

# Production of D-Amino Acid Using Whole Cells of Recombinant *Escherichia coli* with Separately and Coexpressed D-Hydantoinase and N-Carbamoylase

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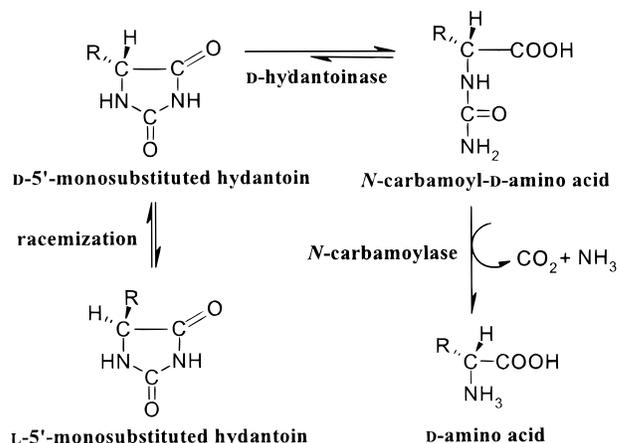
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We developed a fully enzymatic process employing D-hydantoinase and N-carbamoylase for the production of D-amino acid from 5'-monosubstituted hydantoin. For the comparison of the reaction systems using two sequential enzymes, D-hydantoinase of *Bacillus stearothermophilus* SD1 and N-carbamoyl-D-amino acid amidohydrolase (N-carbamoylase) of *Agrobacterium tumefaciens* NRRL B11291 were separately expressed in each host cell and coexpressed in the same host cell. A high level and constitutive expression of both enzymes in *Escherichia coli* in a soluble form was achieved using a promoter derived from *B. stearothermophilus* SD1. The expression levels of both enzymes ranged from 17% to 23% of the total soluble protein, depending on the expression system. In the case of employing separately expressed enzymes, the product yield of D-hydroxyphenylglycine from D,L-*p*-hydroxyphenylhydantoin and productivity were 71% and 2.57 mM/g-cell/h in 15 h, respectively. The accumulation of N-carbamoyl-D-hydroxyphenylglycine was significant over the reaction time. On the other hand, use of coexpressed enzymes resulted in 98% product yield of D-hydroxyphenylglycine in 15 h, minimizing the level of intermediates in the reaction mixture. The productivity of coexpressed whole cell reaction was estimated to be 6.47 mM/g-cell/h in 15 h. The coexpressed system was tested for an elevated concentration of D,L-*p*-hydroxyphenylhydantoin, and efficient production can be achieved.

## Introduction

Optically active D-amino acids are widely used as intermediates for the synthesis of semisynthetic antibiotics, peptide hormones, pyrethroids, and pesticides. In the D-hydantoinase-catalyzed process, the chemically synthesized D,L-5'-monosubstituted hydantoin is enantioselectively hydrolyzed to the corresponding N-carbamoyl-D-amino acid by D-hydantoinase, and this intermediate is further converted to the free D-amino acid by N-carbamoyl-D-amino acid amidohydrolase (N-carbamoylase) or chemical decarbamoylation (Figure 1). Because chemical decarbamoylation has several problems such as low yield and large disposal of waste, much attention has been paid to N-carbamoylase for development of an economic process. So far, several N-carbamoylases have been screened and characterized from *Agrobacterium* (Runser et al., 1990), *Pseudomonas* (Ikenaka et al., 1998), *Comamonas* (Ogawa et al., 1993), and *Arthrobacter* (Möller et al., 1988). Recently, the gene encoding N-carbamoylase of *Agrobacterium* was cloned and expressed in recombinant *Escherichia coli*, and the possibility of coexpression (Grifantini et al., 1998) or immobilization of the relevant enzymes was attempted (Nanba et al., 1998). However, quantitative analysis of a reaction system using both D-hydantoinase and N-carbamoylase remains to be conducted in detail.

In an effort to develop the D-hydantoinase-catalyzed process for the production of D-amino acid, we previously



**Figure 1.** Reaction scheme for the hydrolysis of 5'-monosubstituted hydantoin to D-amino acid using D-hydantoinase and N-carbamoylase; R indicates the functional group.

isolated and characterized the thermostable D-hydantoinase from *Bacillus stearothermophilus* SD1 (Lee et al., 1995). The gene encoding the enzyme was cloned and overexpressed in *E. coli* using a constitutive expression system (Lee et al., 1997; Kim et al., 1997b). Recently, we optimized a reaction system using mass-produced D-hydantoinase for the production of N-carbamoyl-D-*p*-hydroxyphenylglycine from D,L-*p*-hydroxyphenylhydantoin at high substrate concentration (Lee et al., 1998).

In this paper, we report the development of a whole cell process using D-hydantoinase and N-carbamoylase

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for the production of D-amino acid from D,L-5'-monosubstituted hydantoin. Of various D-amino acids, we focused on D-*p*-hydroxyphenylglycine (D-HPG) as a result of its high commercial demand. D-Hydantoinase from *B. stearo-thermophilus* SD1 previously studied in our laboratory was used for this purpose (Lee et al., 1999). As a second-step enzyme, *N*-carbamoylase from *Agrobacterium tumefaciens* NRRL B11291 (Olivieri et al., 1981) was cloned and expressed in *E. coli* using the same constitutive expression system as for D-hydantoinase. As for employing both D-hydantoinase and *N*-carbamoylase, two different systems were attempted. First, we separately expressed *N*-carbamoylase in *E. coli* and combined with a D-hydantoinase-expressing host strain. Second, both enzymes were coexpressed in the same host and used as a biocatalyst for the enzyme reaction. The two systems were compared and analyzed with respect to the product yield, intermediate accumulation, and performance. Details are reported here.

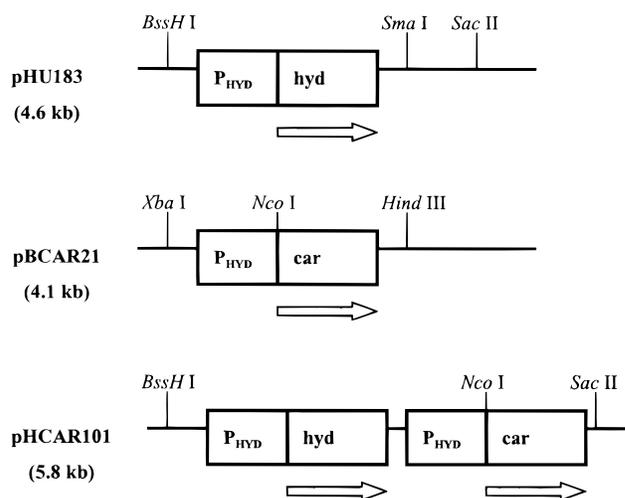
## Materials and Methods

**Enzymes and Reagents.** A thermophilic DNA polymerase for PCR was purchased from New England Biolabs Inc. (Beverly, MA). The restriction enzymes were purchased from Promega Co. (Madison, WI). D,L-*p*-Hydroxyphenylhydantoin (D,L-*p*-HPH) was purchased from Tokyo Kasei Kogyo Co. (Chuo-Ku, Tokyo). *N*-Carbamoyl-D-hydroxyphenylglycine (NC-D-HPG) was obtained from D,L-*p*-HPH by using D-hydantoinase as described elsewhere (Lee et al., 1998). D-Hydroxyphenylglycine (D-HPG) and all other chemicals were of analytical grade and purchased from Sigma (St. Louis, MO).

**Screening of Clones Expressing D-Hydantoinase and *N*-Carbamoylase.** D-Hydantoinase-expressing colony was screened on an agar plate containing 0.5% (w/v) D,L-*p*-HPH, 0.01% (w/v) phenol red, and 1 mM MnCl<sub>2</sub> as described in our previous work (Kim et al., 1997a). For the screening of *N*-carbamoylase-expressing colony, an agar plate containing 0.5% NC-D-HPG, 0.01% phenol red, 1 mM dithiothreitol (DTT) was used. The final pH of the solution was adjusted to 6.3. The transformants were grown for 12 h on a LB agar plate and transferred onto a nitrocellulose filter. The nitrocellulose filter was overlaid on the activity screening plate and incubated for 1 h at 37 °C. *N*-Carbamoylase-producing colony transforms the *N*-carbamoyl-D-amino acid to free D-amino acid, increasing pH, and a red color appears around the *N*-carbamoylase-producing colony.

**Construction of Constitutive Expression Vector.** *B. stearo-thermophilus* SD1 previously isolated in our laboratory and *A. tumefaciens* NRRL B11291 obtained from the culture collection were used as the source of the D-hydantoinase and *N*-carbamoylase genes, respectively. *E. coli* XL1-Blue (*supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA46*, *thi-1*, *relA1*, *lac*, [F' *proAB*, *laqI<sup>q</sup>ZΔM15*, Tn10(*tet<sup>r</sup>*)]) purchased from Stratagene Cloning Systems (LA Jolla, CA) was used as a host for the expression. The promoter region derived from *B. stearo-thermophilus* SD1 (Kim et al., 1997b) was amplified by PCR with the primers *sup1* (5'-AGCCTTAAGCAT-3') and *sup2* (5'-AAAACGGTACCG-3'). The amplified fragment of 350 bp was then cloned into the pBluescript II SK (Stratagene Cloning Systems), which had been digested with the restriction enzymes *Sma* I and *EcoRV*, by blunt-end ligation to construct the constitutive expression vector pBPR101.

**Constitutive Expression of *N*-Carbamoylase from *A. tumefaciens* NRRL B11291.** Chromosomal DNA was isolated from *A. tumefaciens*, and the gene encoding the



**Figure 2.** Genetic maps of the plasmids pHU183, pBCAR21, and pHCAR101. P<sub>HYD</sub> represents the promoter region from *B. stearo-thermophilus* SD1. Hyd and car refer to the coding regions of D-hydantoinase and *N*-carbamoylase, respectively. Arrows indicate the direction of the transcription.

*N*-carbamoylase (Grifantini et al., 1996) was amplified with the primers *car1* (5'-GTCCATGGCACGTCAG-3') and *car2* (5'-CGGATCCTTCAGAATTCGCG-3'). An amplified fragment of 0.9 kb was isolated from low melting agarose and inserted into the pBPR101 using the restriction enzymes *Nco* I and *BamH* I to produce plasmid pBCAR21. A *N*-carbamoylase-producing colony was selected by using the activity screening method, and the cloned gene was confirmed by DNA sequencing.

**Coexpression of D-Hydantoinase and *N*-Carbamoylase.** To construct the coexpression vector, plasmid pBCAR21 was digested with *Pvu* II, and plasmid pHU 183 (Lee et al., 1996) was digested with *Sma* I. The fragment cleaved from pBCAR21 was inserted into pHU183 by blunt-end ligation, and the resulting plasmid pHCAR101 was transformed into recombinant *E. coli* XL1-Blue using Gene Pulser II (Bio-Rad Inc., Hercules, CA). A colony producing both D-hydantoinase and *N*-carbamoylase constitutively was selected by using the activity screening method.

Genetic maps of the constructed plasmids pHU183, pBCAR21, and pHCAR101 are depicted in Figure 2.

**Enzyme Preparation.** Luria-Bertani (LB) medium containing 125 μg/mL of ampicillin was used for the production of D-hydantoinase and *N*-carbamoylase. Cells were cultivated in a 5 L jar containing 3 L of LB medium for 12 h. Temperature and initial pH for the cultivation were 30 °C and 6.8, respectively. Cells were harvested by centrifugation and washed twice with PBS (phosphate-buffered saline) solution containing 1 mM DTT and used for the whole cell enzyme reaction. When the free enzyme was used, cells (3 mg/mL) were disrupted by sonication using LABSONIC U (B. Braun Biotech International, Germany) for 2 min in the ice and centrifuged at 12,000g for 60 min. Supernatant was used for free enzyme reaction.

**Purification of D-Hydantoinase and *N*-Carbamoylase.** D-Hydantoinase was purified to homogeneity according to the previously reported procedure (Lee et al., 1995). Purification of *N*-carbamoylase was carried out by using a FPLC system (Amersham Pharmacia, Sweden). Crude extract of *N*-carbamoylase-producing cells was applied to a Mono-Q HR5/5 column (Amersham Pharmacia, Sweden) and then eluted with a linear gradient of 0 to 1.0 M NaCl in 20 mM sodium phosphate buffer.

*N*-Carbamoylase was eluted at 250 mM NaCl, and active enzyme fractions were pooled. Na<sub>2</sub>SO<sub>4</sub> was added to a final concentration of 20% (w/v), and the resulting solution was loaded onto a Phenyl-superose HR 5/5 (Amersham Pharmacia, Sweden) preequilibrated with 20% Na<sub>2</sub>SO<sub>4</sub>. The protein was eluted with a gradient of 20% to 0% Na<sub>2</sub>SO<sub>4</sub>, and a major activity peak was detected at 1% Na<sub>2</sub>SO<sub>4</sub>. Fractions were again pooled and concentrated by Centricon (Amicon).

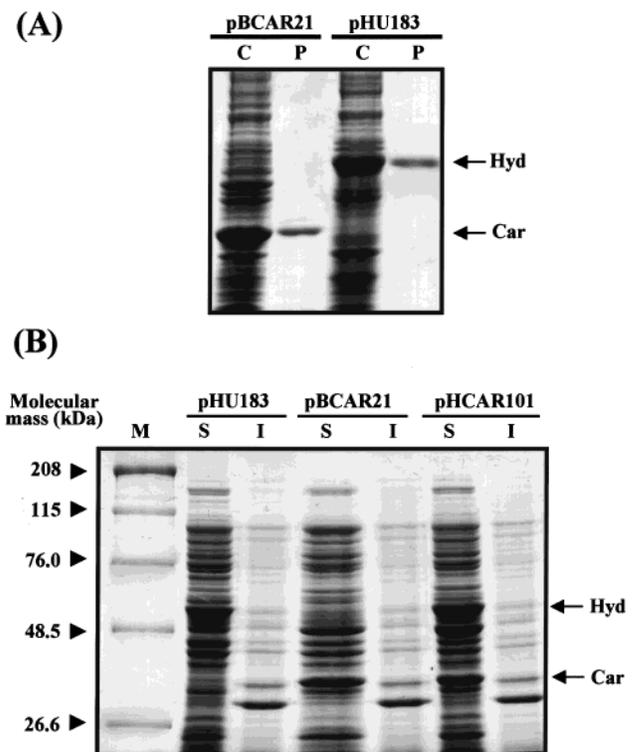
**Enzyme Reaction.** A stirred tank-type reactor equipped with a propeller-type impeller was used for *D*-HPG production from *D,L-p*-HPH. The initial volume of the reaction mixture was 1 L, and distilled water was used as the reaction medium. One millimole of MnCl<sub>2</sub> and DTT were added to reaction mixture to maintain the enzyme activity. The reaction was conducted at 45 °C, and the pH of the reaction mixture was controlled at 7.0 with 1 N HCl and 1 N NaOH during the reaction. Nitrogen gas was sparged with a flow rate of 0.5 vvm for the prevention of substrate oxidation (Lee et al., 1998). At intervals, aliquots were taken and analyzed by HPLC.

**Assay of Enzyme Activity.** The reaction mixture (1 mL) for the determination of *D*-hydantoinase activity contained 15 mM *D,L-p*-HPH as a substrate and 1 mM MnCl<sub>2</sub> in 100 mM Tris-HCl buffer (pH 8.0). In the case of *N*-carbamoylase activity, the reaction mixture (1 mL) was composed of 15 mM NC-*D*-HPG as a substrate and 1 mM DTT in 100 mM potassium phosphate buffer (pH 7.0). The free enzyme was added to the reaction mixture and incubated at 45 °C for 30 min and then stopped by addition of 0.5 mL of 12% trichloroacetic acid. The precipitate was removed by centrifugation, and the amounts of NC-*D*-HPG and *D*-HPG produced were determined using HPLC. One Unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of product from substrate per minute under the specified condition.

**Analysis.** The concentrations of *D,L-p*-HPH, NC-*D*-HPG, and *D*-HPG were determined using HPLC (Shimadzu, Japan). The column used was CLC-ODS (4.6 mm × 250 mm, Shimadzu, Japan). Ten percent (v/v) acetonitrile solution (pH 3.0) was used as the mobile phase, and the flow rate was 1.0 mL/min. The column eluent was detected at 214 nm. Biomass concentration was estimated by measuring the absorbance at 600 nm. The concentration of protein was determined by Bradford's method (Bradford, 1976) using bovine serum albumin as the standard. Analytical SDS/PAGE in slab gel was performed according to Laemmli's method (Laemmli, 1970). The standard proteins used were myosin (20.8 kDa), β-galactosidase (11.5 kDa), bovine serum albumin (76 kDa), fumarase (48.5 kDa), and triosephosphate isomerase (26.6 kDa). Coomassie brilliant blue R-250 was used for the staining of proteins. The relative density of the protein band was analyzed by using Gel Doc 2000 (Bio-Rad, Hercules, CA).

## Results

**Production of *D*-Hydantoinase and *N*-Carbamoylase.** *D*-Hydantoinase and *N*-carbamoylase were purified to homogeneity from recombinant *E. coli* cells harboring plasmids pHU183 and pBCAR21, respectively, and analyzed on SDS/PAGE (Figure 3A). The specific activities of *D*-hydantoinase and *N*-carbamoylase were determined to be 10.5 and 10.1 Units/mg protein, respectively, under standard assay conditions when *D,L-p*-HPH and NC-*D*-HPG were used as the substrates. Crude extracts of cells harboring plasmids pHU183 and pBCAR21 were also



**Figure 3.** (A) SDS/PAGE analysis of the crude extract and purified enzymes from recombinant *E. coli* cells harboring plasmids pBCAR21 and pHU183; C and P denote crude extract and purified enzyme, respectively. Plasmids pBCAR21 and pHU183 contain the genes encoding the *N*-carbamoylase and *D*-hydantoinase, respectively. (B) SDS/PAGE analysis of the soluble and insoluble fractions of cells harboring each plasmid. Cultivated cells were disrupted by sonification and centrifuged at 13,000 rpm. Insoluble fractions were resuspended in the same volume of 2× loading buffer and loaded onto SDS/PAGE. The soluble fractions were directly loaded; M represents the standard proteins, and S and I indicate the soluble and insoluble fractions, respectively. Hyd and Car represent *D*-hydantoinase and *N*-carbamoylase, respectively.

analyzed on SDS/PAGE, and *D*-hydantoinase and *N*-carbamoylase were detected as the major band (Figure 3A).

To investigate the expression levels and state of each enzyme, the soluble and insoluble fractions from *E. coli* cells harboring plasmids pHU183, pBCAR21, and pHCAR101 were analyzed on SDS/PAGE (Figure 3B). The activities of *D*-hydantoinase and *N*-carbamoylase were detected only in the soluble fraction, which indicates that most of the enzymes are expressed as a soluble form. The expression levels of *D*-hydantoinase in XL1-Blue/pHU183 and *N*-carbamoylase in XL1-Blue/pBCAR21 were estimated to be 17% and 16% of the total soluble proteins, respectively. In the case of *E. coli* harboring pHCAR101, both *D*-hydantoinase and *N*-carbamoylase were also expressed in a soluble state in the cytoplasm (Figure 3B). The expression levels of *D*-hydantoinase and *N*-carbamoylase were analyzed to be 23% and 18% of total soluble proteins, respectively, even when coexpressed in XL1-Blue/pHCAR101. These results strongly imply that the promoter derived from *B. stearothermophilus* SD1 effectively functions for the constitutive expression of enzymes as a soluble form.

The expression level of foreign proteins was reported to be dependent on the host cells (Hellebust et al., 1989). We tested several host strains including DH5α, JM109, BL21, and HB101 and compared the expression levels of each enzyme. However, no significant difference in the

**Table 1. Specific Activities and Expression Levels of Enzymes in Different Host Strains**

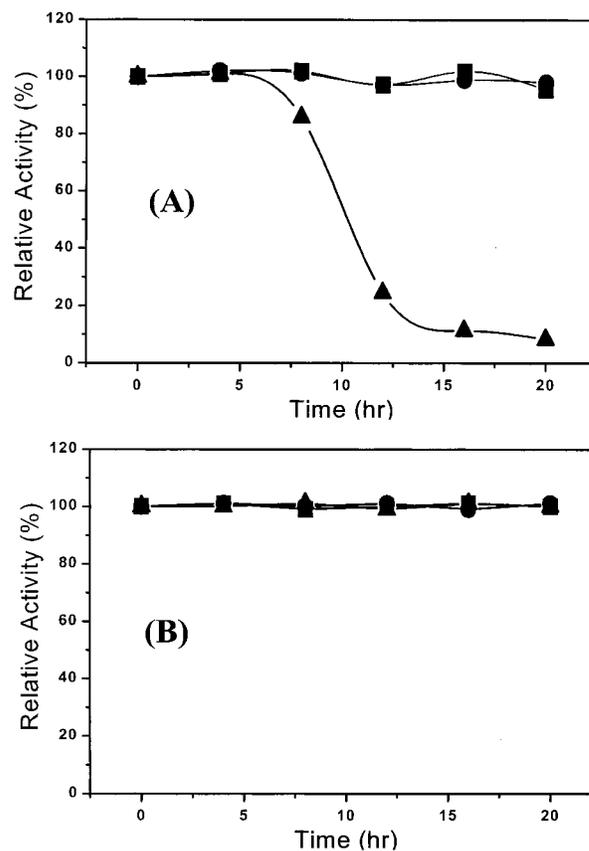
	strain	specific activity (Units/mg-DCW)
D-hydantoinase	<i>B. stearothermophilus</i> SD1	0.05
	XL1 Blue/pHU183	1.04
	XL1 Blue/pHCAR101	0.97
<i>N</i> -carbamoylase	<i>A. tumefaciens</i> NRRL B11291	0.01
	XL1 Blue/pBCAR21	1.14
	XL1 Blue/pHCAR101	1.16

expression level was observed in all cases when compared with XL1 Blue. The specific activities of *E. coli* XL1-Blue containing each plasmid are summarized in Table 1. We previously reported mass production of D-hydantoinase with its own promoter in a recombinant *E. coli* (Lee et al., 1997). *N*-Carbamoylase was also constitutively expressed with promoter from *B. stearothermophilus* SD1. Plasmid stability was analyzed during serial subcultivation up to 100 generations, and segregational or structural instability was found to be negligible (data not shown).

**Optimal Conditions for the Reaction Using Free D-Hydantoinase and *N*-Carbamoylase.** D-Hydantoinase from *B. stearothermophilus* SD1 and *N*-carbamoylase from *A. tumefaciens* NRRL B11291 have different optimal pH and temperature (Lee et al., 1995; Chao et al., 1999). We previously observed a serious modification of substrate D,L-*p*-HPH at temperatures above 45 °C (Lee et al., 1998). The activity of *N*-carbamoylase was stably maintained for more than 15 h at 45 °C but rapidly decreased in 10 h at 50 °C (Figure 4A). The D-hydantoinase remained stable for more than 20 h even at 50 °C (Figure 4B). The enzyme reaction rate increased with increasing temperature, but rapid deactivation of *N*-carbamoylase and substrate modification occurred at temperatures above 45 °C. In this work, the optimal reaction temperature was determined to be 45 °C by taking the above points into account.

Optimal pH of D-hydantoinase was found at pH 8.5 (Lee et al., 1998). On the other hand, *N*-carbamoylase showed a maximum activity at pH 7.0. The product yield of D-HPG and intermediate (NC-D-HPG) accumulation are important factors to be considered when the two-step enzyme reaction is employed. To determine the optimal pH, we investigated the product yield and amount of intermediate accumulated using free enzymes in the range of pH 7.0–8.0. The total activities of two enzymes were adjusted at the same level in the reaction mixture. As shown in Figure 5A, the product yield of D-HPG reached about 98% in all cases, but in the case of pH 8.0, the intermediate was accumulated up to 3.5 mM and production rate of D-HPG was reduced compared with that at pH 7.0 (Figure 5B). This result implies that production rate of D-HPG and accumulation of intermediate is largely affected by the reaction condition of the second-step enzyme *N*-carbamoylase. It was reported that the activity of *N*-carbamoylase is inhibited by ammonia more seriously at alkaline pH than neutral or acidic condition (Olivieri et al., 1981; Runser et al., 1990). The effect of ammonia on the *N*-carbamoylase activity was investigated, and as shown in Figure 6, inhibition of *N*-carbamoylase by ammonia was more serious at pH 8.0 than at neutral pH. From this observation, the reaction using D-hydantoinase and *N*-carbamoylase was performed at pH 7.0 in this work.

**Whole Cell Reaction Using Separately Expressed D-Hydantoinase and *N*-Carbamoylase.** As for a reaction system using whole cells containing D-hydantoinase

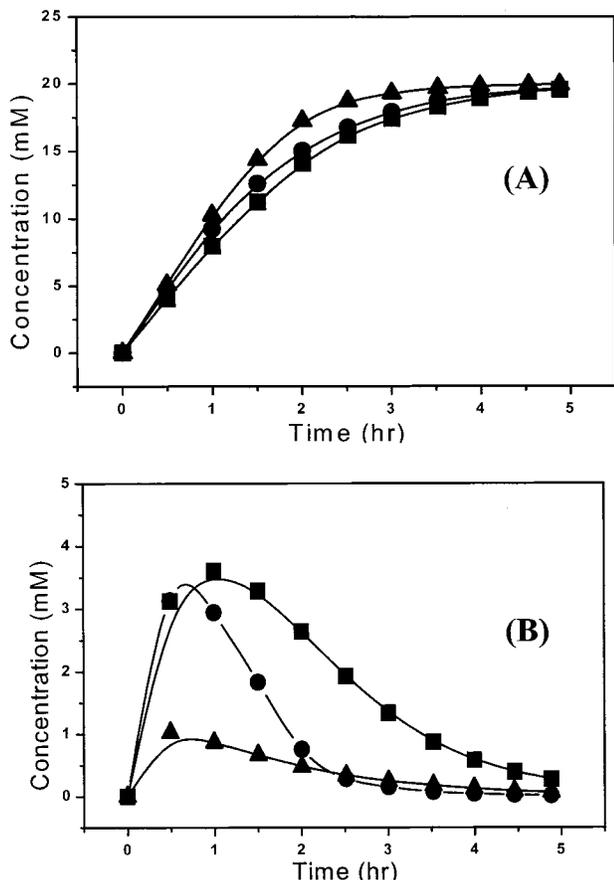


**Figure 4.** Time course of residual activity of *N*-carbamoylase (A) and D-hydantoinase (B) at different temperatures; 30 mg of cells in 10 mL of buffer was incubated at indicated temperature, and the residual activity was determined under standard condition. 100 mM Tris-HCl (pH 8.0) and Na-phosphate buffers (pH 7.0) were used for D-hydantoinase and *N*-carbamoylase, respectively. One hundred percent of activity of D-hydantoinase and *N*-carbamoylase correspond to 1.04 Units/mg-DCW and 1.14 Units/mg-DCW, respectively. Symbols: ▲, 50 °C; ●, 45 °C; ■, 40 °C.

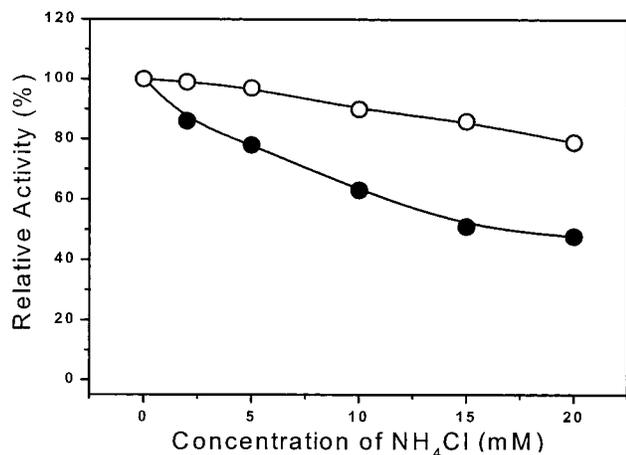
and *N*-carbamoylase, two different systems are possible. One is to use two host cells expressing each enzyme separately, and the other is employing a single host cell expressing both two enzymes. To compare the two systems, we first carried out the reaction using separately expressed enzymes. When separately expressed D-hydantoinase and *N*-carbamoylase were used, the ratio of the activities between the two enzymes was controlled by changing the loading of individual cells. Figure 7A shows the production profile when the activities of the two whole cell enzymes were the same, 200 Units/L. The product yield of D-HPG reached 71% in 15 h, and the intermediate, NC-D-HPG, accumulated up to 13 mM in the reaction mixture. The initial production rate of D-HPG was estimated to be 0.85 mM/h, and the intermediate remained at a high level over the entire reaction time.

When the activity of *N*-carbamoylase increased to 400 Units/L, the initial production rate of D-HPG increased up to 1.95 mM/h. The final conversion was about 90% in 15 h, and the intermediate accumulated up to 10.4 mM at the beginning of reaction (Figure 7B).

**Whole Cell Reaction Using Coexpressed D-Hydantoinase and *N*-Carbamoylase.** The reaction was carried out using whole cells coexpressing both D-hydantoinase and *N*-carbamoylase. In this case, the ratio of the two enzymes could not be controlled in the constructed expression system used. After the recombinant *E. coli* XL1 Blue/pHCAR101 was harvested, the specific activity

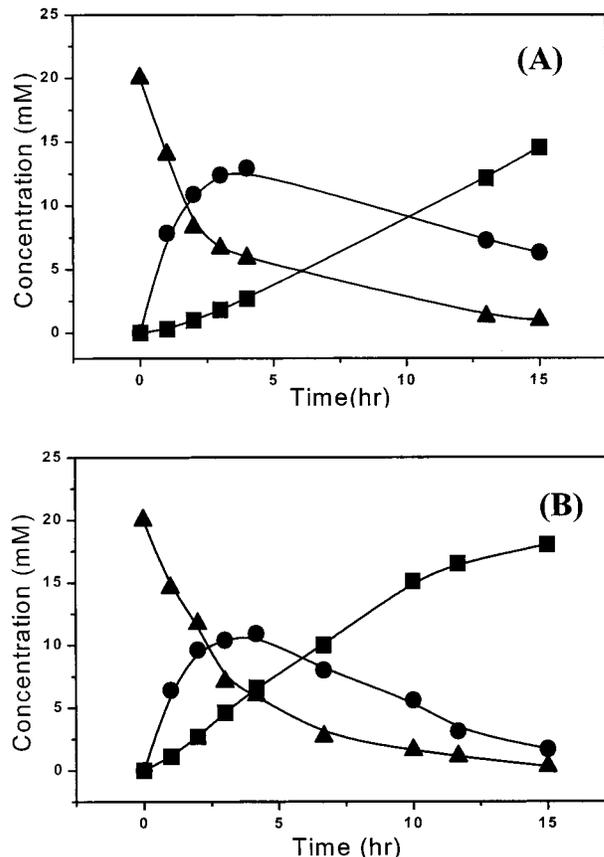


**Figure 5.** Profiles of D-HPG production (A) and intermediate accumulation (B) at various pH values. Initial concentration of substrate (*D,L-p*-HPH) was 20 mM in a 1 L reaction mixture. Reaction was performed at 45 °C, and pH was controlled at the specified value using 1 N HCl and 1 N NaOH. The total activities of D-hydantoinase and *N*-carbamoylase in the reaction mixture were the same, 500 Units. Symbols: ▲, pH 7.0; ●, pH 7.5; ■, pH 8.0.



**Figure 6.** Effect of ammonia on the *N*-carbamoylase activity. The concentration of ammonium chloride was varied from 0 to 20 mM. *N*-Carbamoylase (50 mg of crude extract) was added to the reaction mixture (5 mL) containing 100 mM Na-phosphate buffer and a predetermined concentration of ammonium chloride. After incubation for 30 min, the activity was determined at specified pH under standard condition. Symbols: ○, pH 7.0; ●, pH 8.0.

of each enzyme was measured under standard assay conditions. The ratio of activities between D-hydantoinase and *N*-carbamoylase per gram of cells was estimated to be 1:1.2. For the comparison with the reaction system



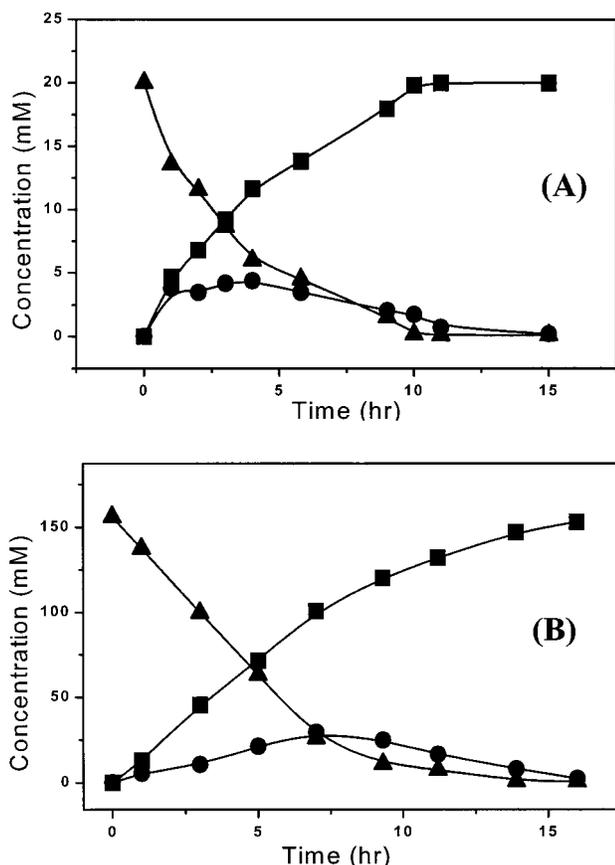
**Figure 7.** Profiles of D-HPG production using the separately expressed enzymes. The total activities of D-hydantoinase and *N*-carbamoylase were (A) 200 (192 mg of XL1-Blue/pHU183) and 200 Units/L (175 mg of XL1-Blue/pBCAR21); (B) 200 (192 mg of XL1-Blue/pHU183) and 400 Units/L (350 mg of XL1-Blue/pBCAR21), respectively. Initial substrate concentration was 20 mM, and total reaction volume was 1 L. Symbols: ▲, *D,L-p*-HPH; ●, NC-D-HPG; ■, D-HPG.

using separately expressed enzymes, the amount of cell loading was determined on the basis of D-hydantoinase activity. When the activities of D-hydantoinase and *N*-carbamoylase were fixed at 200 and 240 Units/L in the reaction mixture, the product yield of D-HPG reached 98% in 15 h, and a much lower level of intermediate accumulated in the reaction mixture, compared with that using the separately expressed whole cells (Figure 8A).

From an economical standpoint of view, an enzyme reaction at high substrate concentrations is desirable. The solubility of the starting substrate, *D,L-p*-HPH, is about 60 mM in aqueous solution at 45 °C, and the enzyme reaction proceeds in the heterogeneous system containing solid substrate particles when the substrate concentration is beyond its solubility (Lee and Kim, 1988). We investigated the possibilities of using the coexpressed system for high substrate concentration. The concentration of substrate was 165 mM (30 g/L), and the activities of D-hydantoinase and *N*-carbamoylase were 1200 and 1440 Units/L, respectively, in the reaction mixture. As shown in Figure 8B, the product yield of D-HPG reached about 96% in 15 h, and the intermediate level was low.

## Discussion

In the D-hydantoinase-catalyzed process for the production of optically active D-amino acids, *N*-carbamoylase has recently been of great interest, because enzymatic decarbamoylation offers several advantages over the



**Figure 8.** Profiles of D-HPG production using the whole cells coexpressing the enzymes at different reaction conditions. (A) Initial substrate concentration was 20 mM. The total activities of D-hydantoinase and *N*-carbamoylase in the reaction mixture were 200 and 240 Units/L (206 mg of XL1 Blue/pHCAR101), respectively. (B) Initial substrate concentration was 165 mM (30 g/L). The total activities of D-hydantoinase and *N*-carbamoylase were 1200 and 1440 Units/L (1.24 g of XL1 Blue/pHCAR101), respectively. Symbols are the same as in Figure 7.

chemical method. In this work, we demonstrated that the coexpression of *N*-carbamoylase with D-hydantoinase in the same host cell led to the efficient production of D-amino acid from 5'-monosubstituted hydantoin.

To provide an economically feasible process, cost-effective production of the biocatalyst is a prerequisite. Much attention has been paid to the overproduction of pharmaceutical proteins by recombinant microorganisms. Previously, we successfully mass-produced D-hydantoinase from *B. stearothermophilus* SD1 using a constitutive expression system in *E. coli* (Lee et al., 1997). To take advantage of the constitutive expression system in the presence work, *N*-carbamoylase was also expressed in *E. coli* using the same promoter derived from *B. stearothermophilus* SD1. As a result, most of *N*-carbamoylase was expressed as a soluble form, and the harvested cells could be directly employed as a biocatalyst in the enzymatic process. Recently, Grifantini et al. (1998) reported the construction of a recombinant *E. coli* strain expressing both D-hydantoinase and *N*-carbamoylase of *A. tumefaciens* NRRL B11291. In their case, a large portion of enzymes was produced as an insoluble aggregate when recombinant *E. coli* was cultivated at 37 °C, and the active soluble fraction of both enzymes was slightly increased at low temperature such as 25 °C. It is noteworthy that a relatively high expression level of D-hydantoinase and *N*-carbamoylase was achieved by

using the constitutive expression system constructed in this work, and this system appears to be effectively used for other proteins.

*N*-Carbamoylase from *A. tumefaciens* is labile against oxidation, requiring a reducing agent such as dithiothreitol and L-cysteine to maintain its activity, and this seems to be due to the crucial role of Cys<sup>172</sup> in the enzyme catalysis (Grifantini et al., 1996). The starting substrate, D,L-*p*-HPH, has a very low solubility in aqueous solution, and a heterogeneous reaction system is desirable for the efficient production of D-HPG as previously described (Lee et al., 1999). However, deactivation of free enzymes by substrate particles in the heterogeneous system (Lee and Kim, 1998; Deeble and Lee, 1985) and titrant such as the NaOH solution (Lee et al., 1999) were observed to be serious. For these reasons, we attempted to use whole cell enzymes instead of free enzymes. In the reaction catalyzed by two sequential enzymes, two different systems can be considered. First, two enzymes are separately expressed in each host cell and combined together in the bioreactor. In this case, the activity ratio is controlled by changing the loading of each enzyme. However, enzyme production needs two individual cultivations, and transport of reaction intermediate might be a limiting factor. As an alternative, both enzymes can be coexpressed in the same host cell and directly used as a biocatalyst. Coexpression system needs only a single cultivation step for enzyme production, and transport barrier of intermediate posed by cell membranes can be minimized, because sequential reaction takes place inside the same cells. However, it seems difficult to control the activity level of each enzyme. Regulation of the expression level by using different promoters with different strength or improving the specific activity of the limiting-step enzyme may be an effective way to solve this problem. Recently, directed evolution has been proven to be a powerful tool for improving the properties of enzymes (Stemmer, 1994; Zhao, 1998), and this approach may be applied to *N*-carbamoylase and D-hydantoinase. In fact, *N*-carbamoylase has much lower thermal stability compared with D-hydantoinase, and improvement of catalytic property is required.

In this work, we compared the two systems in terms of the product yield of D-HPG and intermediate (NC-D-HPG) accumulation. It was observed that NC-D-HPG has a low permeability through the cell membrane of *E. coli* (Lee et al. 1999). When the separately expressed system was used, intermediate accumulated at a high level, and consequently the product yield of D-HPG was relatively low. Moreover, the intermediate level slightly decreased even though the loading of *N*-carbamoylase was doubled. These results strongly suggest that the second-enzyme step limited the overall process, and consequently, the intermediate accumulated at a high level in the reaction mixture. On the other hand, use of cells coexpressing both enzymes resulted in high yield of product, up to 98% in 15 h, leading to a low level of intermediate compared with the separately expressed system.

From the above results, it is evident that the coexpressed whole cell system is more efficient in the production of D-amino acid from hydantoin derivative, when compared with the separately expressed whole cell system. The approach attempted in this work may be applied to other reactions requiring more than a single enzyme.

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