and calibration curves were constructed and used as described in section 6.3.2. Quantification of 5-methylhydantoin was carried out by high performance liquid chromatography (HPLC) using a Macherey Nagel Reverse-Phase Nucleosil 100-5, C-18 column. The flow rate was 1ml/min with detection at 220nm. The mobile phase used was degassed, 0.22μm-filtered acetonitrile/water (1:1). All samples were filtered through 0.22μm filters, and injected manually into a 20μl loop. NCA and protein concentrations were determined as described in section 3.1.4.

6.3.4 The investigation of partition effects on enzyme activity by alteration of aqueous phase:solvent phase ratio

10ml organic solvent were added to 500μl crude extract in 50mM MH, to give a 1:20 ratio. Incubation was conducted with shaking for 3 hours at 50°C in capped vials. 8ml of the organic solvent were then evaporated off using a gentle stream of nitrogen gas. 1ml 0.05M potassium phosphate buffer, pH10 was used to reconstitute any residue. MH in the reconstituted residue was quantified by HPLC as in section 6.3.3. NCA and ALA concentrations in the reconstituted residue were determined as in section 3.1.4

6.3.5 Peptide synthesis by crude extract in the presence of water-immiscible organic solvents

2ml aliquots of lyophilized extract in 20mM D,L-alanine were incubated with an equal volume of organic solvent at 50°C for 3 hours with shaking in capped vials. After settling, the aqueous phase

was sampled and analyzed for peptide formation by the Biuret method (section 2.1.1, Appendix).

Specific activity (SA) was expressed as units per milligram of protein, where one unit (U) was defined as the amount of enzyme required to produce 1μmol of product in one minute under the specified conditions. The data is presented as specific activity, unless otherwise stated.

Results

6.2 Water-miscible organic solvent studies

6.2.1 The effect of water-miscible organic solvents at 1-40% (v/v) concentration

The effect of water-miscible organic solvents was tested on the biocatalytic activity of unlyophilised ("wet") RU-KM3$_L$ crude extract, and the result is shown in Figure 6.2.1.

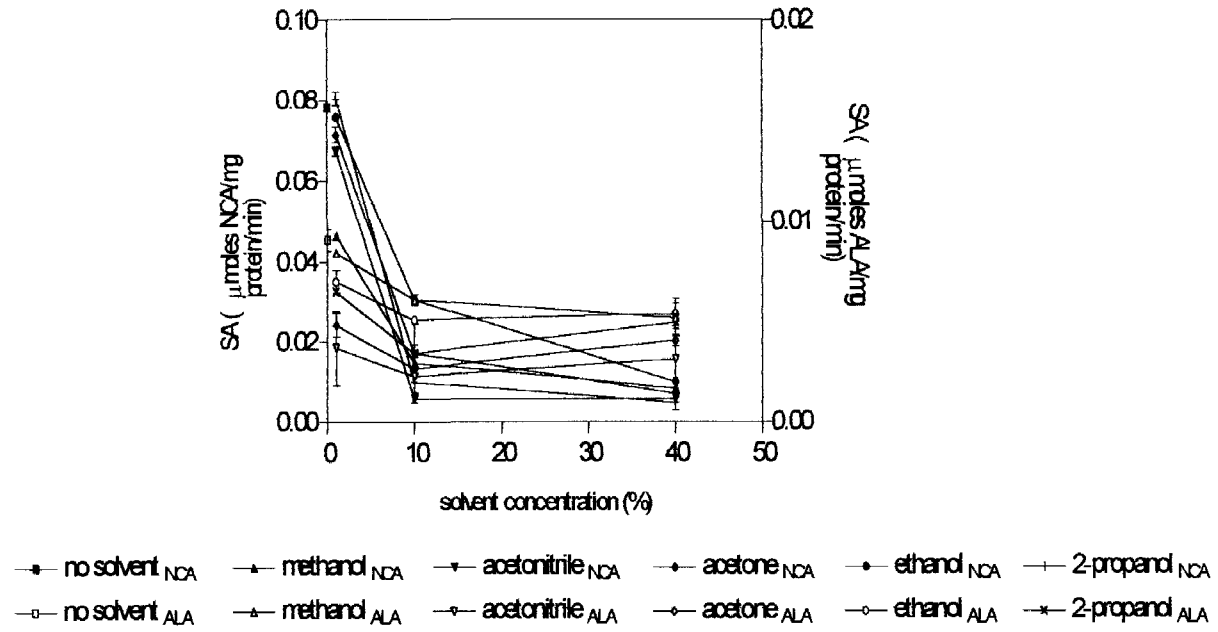


Figure 6.2.1 Conversion of MH by RU-KM3$_L$ crude extratct in the presence of water-miscible organic solvents at 1-40% (v/v) concentration. NCA (*N*-carbamylalanine), ALA (alanine). These date represent the mean ±SEM of triplicate determinations.

6.2.2 The effect of water-miscible organic solvents at 1-10% (v/v) concentration

It was thought that the optimal solvent concentration for enzyme activity may occur between 1 and 10%, at which conversion of substrate would be greater than in the absence of solvent. The effect of 1-10% (v/v) water-miscible organic solvent on substrate conversion by the hydantoinase and carbamoylase enzymes is shown in Figure 6.2.2.

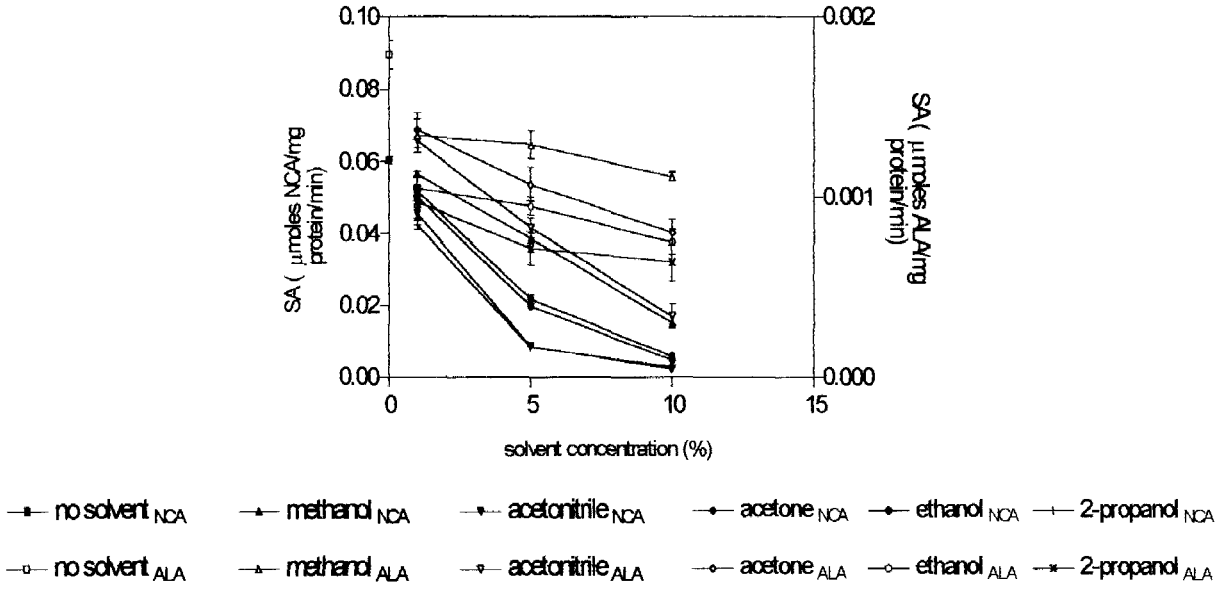


Figure 6.2.2 Conversion of MH by RU-KM3$_L$ crude extract in the presence of water-miscible organic solvents at 1-10% (v/v) concentration. NCA (*N*-carbamylalanine), ALA (alanine). These data represent the mean ±SEM of triplicate determinations.

6.3 Water-immiscible organic solvent studies

6.3.1 Substrate and product solubilities in water-immiscible organic solvents

Establishing whether substrates and products were soluble in water-immiscible organic solvents was important for the experimental design. If solubility was demonstrated, the substrates could be dissolved in these solvents for conversion to products by crude extract suspended in the reaction mixture. Biocatalyst could be removed by centrifugation or filtration to afford a solvent supernatant or filtrate containing the products of interest. The solvent could be evaporated off, the residue reconstituted in buffer, and products determined.

The substrates were found to be insoluble in the different organic solvents. The *N*-carbamylamino acids had partitioned into the lower aqueous layer for all solvents except chloroform (upper aqueous layer). This was deduced from the appearance of the yellow colour formed by reaction with the *p*-dimethylaminobenzaldehyde solution.

6.3.2 The effect of water-immiscible organic solvents on substrate specificity of lyophilised RU-KM3$_L$ crude extact

The conversion of various substrates by biocatalyst in the presence of water-immiscible organic solvent was investigated in a two-phase, aqueous-organic system, with substrates dissolved in the aqueous phase. The result of incubation of extract with substrate in the presence of water-immiscible organic solvent is shown in Figure 6.3.2.

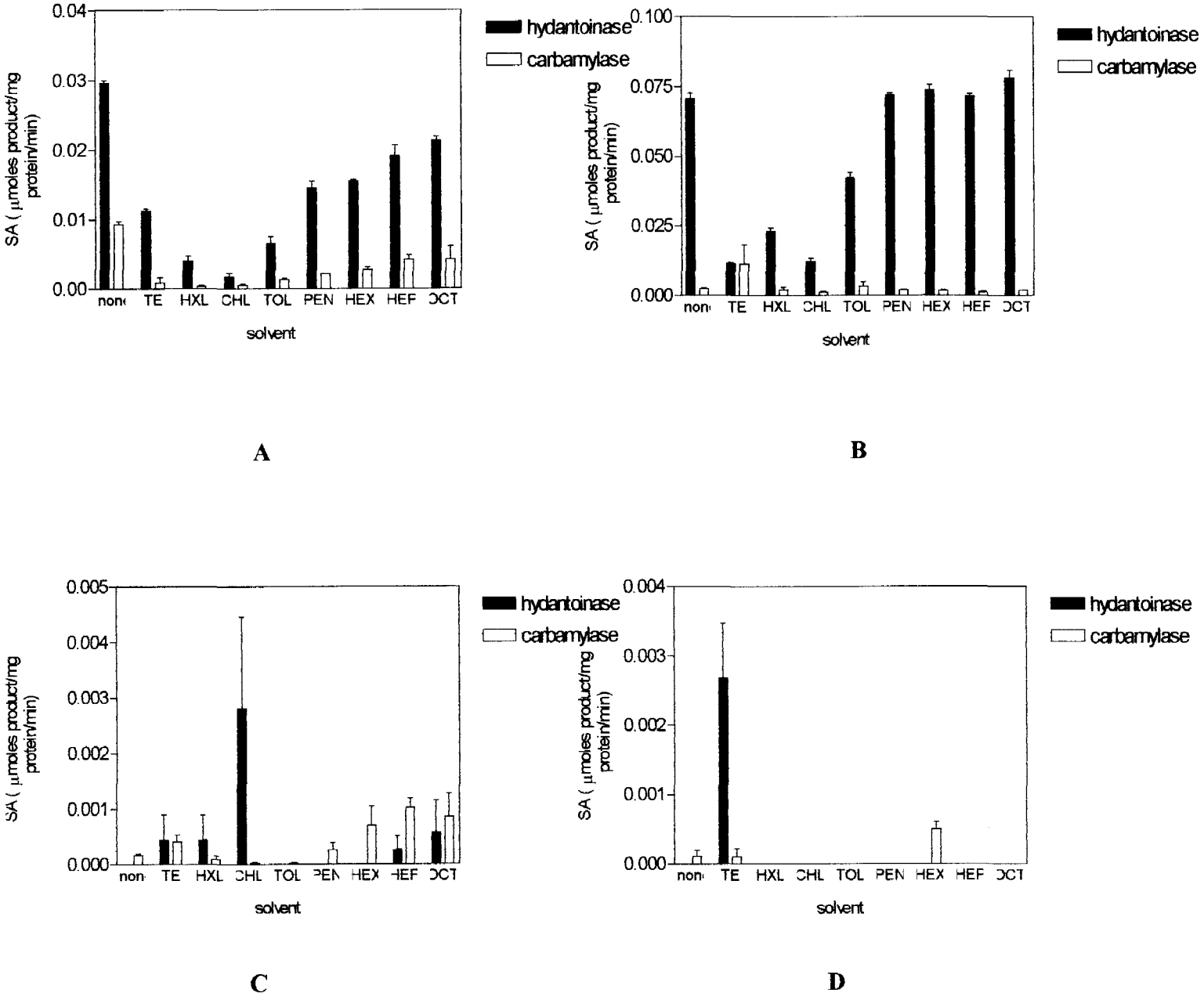


Figure 6.3.2 Conversion of substrate by crude extract in the presence of water-immiscible organic solvent. (A) HYD (hydantoin) as substrate (B) MH (5-methylhydantoin) as substrate (C) DMH (5,5-dimethylhydantoin) as substrate (D) BH (5-benzylhydantoin) as substrate. None (no solvent), TE (triethylamine), HXL (1-hexanol), CHL (chloroform), TOL (toluene), PEN (n-pentane), HEX (hexane), HEP (n-heptane) and OCT (n-octane). These date represent the mean ±SEM of triplicate determinations.

6.3.3 Reversal of the hydantoinase reaction by water-immiscible organic solvents

The possibility of reversing the hydantoinase reaction using alanine and NCA as substrates for the carbamylase and hydantoinase enzymes was considered, and it was envisaged that NCA and MH would be the respective products formed.The result for reversal of the hydantoinase reaction by water-immiscible organic solvents using substrates D,L-alanine (expect the carbamylase enzyme to yield NCA and the hydantoinase enzyme to yield MH) or NCA (expect the hydantoinase enzyme to yield MH) is shown in Tables 6.3.3a and b respectively.

Table 6.3.3a Reaction reversal using D,L-alanine as substrate

Solvent	Specific activity ($U.mg^{-1}$)	
	NCA[a]	MH[b]
no solvent	0	0.011402
TE	0	0
HXL	0	0.0074
CHL	0	0.022377
TOL	0	0.001541
PEN	0	0.003921
HEX	0	0.006654
HEP	0	0.003898
OCT	0	0.002461

NCA[a] (*N*-carbamylalanine) representing the specific activity of the the carbamylase enzyme for NCA production, MH[b] (5-methylhydantoin) representing the specific activity of the hydantoinase enzyme for MH production. TE (triethylamine), HXL (1-hexanol), CHL (chloroform), TOL (toluene), PEN (n-pentane), HEX (hexane), HEP (n-heptane) and OCT (n-octane).

Table 6.3.3b Reaction reversal using NCA as substrate

Solvent	Specific activity ($U.mg^{-1}$)
	MH
no solvent	0.035 (±0.0068)
TE	0.004 (±0.0037)
HXL	0.003 (±0.0029)
CHL	0.005 (±0.0045)
TOL	0.004 (±0.0039)
PEN	0.016 (±0.0112)
HEX	0.026 (±0.0218)
HEP	0.006 (±0.0041)
OCT	0.02 (±0.0198)

MH[a] (5-methylhydantoin) representing the specific activity of the hydantoinase enzyme for MH production. TE (triethylamine), HXL (1-hexanol), CHL (chloroform), TOL (toluene), PEN (n-pentane), HEX (hexane), HEP (n-heptane) and OCT (n-octane). These date represent the mean ±SEM of triplicate determinations.

6.3.4 The investigation of partition effects on enzyme activity by alteration of aqueous phase:solvent ratio

Changing aqueous phase:solvent phase ratios was envisaged as a possible way to effect substrate and/or product partitioning into the organic phase. This would in turn affect reaction rates by promoting substrate extraction and therefore unavailability of substrate to the biocatalyst, or product removal from the vicinity of the biocatalyst and therefore averting product inhibition. The result of using a 1:20 biocatalyst/substrate:organic solvent ratio on MH conversion to NCA and alanine is shown in Table 6.3.4.

Table 6.3.4 MH conversion by a 1:20 biocatalyst/substrate: organic solvent ratio

	Amounts (μmoles/ml)			
	PEN	HEX	HEP	OCT
MH	0.032	0.004	0.007	0
NCA	0	0	0	0
ALA	0	0.002	0	0

MH (5-methylhydantoin), NCA (*N*-carbamylalanine), ALA (D,L-alanine) in μmoles/ml, PEN (n-pentane), HEX (hexane), HEP (n-heptane), OCT (n-octane).

6.3.5 Peptide synthesis by crude extract in the presence of water-immiscible organic solvents

It was thought that peptide synthesis in the presence of organic solvents may be possible by formation of amino acid residue chains, and the result is shown in Table 6.3.5.

Table 6.3.5 Peptide synthesis by crude extract in the presence of organic solvent

	no solvent	TE	HXL	CHL	TOL	PEN	HEX	HEP	OCT
peptide (mg/ml)	0	0	0	0	0	0	0.411	0.401	0

TE (triethylamine), HXL (1-hexanol), CHL (chloroform), TOL (toluene), PEN (n-pentane), HEX (hexane), HEP (n-heptane) and OCT (n-octane).

Discussion

The conversion of substrate by "wet" (unlyophilized) crude extract in the presence of water-miscible organic solvents was studied. As indicated in Figure 6.2.1, water-miscible solvents inhibited enzyme activity at higher concentration, and this inhibition was more pronounced for the hydantoinase enzyme than the carbamylase enzyme. Addition of 2-propanol appeared to increase the hydantoinase activity when compared to the control. The possibility of optimal activity between 1 and 10% water-miscible organic solvent was investigated, because it was thought that there may be an optimal water-miscible solvent concentration between 1 and 10% (v/v) at which conversion of substrate would be greater than in the absence of solvent. As suggested in Figure 6.2.2, 1% (v/v) organic solvent was observed to have the least lowering effect on activity, for the solvents tested. Water-miscible organic solvents at less than 1% (v/v) could be tested, but a significant effect at such low concentrations is unlikely, and not useful. Thus study of the effect of these solvents on enzyme activity was not considered further.

The detrimental effect on both enzyme activities by water-miscible organic solvents was expected, due to partitioning of the solvent into the membrane causing disruption of membrane-associated activities, or displacement of boundary phospholipids necessary for function of membrane-associated enzymes. Such displacement might result in unfavourable conformation and orientation of enzymes. The amount of solvent required for this effect would be very low, possibly less than 1% (v/ v). Loss of enzyme activity through "stripping" of the essential water monolayer around the protein by organic solvents is also possible, especially at high concentrations of the organic solvents.

It was essential to establish whether substrates and products were soluble in the water-immiscible solvents of choice, because knowledge of the solubility of these compounds would allow for assays in the appropriate medium. The observed insolubility of the substrates and products in water-immiscible organic solvents suggested the use of a two-phase system, where buffer and organic solvent would be used in the same reaction vessel at a ratio of 1:1, as a first approach. The buffer phase would contain dissolved substrate and suspended biocatalyst. As the *N*-carbamyl amino acids were found to occur in the aqueous phase, samples for analysis were taken from this phase. This approach was also used for the amino acids owing to their hydrophilic nature.

The effect of water-immiscible solvents on the conversion of hydantoin, 5-methylhydantoin, 5,5-dimethylhydantoin and 5-benzylhydantoin was investigated. Conversion of hydantoin, as measured in the aqueous phase, was observed to be proportional to the hydrophobicity of the organic solvents present as solvent phase (Figure 6.3.2A). The more hydrophobic solvents were not deleterious with regard to enzyme activity, but activity in the most hydrophobic solvent (n-octanol) did not exceed that in aqueous medium. The same trend was observed when 5-methylhydantoin was used as substrate (Figure 6.3.2B) except that (i) conversion was greater and (ii) conversion in the presence of n-pentane, hexane, n-heptane and n-octane was similar and slightly higher than in aqueous medium. 5,5-dimethylhydantoin was poorly metabolized in aqueous medium, and it was thought that its conversion would be improved in the presence of water-immiscible organic solvents (Figure 6.3.2C). Chloroform and n-octanol were the only two solvents in the presence of which hydantoinase activity was observed to be higher than that of the aqueous medium. Carbamylase activity was detected in the presence of the final four organic solvents (n-pentane, hexane, n-heptane, and n-octane), and was

observed to be substantially higher than in only aqueous medium. 5-benzylhydantoin was not readily soluble in aqueous medium, and had to be heated to dissolve, the effect of which may have rendered it non-reactive. Poor hydrolysis of 5-benzylhydantoin was observed in the presence of all organic solvents with the exception of triethylamine. It is possible that solvents less hydrophobic than triethylamine, for example DMSO, may be suitable as organic media for substrates such as 5-benzylhydantoin and *p*-hydroxyphenylhydantoin that are not readily soluble in aqueous media.The occurrence of carbamylase activity in the presence of hexane was ascribed to experimental error (Figure 6.3.2D).

The biocatalyst and substrate were present mainly in the aqueous phase, suggesting that the results observed were due to reaction(s) occurring in the aqueous phase or at least at the liquid-liquid interphase, and not in the organic phase. It is proposed that the more hydrophobic solvents did not gain access to the membrane interior to exert an effect. In the event that contact with protein did occur, stripping of the monolayer of water would not be achieved by these water-immiscible organic solvents. Saturating aqueous phase concentrations of the more hydrophobic solvents may have no effect on enzyme activity, since they would not reach a critical toxic membrane concentration required to exert an effect. A similar argument has been proposed by Osborne *et al.* (1990) for the effect of organic solvents on the 11α-hydroxylase enzyme from *Rhizopus nigricans*. Maximum aqueous phase concentration of organic solvents is to some extent inversely proportional to carbon chain length, so that the effects of triethylamine, 1-hexanol, chloroform and perhaps toluene (these solvents have shorter carbon chain lengths) are due possibly to direct contact of these solvents with the membrane for a deleterious effect (a feature characteristic of low log P value solvents), or the hydrophobic

protein regions after accessing the aqueous phase.

Condensation reactions may be favoured over hydrolysis-type reactions in the presence of organic solvents (Dordick, 1989). It was thought that organic solvents could effect the catalysis of NCA and 5-methylhydantoin production by carbamylase and hydantoinase enzymes using D,L-alanine and NCA respectively as substrates. Table 6.3.3a shows that NCA was not produced when ALA was used as the substrate in the presence of organic solvent. The amounts of MH produced under these conditions were lower compared to MH production when NCA was used as substrate (compare Table 6.3.3b). A trend correlating MH production and solvent hydrophobicity was not observed.

It was thought that changing aqueous:solvent phase ratios may affect conversion rates by effecting partitioning of substrate or product into the organic phase. The data in Table 6.3.4 indicates that negligible amounts of MH were detected in n-pentane, hexane and n-heptane, and it was not detected in n-octane. NCA was not detected in any of the solvents. Negligible amounts of D,L-ALA were detected in hexane, and none in n-heptane or n-octane. The very low amounts extracted into organic phase could be explained by considering that the substrates and products are polar, and would not normally partition into non-polar solvents. Thus it is proposed that the amounts extracted into the organic phases would not influence reaction rates.

It was thought that peptide synthesis by formation of amino acid residue chains might be possible in the presence of organic solvent, and peptide synthesis appeared to have occurred in the presence of hexane and n-heptane (Table 6.3.5) although the amounts observed were relatively low.

Conclusion

The data showed that substrate conversion was significantly reduced in the presence of water-miscible organic solvents at concentrations in excess of 1%. The activity of the hydantoinase and carbamylase enzymes in the presence of water-immiscible organic solvents was not significantly higher than in aqueous medium alone. The substrate specificity of the hydantoinase and carbamylase enzymes was not altered by the presence of organic solvent. The hydantoinase reaction was apparently reversed by the more hydrophobic organic solvents. It was shown that the presence of water-immiscible organic phase in excess of aqueous phase did not result in sufficient substrate or product extraction into the organic phase to affect reaction rate. The amounts of peptide measured were not sufficiently high to allow the confident statement that peptide synthesis had occurred.

GENERAL DISCUSSION

Initially, this study was aimed at isolating thermophilic hydantoinase-producing bacterial organisms, and characterising the thermostable enzymes isolated from them. The isolation of a number of thermophilic organisms was achieved, but the capability of these strains to hydrolyse hydantoin was low, even when resting cell reaction conditions were varied. The initial enrichment conditions were identified as an area requiring considerable attention with respect to optimization, and in view of this, an alternative approach was taken. The biotransformation conditions for the activity of the hydantoinase and carbamylase enzymes of a previously-isolated mesophilic strain, RU-KM3$_L$, were thus optimised, with respect to the induction of the enzymes, substrate specificity, temperature for enzyme activity, optimal time for conduction of resting cell reactions, pH requirements and enzyme activity in the presence of metal ions. The specific activity of crude extract of RU-KM3$_L$ achieved under optimised conditions was 0.5-0.6 U.mg^{-1}, which would give a 40-45% conversion of 5-methylhydantoin in 3h.

The hydantoinase and carbamylase enzymes of RU-KM3$_L$ were suspected to be membrane-associated, and various methods were investigated to obtain active enzyme in fractions without cell debris. Sonication proved successful as a means of separating enzyme activity from cell debris-containing fractions. The release of the hydantoinase enzyme by sonication requires further study, especially with respect to maintenance of the activity of the released enzyme at alkaline pH. It was not considered a disadvantage that the use of detergents to release enzymes was unsuccessful, in that removal of detergents would be difficult, and could lead to loss of enzyme activity during the detergent removal process.

It was observed that losses in enzyme activity were caused by treatments where lyophilisation was used to obtain active enzyme in lyophilised form. The preservation of enzyme activity during the lyophilisation of crude extract was achieved by the incorporation of 50% sucrose. In the absence of sucrose, hydantoinase and carbamylase activities were decreased by 25 and 53% respectively, after lyophilisation. However, upon the addition of 50% sucrose to the fractions to be lyophilised, the hydantoinase and carbamylase enzyme activities were observed to be increased by 54 and 58% respectively. This successful result facilitated the use of active crude extract in powder form as the biocatalyst in organic solvent studies.

Water-miscible organic solvents at 1% concentration were shown to inhibit enzyme activity, and this effect was observed to increase with increase in organic solvent concentration. The proposals that (a) these solvents removed the essential water layer from around the hydantoinase and carbamylase enzymes to cause loss in activity, or (b) these solvents reached a critical concentration in the membrane to inhibit enzyme activity, are in agreement with current, published opinions.

Substrate conversion in the presence of water-immiscible organic solvents was observed to be proportional to the hydrophobicity (log P) of the solvents selected. Since an aqueous phase-solvent phase heterogeneous system was used, it was proposed that less interference by the more hydrophobic solvents would occur in the reaction, and indeed this trend was observed. The hydrophilicity of the substrates used was of major concern, as this could influence any possible effect by the hydrophobic solvents. The hydrophilicity of the substrates used would restrict them to the aqueous phase, and this would favour catalysis of the hydantoinase reaction in that phase. The substrates available (hydantoin, 5-methylhydantoin, 5,5-dimethylhydantoin, and 5-benzylhydantoin) are commonly used to characterise hydantoinase and carbamylase activities, but it would be useful

to consider more hydrophobic substrates in further studies.

The reversal of the hydantoinase reaction was attempted, and the results suggest that this is possible. The partitioning of substrates into the organic phase was investigated, and was shown not to affect the rate of the hydantoinase reaction. The possibility of peptide synthesis was investigated, but was shown not to occur in significant amounts. However, the reversal of the hydantoinase reaction and the possibility of synthesizing peptides will require further study.

It would be of interest to confirm the suggested association of the enzyme activities with the cell membrane, as this would be a novel result. All other literature has reported the association of the enzyme activities with the supernatant fraction of the crude cell extract. The requirement of up to 50% sucrose for the retention (at least) and activation of hydantoinase and carbamylase enzyme activities during the lyophilisation of crude enzyme extract is also proposed to be novel.

APPENDIX

1. Media/ reagents

1.1 Basal medium broth/ plates (HMM, hydantoin minimal medium)

10x M9 salts[a]	100ml
40% glucose	25ml
4% hydantoin	250ml
trace elements[b]	10ml
1M $MgCl_2$	200µl
1M $CaCl_2$	200µl
distilled water	115ml

10x M9 salts[a]

per litre deionized water, autoclaved separately:

Na_2HPO_4	300g
KH_2PO_4	150g
NaCl	25g

trace elements[b]

per litre deionized water

boric acid	50mg
$MnSO_4 \cdot 7H_2O$	40mg

$ZnSO_4$	40mg
$(NH_4)_6MO_7O_{24}\cdot 4H_2O$	20mg
KI	10mg
$CUSO_4$	4mg

Dissolve all components in 990ml deionized water and autoclave. Make up and autoclave separately 20mg $FeCl_3$ in 10ml deionized water. Add to other components of trace elements solution.

1.2 Biuret reagent (Plummer, 1987)

Dissolve 3g $CuSO_4\cdot 5H_2O$ and 9g $NaKC_4H_4O_6\cdot 4H_2O$ (sodium potassium tartrate) in 500ml 0.2M NaOH. Add 5g KI and make up to 1L with 0.2M NaOH.

BSA (bovine serum albumin) stock: 5mg/ml in distilled water.

1.3 Bradford reagent (Bradford, 1976; Bollag *et al.*, 1996)

Dissolve 100mg Coomassie Brilliant Blue G-250 in 50ml 95% ethanol. Add 100ml 85% phosphoric acid. Make up to 1L with distilled water. Filter using Whatman No 1filter paper. Store at 4°C.

BSA (bovine serum albumin) stock: 0.5mg/ml in distilled water.

1.4 p-dimethylaminobenzaldehyde solution (Ehrlich's reagent)

Make up 10% in 6N HCl

1.5 Ninhydrin reagent (Plummer, 1987)

Prepare fresh:

Dissolve 0.8g Ninhydrin (triketohydrindene hydrate) and 0.12 hydrindantin in 30ml methyl cellosolve (ethylene glycol monomethyl ether). Add 10ml 4M acetate buffer, pH5.5 and store in a brown bottle.

2. Analytical

2.1 Colorimetric methods

2.1.1 Biuret method (Plummer, 1987)

Add 3ml Biuret reagent to 2ml protein sample. Mix immediately and incubate at 37°C for 10 minutes. Read absorbance at 540nm against a calibration curve of BSA standards in the 0-5mg/ml protein range.

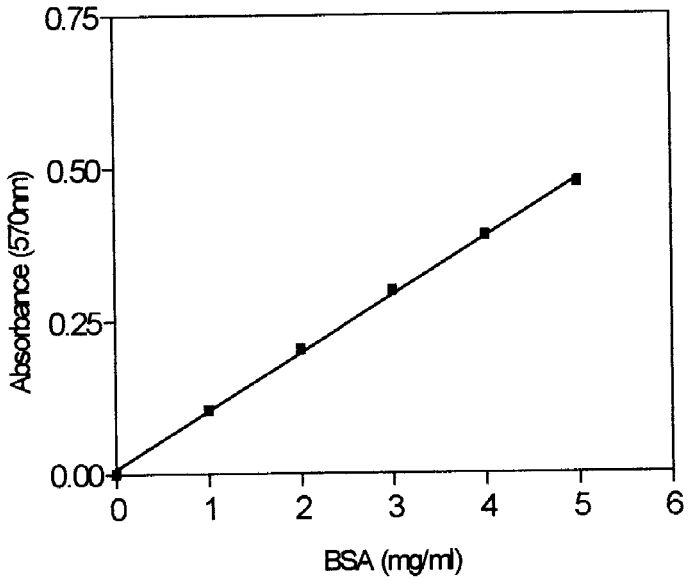


Figure 1 Standard curve for the Biuret method for peptide determination, r^2=0.99874. The data represent the mean ±SEM of duplicate determinations.

2.1.2 Bradford protein binding assay (Bradford, 1976; Bollag *et al.*, 1996)

Add 1ml to Bradford reagent to protein sample made to 100µl with 0.15M NaCl. Incubate at room temperature for 2 minutes. Read absorbance at 595nm against a calibration curve of BSA standards in the 0-20µg protein range.

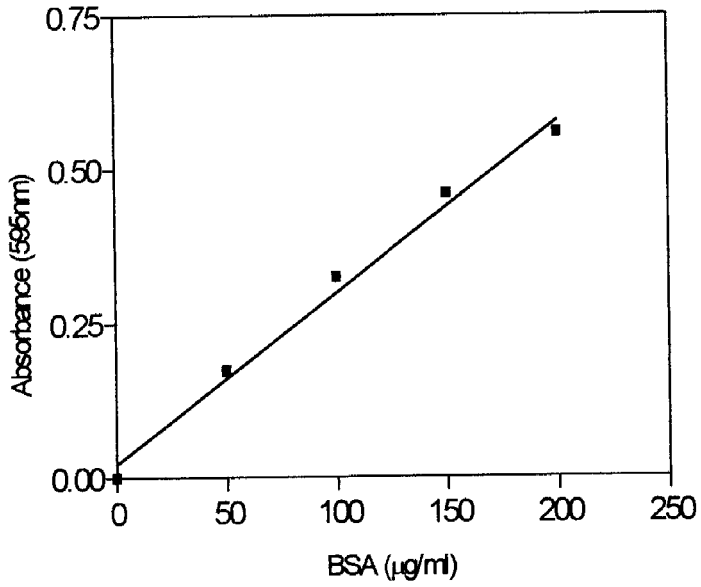
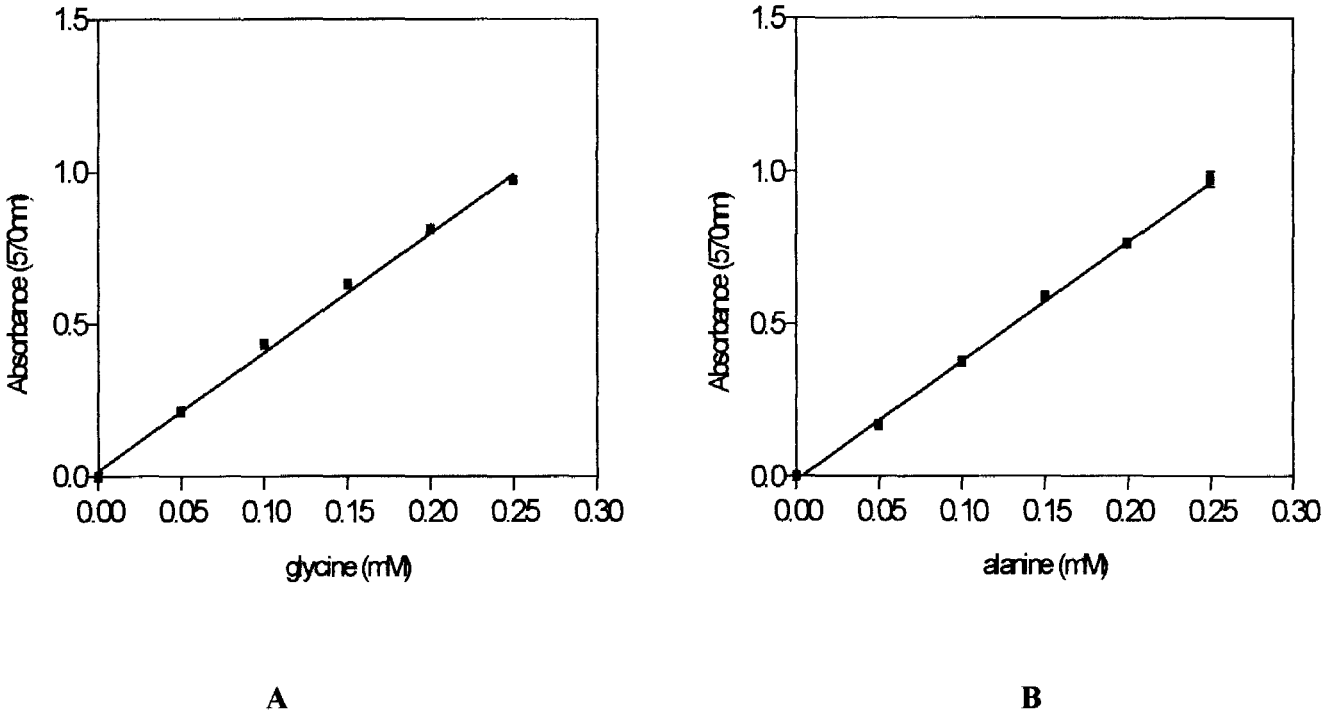


Figure 2 Typical standard curve for protein determination by the Bradford method. r^2=0.989939. The data represent the mean ±SEM of duplicate determinations.

2.1.3 Ninhydrin assay for amino acids (Plummer, 1987)

Add 1ml Ninhydrin reagent to supernatant diluted 1:20 or 1:50 in 1ml buffer. Boil for 15 minutes and cool at room temperature. Add 3ml 50% ethanol and incubate at room temperature for 10 minutes. Read extinction at 570nm against a calibration curve of amino acid standards ranging from 0-0.25mM.



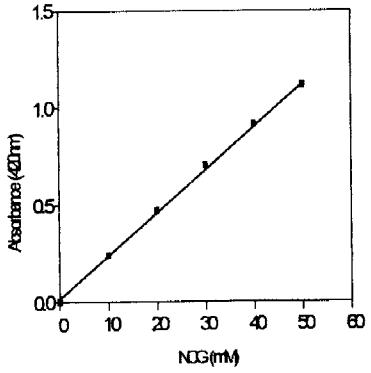
A B

Figure 3 Typical standard curves for (a) glycine, r^2=0.996814 and (b) alanine, r =0.998984 determination by the Ninhydrin method for amino acid determination. The data represent the mean ±SEM of determinations from two representative assays.

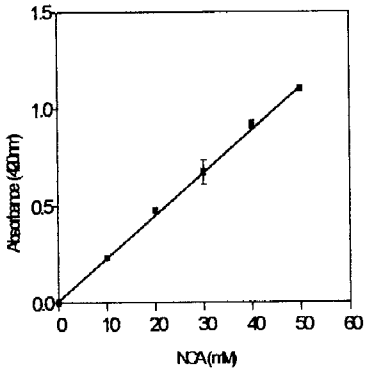
2.1.4 Whole cell biocatalytic assay

Harvest cells induced in nutrient broth or basal medium by centrifugation in Beckman JA-14 or JA-10 centrifuge rotors at 8000rpm for 10 minutes at 4°C using pre-weighed centrifuge tubes. Resuspend pelleted cells in cold buffer at half the induction volume and repeat the washing and centrifugation twice. Resuspend the final weighed pellet (xmg) in cold buffer for the required cell mass (mg) per reaction. For 100mg cells per reaction, resuspend final pellet in (xmg pellet/100mg)x2.5ml buffer, and use 2.5ml with an equal volume of substrate. Incubate under the specified conditions, and obtain supernatant by microcentrifugation at 1200rpm for 3 minutes at room temperature. Deproteinize 1ml supernatant by adding to 0.5ml 12% TCA (trichloroacetic acid) and

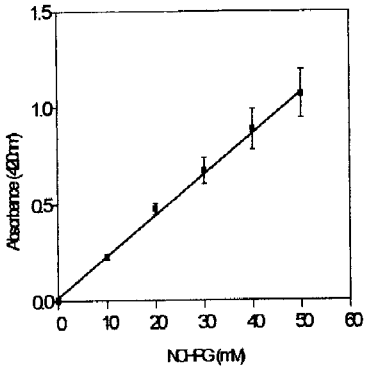
mix in by vortex. Add 3ml deionized water and 0.5ml acidic 10% *p*-dimethylaminobenzaldehyde solution (Ehrlich's reagent) and mix in. Incubate for 20 minutes at room temperature and read extinction at 420nm against a calibration curve of standards of the corresponding *N*-carbamylamino acid in the 0-50mM range. Conversion of absorbance readings to mM product or mg/ml protein was using a Quattro Pro Version 6.01 spreadsheet.



A



B



C

Figure 4 Typical standard curves for *N*-carbamylamino acid determination (a) NCG (*N*-carbamyl glycine), r^2=0.998892 (b) NCA (*N*-carbamyl alanine), r^2=0.998441 (c) NCHPG (*N*-carbamyl hydroxyphenylglycine, r^2=0.997723. The data represent the mean ±SEM of determinations from two representative assays.

2.2 Chromatographic methods

2.2.1 HPLC (high performance liquid chromatography)

Column: Macherey-Nagel Reverse Phase Nucleosil® 100-5, C_{18}

Mobile phase: 50% acetonitrile

Flow rate: 1ml/min

Run time: 20 minutes

Detection wavelength: 220nm

Apparatus: Beckman System Gold™ 126 Programmable Solvent Module, 168 Diode Array Detector Module. Beckman System Gold™ Software, Version 6.0

Retention times(detection wavelength): 5-methylhydantoin : 2.26min (220nm)

:*N*-carbamylalanine: 1.8 min (210nm)

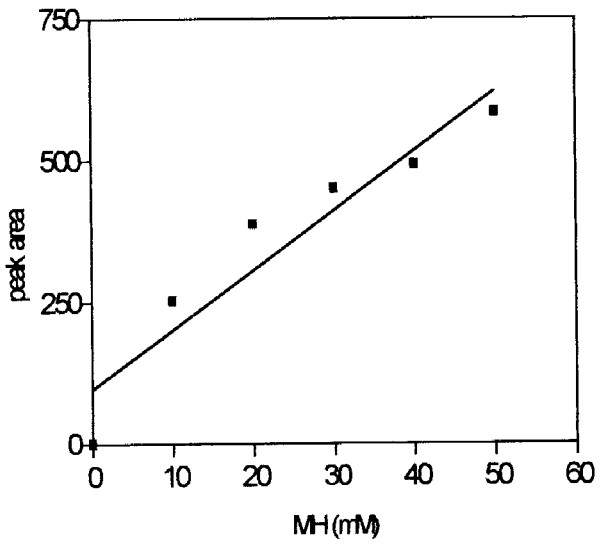


Figure 5 Standard curve for 5-methylhydantion determination by HPLC, r^2=0.898506

2.3 Analysis of 5-methylhydantion synthesized

2.3.1 HPLC

A sample of the product was analyzed by HPLC using the conditions in 2.2.1 above.

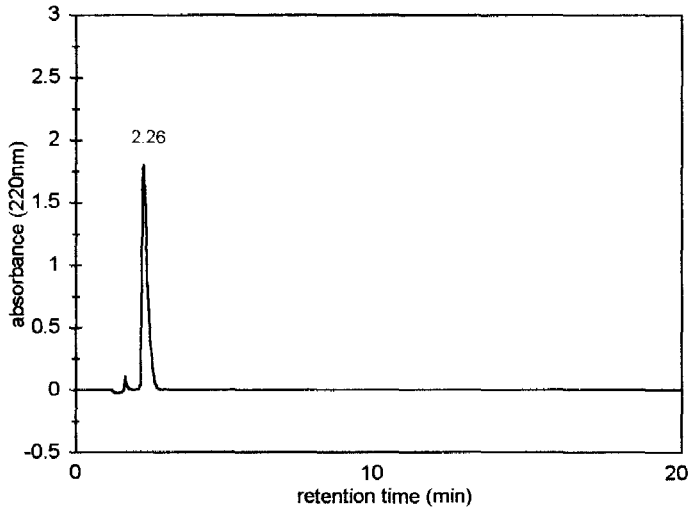


Figure 6 HPLC trace of 5-methylhydantoin synthesized

2.3.2 Melting point determination

Melting point of 5-methylhydantoin was determined as 145-147°C, and 144-146°C. Comparison with literature: 145-146°C (Pollock and Stevens, 1965).

2.3.3 Nuclear Magnetic Resonance Spectroscopy (NMR)

The synthesized product was identified as 5-methylhydantoin.

^{1}H NMR (DMSO, d_6, 400MHz)

δ 10.60 (NH, s), 7.81 (NH, s), 4.01 (1H, q), 1.21 (Me, d, J=6.9)

^{13}C NMR (DMSO, d_6, 100MHz)

δ 178.2 (s), 158.2 (s), 54.3 (d), 17.6 (q)

Spectra were recorded on a Bruker AMX 400 spectrometer at 30°C.

2.4 Other substrates for the hydantoinase reaction

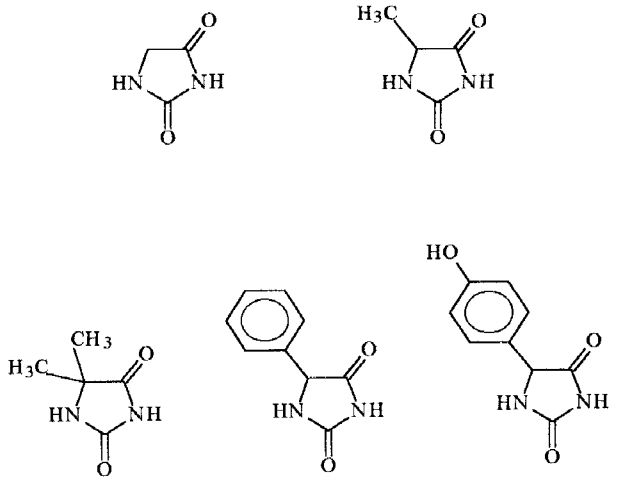


Figure 7 Some substrates for the hydantoinase enzyme system. Clockwise from top left: hydantoin, 5-methylhydantoin 5-hydroxyphenylhydantoin, 5-phenylhydantoin, 5,5-dimethylhydantoin

3. 5-methylhydantoin synthesis (Bucherer and Steiner, 1934)

4g acetaldehyde (D=0.79g/cm^3) were dissolved in 50% ethanol. 18.2g ammonium carbonate and 5.2g KCN were added and the reaction warmed at 60°C under reflux for 2-3 hours. The reaction mixture was concentrated to 2/3 volume by rotary evaporation at 60°C, cooled on ice and acidified to pH2 with HCl . Lyophilization was overnight followed by re-constitution in 100ml absolute ethanol. The precipitate was removed by filtration over vacuum, and the filtrate concentrated by rotary evaporation at 60°C. Viscosity of the concentrated filtrate was reduced by addition of 1-2ml deionized water, and the product re-crystallized overnight at 4°C. Crystals were filtered over vacuum , washed in diethylether (over vacuum) and dried in a vacuum desiccator. A yield of about 70% was achieved.

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