Microbial transformations: Production of D-amino acids using hydantoinase

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D-amino acids have become important chiral-building blocks for many pharmaceutical and food products. Though numerous routes for their production exist, their production by microbial D-hydantoinase offers many advantages. Major ones being ease of production of precursor molecules, the strict D-specificity of the enzymes, and spontaneous racemization of the precursor DL-mixture under alkaline conditions. This review aims at covering all the important biochemical aspects of the hydantoinases known so far, and shed some light on the genesis of this class of enzyme in the evolutionary chain.

MICROBES and their products have benefited man and animals in innumerable ways. In the area of commodity chemicals and pharmaceuticals, viz. organic acids, antibiotics, anticancer agents, immunosuppressants, etc. microbes have not only produced rather exotic molecules which would be really hard to synthesize but also produced numerous enzymes which have enriched the toolbox of the synthetic organic chemists as biocatalysts.

Chirality, stereoselectivity, and stereospecific production of chemicals are characteristics of nature. Many of the xenobiotics obtained by organic synthesis are chiral. Contrary to natural products, synthetic chiral compounds are usually obtained as racemates. This situation is rapidly changing with the development of stereospecific catalytic methods, and the application of biotechnological methods which rely on the help of enzymes as catalysts. Stereospecific synthesis and separation of stereoisomers are still laborious tasks. As a consequence, most of the chiral drugs are marketed as racemic mixtures[1]. Enantiomers usually differ greatly in their biological properties. This holds true for both dynamic (desired and undesired action) and kinetic properties, the latter particularly for the rate of enzymatic conversion, transport by carriers, protein binding, and for distribution and elimination[2–4]. Enantiomers, must therefore be considered as different chemical compounds. Usually only one of the isomers fully contributes to the therapeutic action while the other often is to be classified as a medicinal pollutant[5]. Most of the traditional chemical methods to separate enantiomers are inefficient and tedious. Therefore, emphasis today is to develop newer methods to produce optically pure enantiomers. Recent advancements in chemical and physical methods having future potential, is the use of chiral helical peptides immobilized on polystyrene beads, and the use of chiral reverse phase micelles[6]. On the other hand, biological catalysts (enzymes) are becoming more and more important in synthetic organic synthesis[7], especially for chiral resolutions[8,9]. Enzymes offer an efficient, highly specific, and environment friendly alternative to chemical methods. Recent developments in the field of enzymology, viz. immobilization, extremozymes, and site-directed mutagenesis, and most recently cross-linked enzyme crystals[10], yield stable enzymes which are more suitable for commercial organic synthesis and their resolution.

Optically pure amino acids are important as feed and food additives, chiral-building blocks for pharmaceuticals, and agrochemicals. L-amino acids (as feed and food additives) are required in bulk quantities in comparison to the D-amino acids and other speciality amino acids which are required for production of semisynthetic antibiotics, herbicides, pesticides, biologically active peptides, and other drugs. Uses of such L- and D-amino acids are summarized in Table 1.

D-amino acids, D-*p*-hydroxyphenylglycine and D-phenylglycine are required for production of semisynthetic antibiotics (Figure 1), e.g. amoxicillin, cephadroxyl, ampicillin and cephalexin. Amoxicillin is one of the most widely used antibiotics because it is a broad-spectrum antibiotic, and bacterial resistance towards it is limited[11]. Demand for these side-chained D-amino acids is likely to increase in the near future with the introduction of novel semi-synthetic antibiotics like cefbuperzone, aspoxicillin,

Table 1. Commercial applications of optically active α-amino acids

Amino acid	Required isomer	Uses
Methionine	L	Feed additive
Lysine	L	Feed additive
Phenylalanine	L	Artificial sweetener (Aspartame)
Aspartic acid	L	Artificial sweetener (Aspartame)
Alanine	D	Artificial sweetener (Alitame)
Homo-phenylalanine	L	Pharmaceuticals (Enalapril)
Phenylglycine	D	Pharmaceuticals (Ampicillin)
p-Hydroxyphenylglycine	D	Pharmaceuticals (Amoxicillin)
Valine	D	Agrochemicals (Fluvalinate)
Serine	D	Pharmaceuticals (D-cycloserine)

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cefpiramide, etc.[12]. Fermentation has been the method of choice for the production of L-α-amino acids which are required in bulk as food and feed additives[13].

Quantum of amino acid fermentation can be visualized by the fact that L-lysine alone is produced at a scale of more than 100,000 t/annum by fermentation. The other amino acids which are produced at ton scale by fermentation are L-glutamate, L-tryptophan, L-phenylalanine and L-threonine[13]. Recently, some microorganisms have been reported to produce D-alanine during fermentation[14] which might provide another route to be exploited for commercial production of some of the natural D-α-amino acids. Several enzymatic methods have been developed in recent years for the production of speciality amino acids.

Some of these processes are: (i) the aspartase-catalysed amination of fumaric acid for the production of aspartic acid, (ii) synthesis of tryptophan using tryptophanase[15], and (iii) production of D-glutamic acid using glutamate racemase and glutamate decarboxylase[16]. However, these methods are applicable only for the production of a single amino acid. On the other hand, with the use of enzymes with high stereoselectivity and low substrate specificity, such as amino acylase, amidase, esterase, and hydantoinase, the same method can be used for the production of numerous optically active amino acids[17].

Chemical synthesis of natural and unnatural α-amino acids yields a racemic mixture of DL-α-amino acids which needs to be resolved to optically pure form before they can be used. A number of chemical and biological methods have been reported for the resolution of D-amino acids from their racemic mixtures. These methods include chemical resolutions by preferential crystallization of diastereomeric salts, crystallization in optically active solvents, chromatographic methods, and enzymatic methods[18]. The enzymatic methods utilize proteinases, aminopeptidases, aminoacylase, and D-hydantoinases.

DL-5-monosubstituted hydantoin derivatives are used industrially for the production of DL-α-amino acids by alkali hydrolysis. DL-5-monosubstituted hydantoin derivatives can be used as substrates for the enzymatic production of optically pure α-amino acids[17,19]. As hydantoin derivatives can be racemized easily under slightly alkaline conditions by keto–enol tautomerization, enzymatic resolution of these can yield up to 100% of the desired enantiomer.

Conversion of DL-5-monosubstituted-hydantoin derivatives to optically pure α-amino acids can be carried out in two steps (Figure 2). The first step involves a stereoselective enzyme hydantoinase (dihydropyrimidinase EC 3 : 5 : 2 : 2) which can setereoselectively hydrolyse D- or L-enantiomer of hydantoin derivative to optically pure *N*-carbamoyl-α-amino acid. In the second step, the intermediate *N*-carbamoyl-α-amino acid is further hydro-

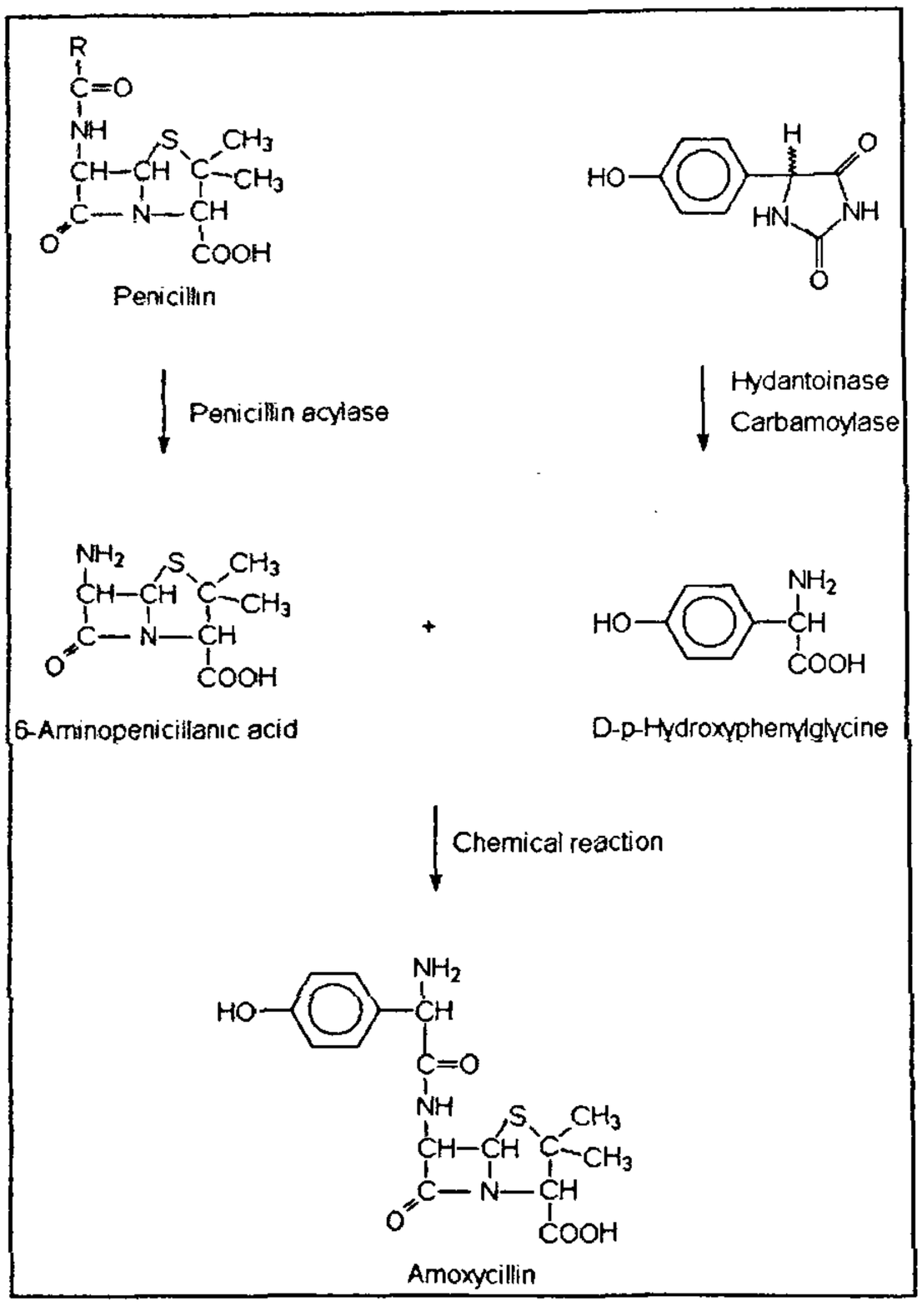

Figure 1. Schematic representation of amoxicillin production.

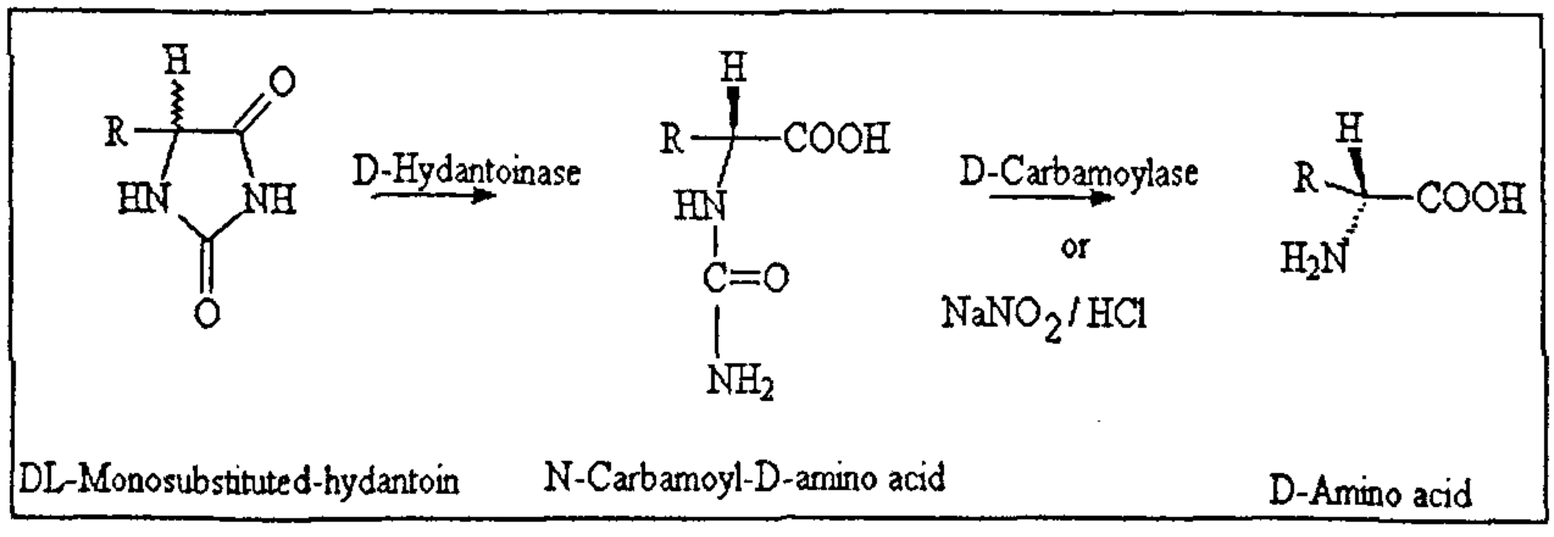

Figure 2. Schematic representation of hydantoinase/carbamoylase process for the production of D-amino acids.

lysed to optically pure α-amino acid either chemically with $NaNO_2$/HCl (ref. 20) or enzymatically with carbamoylase[21].

Synthesis and properties of hydantoin and hydantoin derivatives

In 1861, hydantoin was synthesized by Baeyer by the hydrogenation of allantoin. Presently, 5-monosubstituted-hydantoin derivatives are important precursors for the commercial production of DL-α-amino acids. These hydantoin derivatives can be easily synthesized by various methods, most important of these being the Bucherer–Berg synthesis[22,23] (Figure 3), which utilizes the respective aldehydes, potassium cyanide, and ammonium carbonate to synthesize DL-5-substituted hydantoins. The limitation of this method is the use of highly toxic potassium or sodium cyanide. A method by Suzuki *et al.*[24] utilizes amino acids and potassium cyanate to produce *N*-carbamoyl-amino acid, which can be converted to DL-5-substituted hydantoins by heating in the presence of hydrochloric acid (Figure 4).

However, an efficient and simple method for the synthesis of *p*-hydroxyphenylhydantoin from glyoxalic acid, phenol, and hydrochloric acid (Figure 5) has been reported by Ohashi *et al.*[25]. This method is economically viable and safe as it utilizes cheap reactants and does not require hazardous chemicals like sodium cyanide.

DL-5-monosubstituted hydantoin derivatives racemize under alkaline conditions by keto–enol tautomerism. The substituents cause electronegative inductive effect, and aryl groups when present at C5 position enhance the rate of racemization[17]. The rate of racemization of various 5-substituted hydantoins at pH 8.5 and temperature 40°C was studied[26]. It was observed that hydantoins racemize according to first-order rate law but with different rate constants. Half time for racemization varied from 55.9 h in case of isopropylhydantoin to 16 min in case of phenyl-hydantoin. They concluded that aromatic and some heterosubstituted hydantoins racemize reasonably fast with respect to duration of the biotransformation reaction.

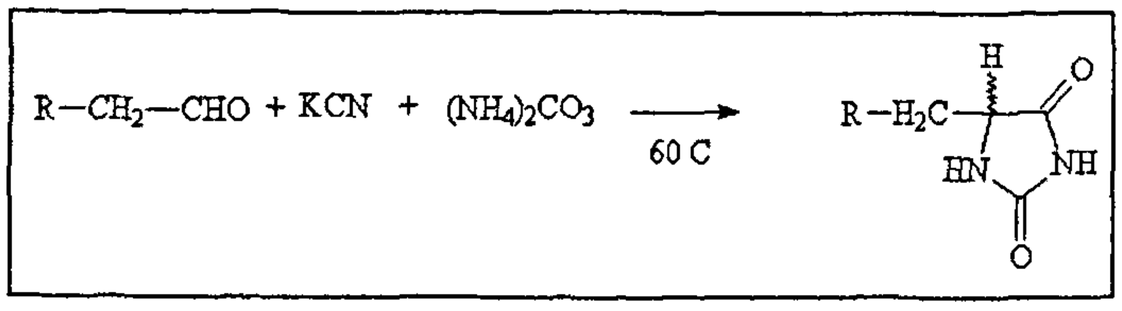

Figure 3. Reaction depicting Bucherer–Berg synthesis of 5-substituted hydantoins.

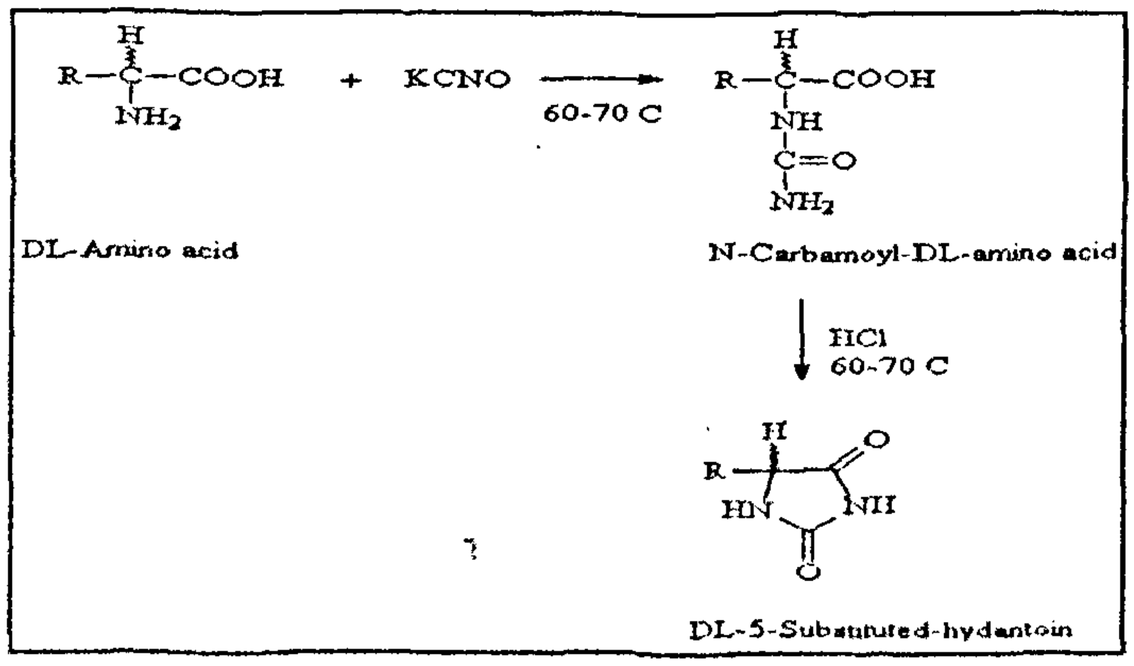

Figure 4. *Reaction depicting the 5-substituted hydantoin synthesis from α-amino acids.*

Screening and production of D-hydantoinase

In 1946, the enzymatic cleavage of hydantoins was first reported from various plant and animal sources[17], followed by a report on the commercial utilization of hydantoinase for the production of D-phenylglycine and D-*p*-hydroxyphenylglycine[27]. Having used the animal and plant sources, it was the turn of the microbial world to be exploited for hydantoinase producers. Yamada *et al.*[19] investigated the hydantoin-hydrolysing activity in micro-organisms and observed that various microbes including bacteria, and fungi, particularly actinomycetes, possessed this activity. These findings resulted in microbial screening by various groups, and many organisms with D- or L-hydantoinase activity were reported. Some of these strains effectively produced only the hydantoinase, viz. *Pseudomonas putida*[19], *Bacillus* sp.[28–30], *Peptococcus anaerobius*, and *Agrobacterium tumefaciens*[31], but a few of them possessed both the enzymes, i.e. hydantoinase and carbamoylase, required for conversion of hydantoin to amino acid. The strains possessing both the enzymes were obtained from *Agrobacterium* sp.[32,33], *Pseudomonas* sp.[34], and *Arthrobacter crystallopoites*[35].

Most of the reported screening methods used enrichment culture as the preliminary step, using various hydantoin derivatives as carbon and/or nitrogen source[34,35]. These microorganisms were then tested for hydantoin-hydrolysing activity on agar plates by specially designed assays[36], or as growing cells in liquid culture, or as resting cells in buffer solution with the specific hydantoin as substrate[19,34,35]. Production of *N*-carbamoyl amino acid was analysed by thin layer chromatography, high performance liquid chromatography, or by colorimetry[28,37]. For screening of D-hydantoinases, nucleotide probe-based colony hybridization assay was described by Lapointe *et al.*[38], and a continuous-culture-based enrichment technique by Morin *et al.*[39]. A rapid and sensitive

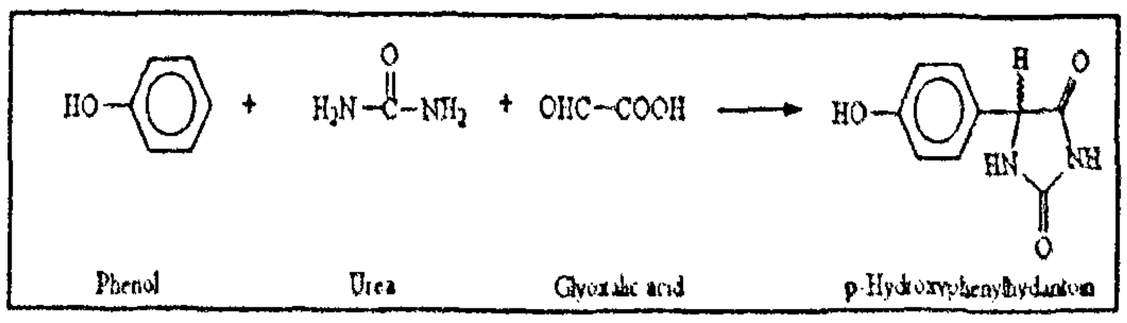

Figure 5. Reaction depicting *p*-hydroxyphenylhydantoin synthesis by Ohashi's method.

method using a microtiter plate assay for detection of hydantoinase has also been reported recently by Meyer and Runser[40].

Optimization of D-hydantoinase production

D-hydantoinase was produced as an inducible enzyme in most of the microbial strains reported. Hydantoinase production in *Pseudomonas* species, *Agrobacterium* species, and *Peptococcus anaerobius* was observed to be induced by various substrates and substrate analogues. D-hydantoinase production was maximally induced by uracil in *Pseudomonas putida*[19], and *Agrobacterium* sp.[33]. An unmetabolizable substrate analogue, 2,4-thiouracil, enhanced hydantoinase production up to five fold in *Agrobacterium* sp.[41]. In *P. fluorescens* and *Bacillus stearothermophilus* SD1, the enzyme was produced constitutively, and substrates or substrate analogues were unable to induce the enzyme production[42,43].

Although production of hydantoinase has been reported in many organisms, only few reports about the medium optimization are available. Meat and beef extract, when used as nitrogen source, enhanced D-hydantoinase production in *P. putida*[19], and *Bacillus* SD1 (ref. 43). This may be due to the high pyrimidine and purine content in these complex meat-derived nitrogen sources[44]. Yeast extract was used as the nitrogen source in *Pseudomonas* sp.[42], and *A. radiobacter*[45]. Glucose or glycerol was used as a carbon source in most of the cases for the production of D-hydantoinase. In a recent study[46], medium optimization studies were carried out for the production of D-hydantoinase in *A. radiobacter* NRRL B 1229, wherein with the use of an empirical modelling technique (response surface method) Achary *et al.* were able to achieve 35 U/ml of enzyme activity and 1.69 mg/ml of biomass in optimized complex medium. The optimized medium contained molasses as the carbon source, and ammonium nitrate as the nitrogen source. Mass production of D-hydantoinase from batch culture of recombinant *E. coli* in minimal medium with glycerol as the sole carbon source has been reported by Lee *et al.*[47]. They achieved 50 g dry cell weight per litre of broth, and an enzyme yield of 38000 U/g DCW in a 50 litre fermenter. They also reported that D-hydantoinase gene, which is expressed under its own promoter, is catabolically repressed by glucose in the recombinant strain.

Purification and characterization

In an attempt to understand more about this enzyme, it was purified from various sources. D-hydantoinase from beef-liver powder was purified using acid treatment and heat treatment, followed by ammonium sulphate and acetone precipitation[48]. The purity of the enzyme was 80% with a yield of 25%. Bovine-liver dihydropyrimidinase was purified to homogeneity[49] with 13% yield. D-hydantoinases were also purified from various microbial sources to homogeneity, using various precipitation and chromatographic steps. D-hydantoinase from *P. striata* was purified to homogeneity after seven steps, including crystallization with a yield of 3.0% (ref. 37). D-hydantoinase from *Agrobacterium* sp. was purified to homogeneity with a purification of 965 fold and a yield of 9.0% after seven steps[50]. Thermostable D-hydantoinase from *B. stearothermophilus* was purified to homogeneity, after a preparative SDS-PAGE step, with a purification of 50 fold and a yield of 1.5% (ref. 51). D-hydantoinase from *B. circulans* has been purified to homogeneity with an overall yield of 12% and a purification of 243 fold[29]. An affinity chromatography-based purification protocol, which utilized polyclonal antibodies against the purified D-hydantoinase, resulted in 60% yield of D-hydantoinase from *B. stearothermophilus* SD1 (ref. 52).

Relative molecular weight and subunits: D-hydantoinase reported from bovine liver[49], *P. putida*[37], *Agrobacterium* sp.[50], and from *B. circulans*[29] had a homotetrameric structure with a native molecular weight of 190 to 250 kDa and a subunit weight of 53 to 62 kDa. The enzyme from *B. stearothermophilus* SD1[51] and dihydropyrimidinase from *Pseudomonas* sp.[53] had a homodimeric structure with a native molecular weight between 115 and 126 kDa and subunit molecular weight of 50 to 55 kDa.

The isoelectric point (pI) of the enzyme was determined for the enzyme from *Agrobacterium* sp.[50], and from *B. stearothermophilus* SD1 (ref. 52) and was reported to be 6.5 and 4.47, respectively. D-hydantoinase from *B. circulans* had a pI of 4.55 (ref. 29).

N-terminal amino acid sequence: *N*-terminal amino acid sequence comparison has been summarized in Table 2. All these enzymes belong to the same family as they show a high level of similarity in their *N*-terminal amino acid sequence. *N*-terminal sequence of the D-hydantoinase from *Agrobacterium* sp. does not match with any known sequence[50].

N-terminal amino acid sequence from different microbial sources showed varying degree of similarity except for the sequence from *Agrobacterium* sp. Sequence from thermophilic gram-positive bacteria and *B. stearothermophilus* showed highest similarity, indicating their similar origin.

Effect of temperature and pH: D-hydantoinase isolated from various *Pseudomonas* species had a temperature optimum between 45 and 55°C and a pH optimum between 7.0 and 9.0. They were unstable above a temperature of 50°C (refs 19, 54, 55). D-hydantoinase from *Agrobacterium* species showed higher temperature optimum, between 60 and 70°C, and a pH optimum in the

alkaline range 8.0–10 (refs 31, 56). These enzymes were relatively stable compared to those from *Pseudomonas* species D-hydantoinase from *Peptococcus anaerobius* had a broad pH and temperature optimum, and was stable for up to 1 h at 60°C. Thermostable enzymes are the enzymes of choice for commercialization because of their longer operational life, and therefore enzymes from thermophilic organisms have been isolated by various groups. The D-hydantoinase from these thermophilic organisms showed higher thermostability; the enzyme from *B. stearothermophilus* had a half life of 30 min at 80°C, a temperature optimum of 70°C, and a pH optimum of 8.0 (ref. 51). D-hydantoinase from *B. circulans* was stable up to 90 min at 60°C even in the presence of 0.5% SDS (ref. 29).

Substrate specificity: All D-hydantoinases and dihydropyrimidinases reported, showed broad substrate specificity, and were able to cleave a number of natural substrates as well as many 5-substituted hydantoin derivatives, including alkyl-substituted or aryl-substituted hydantoins[17]. Substrate specificities of different D-hydantoinases are summarized in Table 3.

Hydantoinase reported from *Pseudomonas* sp.[37] catalysed hydrolysis of dihydrouracil with highest specificity, and the aliphatic-side-chain-substituted hydantoin derivatives, viz. isobutylhydantoin, isopropylhydantoin, methylmercaptoethylhydantoin were hydrolysed at a relative rate of ~ 50%. Aromatic-substituted hydantoins were cleaved with relatively lower specificity. Hydantoinase from *A. radiobacter*[32] converted phenylhydantoin with maximum specificity, and it cleaved *p*-hydroxyphenylhydantoin, benzylhydantoin, methoxyphenylhydantoin, and thienylhydantoin at a relative rate of about 70%. This enzyme cleaved aliphatic-side-chain-containing hydantoin, methylhydantoin, also at the same rate. Hydantoinase from *Pseudomonas* sp.[57] also cleaved both aliphatic-substituted or aromatic-substituted hydantoin. It catalysed hydrolysis of cyanoethylhydantoin at a maximum rate. Hydantoinase from *Arthrobacter* sp.[35], *B. stearothermophilus* SD1 (ref. 51), and *B. circulans*[29] cleaved unsubstituted hydantoin as the preferred substrate, followed by phenylhydantoin and dihydrouracil. The enzymes from *Arthrobacter* species cleaved methylhydantoin also with high specificity, although the *Bacillus* enzymes were less active on this substrate. Enzymes from animal sources[49], plants[58], and from anaerobic bacteria have also been reported to show broad-substrate specificity. The enzyme from *A. radiobacter* was reported to be used at commercial scale by Degussa for production of various speciality D-amino acids, viz. D-alanine, D-norvaline, D-norleucine, D-alloisoleucine, D-cysteine, D-citrulline, D-napthylalanine, and D-*N*-carbamoylthialysine[26]. Most of the D-hydantoinase reported from microbial sources had higher specificity toward the dihydrouracil as substrate, which is a natural substrate for dihydropyrimidinase, thereby classifying them as dihydropyrimidinases[19]. However in

Table 2. Comparison of *N*-terminal amino acid sequence of hydantoinases

Culture	Sequence	Reference
Pseudomonas sp. DSM84	MSLLIRGATVVTHEESYPADV	38
Pseudomonas sp. NS671	MKLFGVDVGGTFTDIIFSDTE	77
Gram-negative bacterium	MPLLIKNGEIITADSRYKADI	80
Agrobacterium sp. IP671	ATDIFTAPLGYISEYGVNSSM	50
Bacillus stearothermophilus	MTKLIKNGTIVTATDIYEADL	79
B. stearothermophilus SD1	MTKIIKNGTIVTATDTYEAHL	81
Gram-positive bacterium Lu 1220	MTKIIKNGTIVTATDTYEADL	80
Blastobacter sp.	STVIKGGTIVAADRSYEADI	59

Table 3. Substrate specificity of various D-hydantoinases

Substrate	*Bacillus* sp. (ref. 51)	*Bacillus* sp. (ref. 29)	*Pseudomonas* sp. (ref. 37)	*Pseudomonas* sp. (ref. 57)	*Agrobacterium* sp. (ref. 50)	*Agrobacterium* sp. (ref. 32)	*Arthrobacter* sp. (ref. 35)
Hydantoin	100	100	13	80	22	–	2
Dihydrouracil	46	–	100	–	0	–	16
Phenylhydantoin	42	98	25	62	–	100	–
p-Hydroxyphenyl hydantoin	17	–	16	61	75	73	16
Methylhydantoin	–	25	45	58	37	72	–
Isopropylhydantoin	5	8	15	15	45	–	–
Isobutylhydantoin	–	26	48	19	50	–	–
Methylthioethyl hydantoin	–	41	48	80	55	–	–
Benzylhydantoin	–	12	25	41	100	72	–
Cyanoethyl hydantoin	–	–	–	100	25	–	–
5-Bromouracil	–	–	–	–	–	–	100

Blastobacter sp. these two activities are distinctly expressed[59], and in *A. radiobacter* the purified D-hydantoinase did not cleave dihydrouracil at all, indicating that it is a true hydantoinase[50].

Metal ion requirement and chemical modifications: D-hydantoinase or dihydropyrimidinase is a metalloenzyme requiring divalent metal ions for its activation. While dihydropyrimidinase from bovine liver required four Zn^{+} ions for its activation, the enzymes from *Pseudomonas* sp.[37,60] required Fe^{+3} ions. While D-hydantoinase from *A. tumefaciens*[50] required Ni^{+2}, that from thermophile *B. stearothermophilus* SD1 (ref. 51) and *B. circulans*[29] required Mn^{+2} ions for their activation. The D-hydantoinase from *Agrobacterium* sp. was metallo-independent[31]. Most of the D-hydantoinases reported are inhibited by metal ion chelators such as EDTA, and *O*-phenanthroline[37,50,60], indicating thereby that metal ion presence is essential for enzyme activity. D-hydantoinase is reported to be inhibited by the cysteine-modifying reagents, such as iodoacetamide, dipyridyl and *N*-ethylmaleimide[29,37,60], indicating that one or more cysteine residues may be playing some role at the catalytic centre of the enzyme. D-hydantoinase from *Agrobacterium* sp. IP671 (ref. 50) and *B. stearothermophilus* SD1 (ref. 51) were reported to be inhibited by diethylpyrocarbonate, which indicates that either histidine residues are present at the active site itself or have some role at the metal-binding site of the enzyme.

Optimization of biotransformation

The most important commercial application of D-hydantoinase is in the production of D-*p*-hydroxyphenylglycine from DL-5-*p*-hydroxyphenylhydantoin (DL-HPH). Since most of the DL-5-monosubstituted hydantoin derivatives have low-water solubility at room temperature and racemize under alkaline conditions, the emphasis is on to carry out this reaction at higher temperatures under alkaline conditions. Most of the dihydropyrimidinases reported from animal sources and from *Pseudomonas* species had a low thermostability and a pH optimum near neutrality, hence the reactions were carried out below optimal conditions. Cecere *et al.*[61] used dihydropyrimidinase from calf liver and carried out biotransformation of DL-HPH at pH 8.5 and temperature 30°C to *N*-carbamoyl-D-*p*-hydroxyphenylglycine in 20 h with a final yield of 72%. Whole cells of *P. desmolyticum*, producing D-hydantoinase, were used to convert DL-phenylhydantoin to *N*-carbamoyl-D-phenylglycine at pH 9.5 and temperature 30°C with a molar yield of 90% (ref. 62). Runser and Ohleyer[56] described a D-hydantoinase from *Agrobacterium* sp. wherein whole cells of this strain were used to convert 30 g/l of DL-benzylhydantoin to *N*-carbamoyl-D-phenylalanine at pH 10 and temperature 60°C with a yield of 96% in only 10 h. D-hydantoinase from *Agrobacterium* sp. was used to convert DL-phenylhydantoin (4.0 mg/ml) to *N*-carbamoyl-D-phenylglycine at pH 9.5 and temperature 45°C with a molar yield of 98% within 2 h (ref. 31).

Presence of organic co-solvents enhanced the solubility of the DL-5-monosubstituted hydantoins. The use of 5% DMSO as organic co-solvent during the bioconversion of DL-*p*-hydroxyphenylhydantoin to *N*-carbamoyl-D-*p*-hydroxyphenylglycine enhanced the activity of immobilized D-hydantoinase two fold, although presence of higher concentrations of solvent inactivated the enzyme completely[63].

Although a number of organisms have been reported to produce both D-hydantoinase and D-carbamoylase, attempts to convert DL-hydantoins directly to D-amino acids were restricted by different pH optima and low thermostability of D-carbamoylase. D-carbamoylases reported from various microbes had a pH optimum near neutrality and were very thermolabile in comparison to D-hydantoinases[64]. It was reported[57] that *Pseudomonas* sp. AJ-11220 was able to convert DL-*p*-hydroxyphenylhydantoin (DL-HPH) to D-*p*-hydroxyphenylglycine (D-HPG) with a molar yield of >90% at pH 8.0 and temperature 30°C in 48 h. A conversion yield of 98% was achieved by adsorptive removal of NH_4^+ ions from reaction mixture during conversion of DL-*p*-hydroxyphenylhydantoin to D-*p*-hydroxyphenylglycine by *Agrobacterium* sp. I671, in comparison to 50% conversion in 27 h when no adsorbent was present[65].

Reaction conditions for one-pot synthesis of D-HPG using partially purified D-hydantoinase and D-carbamoylase from *Agrobacterium* sp. I671, were optimized[64]. These enzymes were able to convert DL-HPH to D-HPG with 95% conversion in 15 h at pH 8.0 and temperature 30°C in 48 h : D-hydantoinase and D-carbamoylase were required in a ratio of 1 : 3 for optimum conversion.

In the near future with the isolation of more thermostable D-hydantoinases[28] and D-carbamoylases[66] and with increase in the yield of these enzymes' production after cloning[43] and with stabilization of these enzymes using site-directed mutagenesis[67], the process efficiency is expected to increase.

Immobilization and characterization of immobilized enzymes

For commercial use, the most desired property of a biocatalyst is its operational stability and reusability. Generally, enzymes in free form are thermolabile and cannot be reused owing to their loss during downstream processing and purification of the product. Immobi-

lization is the most important technique used for enzyme *stabilization* and enhancement of its operational life. Immobilized enzymes can be reused for number of cycles of conversion. Immobilization does not necessarily enhance the enzyme's stability, but this can be achieved by trying different methods of immobilization out of the numerous methods reported[68]. Immobilization can be used to modify other properties of the enzymes such as pH and temperature effect, K_m and V_{max}, and even substrate specificity[69,70]. Number of methods for both cell as well as enzyme immobilization have been reported after the successful application of the L-acylase in industrial production of optically pure amino acids[71]. Penicillin acylase is the most important example of reusability of immobilized enzymes: as immobilized penicillin acylase was used for more than 1000 cycles for production of 6-APA from penicillin[72].

D-hydantoinase, which is a commercially important enzyme, was immobilized by various groups in free cells as well as in whole cells. Whole cells of a D-hydantoinase-producing alkalophilic *Bacillus* sp. were immobilized in polyacrylamide gel beads[73], and were used for the production of D-phenylglycine from DL-phenylhydantoin at pH 8.5 and temperature 33°C for 14 to 18 h. Immobilized cells were able to convert 100% of 1.0% suspension of DL-phenylhydantoin even after 10 cycles. The rate of conversion was 60 to 90% when DL-*p*-hydroxyphenylhydantoin was used as the substrate.

Immobilized nongrowing cells of *P. putida* in alginate beads were used[74] for the conversion of dihydrouracil to *N*-carbamoyl-*β*-alanine in batch mode as well as in continuous mode. Forty to forty-five per cent conversion was obtained in both the cases. Highest productivity was achieved in a packed-bed reactor at a dilution rate of $0.5\ h^{-1}$.

Polyacrylamide-gel-entrapped whole cells of *Pseudomonas* sp. KBEL 101 were used to study the operational stability of D-hydantoinase during the production of D-*p*-hydroxyphenylglycine from DL-*p*-hydroxyphenylhydantoin[75]: the half life of immobilized D-hydantoinase was observed to be 50 h, which they were able to enhance it to almost no loss of activity in 7 days by supplying 0.1% (w/v) glycerol with reaction mixture. When 0.1% (w/v) of yeast extract was provided with the reaction mixture, no loss in activity was seen for 25 days. Partially purified D-hydantoinase from *B. stearothermophilus* SD1 was immobilized on various supports by adsorption. DEAE cellulose was found to be the most effective for activity recovery and the amount of protein bound. DEAE cellulose-immobilized enzyme was used to convert DL-*p*-hydroxyphenylhydantoin in repeated batches, with constantly maintained production rate for nine successive cycles at 55°C and pH 8.0 (ref. 12).

A commercial preparation of D-hydantoinase from thermophilic bacteria immobilized on granulated organic polymer support was used to study various properties of immobilized D-hydantoinase[76]. The half life of immobilized enzyme was reported to be eight batches when used at 50°C and pH 8.5 with 10% w/v substrate. The optimum temperature for conversion was 60°C and the product did not inhibit the activity of immobilized enzyme.

Cloning of D-hydantoinase gene

Gene cloning is one of the most powerful techniques of modern biotechnology, which results in production of protein of interest in high yield. Before the advent of genetic engineering, there were only three options for a researcher to improve yield and characteristics of a biocatalyst. (i) By screening of microbes from soil and water samples to find a new high-yielding isolate. (ii) By medium optimization and process parameters optimization to increase yield in known cultures. (iii) By random mutagenesis and selection of the new overproducing mutants.

Now, with the tools of genetic engineering, it is possible to clone a gene for an already known biocatalyst and overexpress it or modify it for the desired characteristics by site-directed mutagenesis after studying its primary and possible three-dimensional structure.

D-hydantoinase as well as dihydropyrimidinases have been cloned and sequenced from various animal and microbial sources. The first to be cloned[77] was the D-hydantoinase gene from the native plasmid of *Pseudomonas* sp. NS 671. This gene was found to be located on this plasmid with other genes of the DL-hydantoin degradation pathway such as L-hydantoinase, L-carbamoylase, and hydantoin racemase. Another D-hydantoinase gene from *P. putida* DSM84 from chromosomal DNA was also cloned[78]. Amino acid sequence decoded from the gene had regions of similarity with the other known D-hydantoinases, allantoinase and dihydroorotase.

Recently, genes from thermophilic *Bacillus* sp. have been cloned by various groups[43,79]. A gene for nonspecific hydantoinase from *B. stearothermophilus* from genomic DNA which encoded a 471 amino-acid-long peptide was also cloned[79]. The nucleotide sequence of this gene had a homology of 91% with the sequence of D-hydantoinase from a gram-positive organism Lu1220 (ref. 80). D-hydantoinase gene from *B. stearothermophilus* SD1 was cloned and overexpressed in *E. coli*[43]. The cloned enzyme was expressed as 20% of the total protein in *E. coli*. D-hydantoinase protein sequence decoded from the gene sequence of D-hydantoinase from *B. stearothermophilus* SD1 showed 20–28% homology with other hydantoinases, allantoinase and dihydroorotase protein sequences. This sequence exhibited more than 89% identity with hydantoinase from other thermophiles. Among the hydantoinases from thermophiles, C-terminal regions of enzymes were completely different, implying thereby that

C-terminal region plays an important role in the biochemical properties of the enzymes[81]. Dihydropyrimidinase gene cloned by c-DNA cloning from rat liver and human liver showed 40% similarity with the D-hydantoinase gene sequences from various microorganisms.

Diversity and evolutionary relationship between hydantoinase and other related enzymes

Dihydropyrimidinase and D-hydantoinase are the names interchangeably used for the enzymes which hydrolyse six-membered cyclic amides, the dihydropyrimidines, and five-membered hydantoin derivatives (Figure 6). Dihydropyrimidinase has been reported from various animal, plant, and microbial sources. This enzyme is involved in reductive catabolism of pyrimidines. Dihydropyrimidinase from animal sources possesses activity to cleave stereoselectively D-hydantoin derivatives to *N*-carbamoyl-D-amino acids. Later, D-hydantoin-hydrolysing enzymes of microbial origin were shown to cleave dihydropyrimidines with high specificity[19]: production of these enzymes was induced by uracil and its structural analogues[19,41], indicating that D-hydantoinases from microbial sources are similar to dihydropyrimidinase. D-hydantoinases are now being studied from various microbial sources which include: (i) mesophiles, e.g. *Pseudomonas* sp., (ii) anaerobes, e.g. *Peptococcus* sp.; and (iii) the thermophiles, e.g. *B. stearothermophilus*. These microbial hydantoinases form a class of enzymes differing in their substrate specificities. The enzymes from *Bacillus* sp.[28,29] utilize unsubstituted hydantoin as their preferred substrate and cleave dihydrouracil (natural substrate for dihydropyrimidinase) at a relatively lower rate. Only the D-hydantoinase reported from *Agrobacterium* sp. was found to be a true hydantoinase as it did not cleave dihydropyrimidines[50].

There are also a number of other related enzymes reported in literature with similar activity to hydrolyse cyclic amides, viz. dihydroorotase[82], allantoinase[83], barbiturase[84], penicillinase[85], and imidase[85].

These enzymes also play a role in biosynthesis or degradation of purine and pyrimidines[86], and cleave their substrates with L-specificity. *N*-methylhydantoinase, an ATP-dependent hydantoinase which is essentially required for creatinine degradation[87], cleaves some of the 5-substituted hydantoin derivatives with L-selectivity. Due to their broad substrate specificity and a great diversity of these enzymes with similar activities, it is difficult to understand their exact physiological role. Now these enzymes from various sources have been purified and characterized and many of them have been cloned. Hence, at this advanced stage it is possible to evaluate their physiological role and evolutionary significance. Amino acid sequence and nucleotide sequence comparison of D-hydantoinase genes revealed that these enzymes (hydantoinase, dihydropyrimidinase, allantoinase, and dihydroorotase) are closely related, having high segmental homology[78]. Three histidine residues and one aspartic acid residue were found to be highly conserved in all the sequences, and these appeared to be involved in the metal-binding site—as was earlier reported by Brooks *et al.*[49] that these proteins were metalloenzymes. In case of dihydroorotase, site-directed mutagenesis studies actually revealed that these residues indeed formed a metal-binding site which is essential to its catalytic activity[88,89].

Holm and Sander[90], in a recent computer-based study using some crystal structures, amino acid and nucleotide

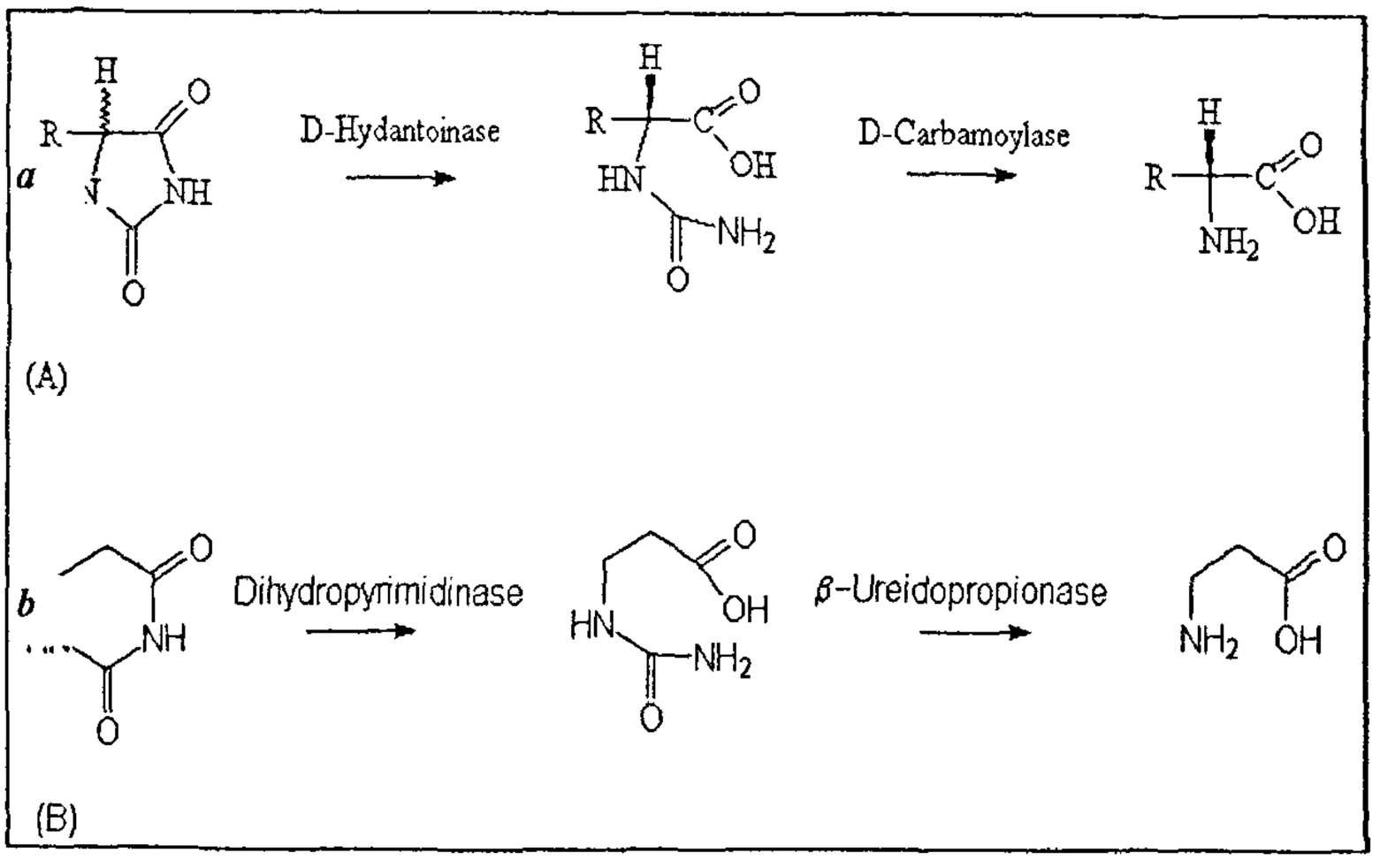

Figure 6. Schematic representation of hydantoinase and dihydropyrimidinase reaction. ***a***, Hydantoinase reaction; ***b***, Dihydropyrimidinase reaction.

sequences, proposed that these enzymes, including urease, adenine deaminase and amino acid acylase, are distantly related and have evolved from a single super family. This super family has a conserved metal-binding site which contains highly conserved histidine residues and an aspartic acid residue. The characterization of these enzymes also revealed the role of metal ions in catalysis. It was also reported that whenever this metal-binding site was missing, the protein lost its catalytic activity. These proteins, although devoid of any catalytic activity, were found to be essential for growth and development in many higher organisms, perhaps they still possessed an active nucleotide-binding site which played some role in the physiology of these organisms by binding to the nucleotides or to the nuclear material.

Conclusion

Though D-hydantoinases are already being used industrially, however to increase the scope of use of this enzyme for the production of other D-amino acids, broader substrate specificity is required. For this, two options available are: (i) screen for new enzymes from microbial isolates where serendipity has a role to play; (ii) modify the existing enzymes to better ones by directed evolution.

1. Sheldon, R. A., *J. Chem. Tech. Biotechnol.*, 1996, **67**, 1–14.
2. Simonyl, M., Fitos, I. and Visy, J., *TIPS*, 1986, **165**, 112–116.
3. Testa, B., *TIPS*, 1986, **165**, 60–64.
4. Walle, T. and Walle, K. U., *TIPS*, 1986, **165**, 60–64.
5. Ariens, E. J., *Med. Res. Rev.*, 1986, **6**, 451–466.
6. Persidis, A., *Nature Biotechnol.*, 1997, **15**, 594–595.
7. Faber, K. and Franssen, C. R. M., *Tibtech.*, 1993, **11**, 461–470.
8. Margolin, A. L., *Enzyme Microb. Technol.*, 1993, **15**, 266–280.
9. Poppe, L. and Novak, L., *Selective Biocatalysis: A Synthetic Approach*, VCH Publ., Germany, 1992.
10. Zelinski, T. and Waldmann, H., *Angew. Chem. Int. Ed. Engl.*, 1997, **36**, 722–724.
11. Louwrier, A. and Knowles, C. J., *Enzyme Microb. Technol.*, 1996, **19**, 562–571.
12. Lee, D. C., Lee, S. G. and Kim, H. S., *Enzyme Microb. Technol.*, 1996, **18**, 35–40.
13. Hodgson, J., *Biotechnology*, 1994, **12**, 152–155.
14. Takeuchi, M. and Yonehara, T., Process for producing D-alanine, US patent 5254464, 1991.
15. Nakazawa, H., Enei, H., Okumura, S. and Yamada, H., *Agric. Biol. Chem.*, 1972, **36**, 2523–2528.
16. Yagasaki, M., Ozaki, A. and Hashimoto, Y., *Biosci. Biotech. Biochem.*, 1993, **57**, 1499–1502.
17. Syldatk, C., Laufer, A., Muller, R. and Hoke, H., *Adv. Biochem. Eng. Biotechnol.*, 1990, **41**, 29–75.
18. Greenstein, J. P. and Winitz, M. (eds), in *Chemistry of the Amino Acids*, Krieger Publisher, USA , 1984, vol. 1, pp. 715–759.
19. Yamada, H., Takahashi, S., Kii, Y. and Kumagai, H., *J. Ferment. Technol.*, 1978, **56**, 484–491.
20. Takahashi, S., Ohashi, T., Kii, Y., Kumagai, H. and Yamada, H., *J. Ferment. Technol.*, 1979, **57**, 328–332.
21. Olivieri, R., Fascetti, E., Angelini, C. and Degen, L., *Enzyme Microb. Technol.*, 1979, **1**, 201–204.
22. Henze, H. R. and Speer, R. J., *J. Am. Chem. Soc.*, 1942, **64**, 522–523.
23. Finkbeiner, H., *J. Org. Chem.*, 1965, **30**, 3414–3419.
24. Suzuki, T., Igarashi, K., Hase, K. and Tuzimura, K., *Agric. Biol. Chem.*, 1973, **37**, 411–416.
25. Ohashi, T., Takahashi S., Nagamachi T., Yoneda K. and Yamada, H., *Agric. Biol. Chem.*, 1981, **45**, 831–838.
26. Bommarius, A. S., Kottenhahn, M., Klenk, H. and Drauz, K., *NATO ASI Ser., Ser. C*, 1992, **381**, 161–174.
27. Dinelli, D., Marconi, W., Cecere, F., Galli, G. and Morisi, F., in *Enzyme Engineering* (eds Bye, E. K. and Weetal, H. H.), Plenum Press, New York, 1978, vol. 3, pp. 477–481.
28. Lee, S. G., Lee, D. C., Sung, M. H. and Kim, H. S., *Biotechnol. Lett.*, 1994, **16**, 461–466.
29. Luksa, V., Starkuviene, V., Starkuviene, B. and Dagys, R., *Appl. Biochem. Biotechnol.*, 1997, **62**, 219–231.
30. Sharma, R. and Vohra, R. M., *Biochem. Biophys. Res. Commun.*, 1997, **234**, 445–448.
31. Durham, D. R. and Weber, J. E., *Biochem. Biophys. Res. Commun.*, 1995, **216**, 1095–1100.
32. Olivieri, R., Fascetti, E., Angelini, C. and Degen, L., *Biotech. Bioeng.*, 1981, **23**, 2173–2183.
33. Runser, S., Chinski, N. and Ohleyer, E., *Appl. Microbiol. Biotechnol.*, 1990, **33**, 382–388.
34. Yokozeki, K., Nakamori, S., Eguchi, C., Yamada, K. and Mitsugi, K., *Agric. Biol. Chem.*, 1987, **51**, 355–362.
35. Moller, A., Syldatk, C. and Wagner, F., *Enzyme Microb. Technol.*, 1988, **10**, 618–625.
36. Morin, A., Hummel, W. and Kula, M. R., *Biotechnol. Lett.*, 1986, **8**, 573–576.
37. Takahashi, S., Kii, Y., Kumagai, H. and Yamada, H., *J. Ferment. Technol.*, 1978, **56**, 492–498.
38. Lapointe, G., Leblanc, D. and Morin, A., *Appl. Microbiol. Biotechnol.*, 1995, **42**, 895–900.
39. Morin, A., TranTrung, N. H., Lapointe, G. and Dubeau, H., *Appl. Microbiol. Biotechnol.*, 1995, **43**, 259–266.
40. Chien, H. R. and Hsu, W. H., *Biotechnol. Lett.*, 1996, **10**, 879–882.
41. Meyer, P. and Runser, S., *FEMS Microbiol. Lett.*, 1993, **109**, 67–74.
42. Morin, A., Hummel, W. and Kula, M. R., *Appl. Microbiol. Biotechnol.*, 1986, **25**, 91–96.
43. Lee, D. C., Lee, S. G., Hong, S. P., Sung, M. H. and Kim, H. S., *Ann. N. Y. Acad. Sci.*, 1996, **799**, 401–405.
44. Zabriskie, D. W., Armiger, W. B., Phillips, D. H. and Albano, P. A., *Traders' Guide to Fermentation Media Formulation*, Traders Protein Inc. USA, 1980.
45. Deepa, S., Sivasankar, B. and Jayaraman, K., *Process Biochem.*, 1993, **288**, 447–452.
46. Achary, A., Hariharan, K. A., Bandhopadhyaya, S., Ramachandran, R. and Jayaraman, K., *Biotechnol. Bioeng.*, 1997, **55**, 148–154.
47. Lee, D. C., Kim, G. J., Cha, Y. K., Lee, C. Y. and Kim, H. S., *Biotechnol. Bioeng.*, 1997, **56**, 449–455.
48. Wallach, D. P. and Grisolia, S., *J. Biol. Chem.*, 1957, **226**, 277–288.
49. Brooks, K. P., Jones, E. A., Kim, B. D. and Sander, E. G., *Arch. Biochem. Biophys.*, 1983, **226**, 469–483.
50. Runser, S. M. and Meyer, P. C., *Eu. J. Biochem.*, 1993, **213**, 1315–1324.
51. Lee, S.G., Lee, D. C., Hong, S. P., Sung, M. H. and Kim, H. S., *Appl. Microbiol. Biotechnol.*, 1995, **43**, 270–276.
52. Lee, S. G., Lee, D. C. and Kim, H. S., *Appl. Biochem. Biotechnol.*, 1997, **62**, 251–266.
53. Xu, G. and West, T. P., *Arch. Microbiol.*, 1994, **161**, 70–74.
54. Morin, A., Leblanc, D., Paleczek, A., Hummel, W. and Kula, M. R., *J. Biotechnol.*, 1990, **16**, 37–48.
55. Yokozeki, K. and Kubota, K., *Agric. Biol. Chem.*, 1987, **51**, 721–728.

56. Runser, S. and Ohleyer, E., *Biotechnol. Lett.*, 1990, **12**, 259–264.
57. Yokozeki, K., Nakamori, S., Yamanaka, S., Eguchi, C., Mitsugi, K. and Yashinaga, F., *Agric. Biol. Chem.*, 1987, **51**, 715–719.
58. Morin, A., *Enzyme Microb. Technol.*, 1993, **15**, 208–214.
59. Ogawa, J. and Shimizu, S., *J. Mol. Catal. B (Enzymatic)*, 1996, **2**, 163–176.
60. Morin, A., Hummel, W., Schutte, H. and Kula, M. R., *Biotechnol. Appl. Biochem.*, 1986, **8**, 564–574.
61. Cecere, F., Galli, G. and Penna, D. G., US patent 4,065,353, 1977.
62. Gokhale, D. V., Bastawde, K. B., Patil, S. G., Kalkote, U. R., Joshi, R. R., Joshi, R. A., Ravindranathan, T., Gaikwad, B. G., Jogdand, V. V. and Nene, S., *Enzyme Microb. Technol.*, 1996, **18**, 353–357.
63. Kim, D. M. and Kim, H. S., *Enzyme Microb. Technol.*, 1993, **15**, 530–534.
64. Kim, G. J. and Kim, H. S., *Enzyme Microb. Technol.*, 1995, **17**, 63–67.
65. Kim, G. J. and Kim, H. S., *Biotechnol. Lett.*, 1994, **16**, 17–22.
66. Ogawa, J., Chung, M. C. M., Hida, S., Yamada, H. and Shimizu, S., *J. Biotechnol.*, 1994, **38**, 11–19.
67. Buson, A., Neggro, A., Grassato, L., Tagliaro, M. and Nuti, M. P., *FEMS Microbiol. Lett.*, 1996, **145**, 55–62.
68. Bernath, F. R. and Venkatasubramanian, K., in *Manual of Industrial Microbiology and Biotechnology* (eds Demain, A. L. and Solomon, N. A.), ASM Publication, USA, 1986, pp. 230–247.
69. Clark, D. S., *Tibtech.*, 1992, **12**, 439–443.
70. Katzir, K. E., *Tibtech.*, 1993, **12**, 439–443.
71. Aida, K., Chibata, I., Nakayama, K., Takinami, K. and Yamada, H., *Biotechnology of Amino Acid Production*, Elsevier, Amsterdam, 1986.
72. Matsumoto, K., in *Industrial Application of Immobilized Biocatalysts* (eds Tanaka, A., Tosa, T. and Kobayashi, T.), Marcel Deckker Inc., New York, 1993, pp. 67–88.
73. Yamada, H., Shimizu, S., Shimada, H., Tani, Y., Takahashi, S. and Takahisa, O., *Biochemie*, 1980, **62**, 395–399.
74. Chevalier, P., Roy, D. and Morin, A., *Appl. Microbiol. Biotechnol.*, 1989, **30**, 482–486.
75. Kim, D. M., Kim, G. J. and Kim, H. S., *Biotechnol. Lett.*, 1994, **16**, 11–16.
76. Lee, C. K. and Lin, K. C., *Enzyme Microb. Technol.*, 1996, **19**, 623–627.
77. Watabe, K., Ishikawa, T., Mukohara, Y. and Nakamura, H., *J. Bacteriol.*, 1992, **174**, 962–969.
78. Lapointe, G., Viau, S., Lablanc, D., Robert, N. and Morin, A., *Appl. Environ. Microbiol.*, 1994, **60**, 888–895.
79. Mukohara, Y., Ishikawa, T., Watabe, K. and Nakamura, H., *Biosci. Biotech. Biochem.*, 1994, **58**, 1621–1626.
80. Jacob, E., Henco, K., Marcenowski, S. and Schenk, G., US patent 4912044, 1990.
81. Kim, G. J., Park, J. H., Lee, D. C., Ro, H. S. and Kim, H. S., *Mol. Gen. Genet.*, 1997, **255**, 152–156.
82. Ogawa, J. and Shimizu, S., *Arch. Microbiol.*, 1995, **104**, 353–357.
83. Buckholz, R. and Cooper, T. G., *Yeast*, 1991, **7**, 913–923.
84. Hyaishi, O. and Komberg, A., *J. Biol. Chem.*, 1952, **197**, 717–732.
85. Ogawa, J., Soong, C. L., Honda, M. and Shimizu, S., *Eur. J. Biochem.*, 1997, **243**, 322–327.
86. Vogels, G. D. and Vanderdrift, C., *Bacteriol. Rev.*, 1976, **40**, 403–468.
87. Yamada, H., Shimizu, S., Kim, J. M., Shinmen, Y. and Sakai, T. A., *FEMS Microbiol. Lett.*, 1985, **30**, 337–340.
88. Williams, N. K., Manthey, M. K., Hambley, T. W., Donoghue, S. O., Keegan, M., Chapman, B. E. and Christopherson, R. I., *Biochemistry*, 1995, **34**, 11344–11352.
89. Zimmermann, B. H., Kemling, N. M. and Evans, D. R., *Biochemistry*, 1995, **34**, 7038–7046.
90. Holm, L. and Sander, C., *Proteins: Struc. Func. Gen.*, 1997, **28**, 72–82.