One-Step Production of D-*p*-Hydroxyphenylglycine by Recombinant *Escherichia coli* Strains

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The gene encoding D-hydantoinase from *Agrobacterium radiobacter* NRRL B11291 was successfully cloned by use of polymerase chain reaction. A positive clone was scored, and its nucleotide sequence was further analyzed. The analysis by deleting various lengths of nucleotides from the amino terminus of the open reading frame revealed the putative regions for promoter and RBS site. By highly expressing both D-hydantoinase and carbamoylase, recombinant *Escherichia coli* strains were able to convert DL-hydroxyphenyl hydantoin (DL-HPH) to D-*p*-hydroxyphenylglycine (D-HPG) with a conversion yield of 97%, accounting for productivity 5 times higher than that obtained by *A. radiobacter* NRRL B11291. Immobilizing the recombinant cells with κ-carrageenan could also achieve a conversion of 93%, while *A. radiobacter* NRRL B11291 attained 20% within the same period of reaction time. These results illustrate the feasibility in employing recombinant *E. coli* to accomplish one-step conversion of DL-HPH to D-HPG. In the process of improving D-HPG production, D-hydantoinase activity was increased 2.57-fold but carbamoylase activity remained constant, which resulted in only a 30% increase in the reaction rate. It suggests that carbamoylase is the step setting the pace of the reaction. Since the reaction substrate is highly insoluble, achieving sufficient agitation appears to be an important issue in this heterogeneous system. This view is further supported by the study on repeated use of cells, which shows that to reach a conversion of more than 90% free cells can be recycled six times, whereas immobilized cells can be used only twice. In conclusion, the poor reusability of immobilized cells is due to the fouling on the gel surface.

Introduction

The increasing demand for antibacterial drugs has called for a mass production of semisynthetic antibiotics. Among the optically active D-amino acids, D-*p*-hydroxyphenylglycine (D-HPG) and D-phenylglycine are the most important precursors used for the synthesis of semisynthetic cephalosporin and penicillin. The preparation of D-HPG is mainly carried out in a two-step reaction (Olivieri et al., 1981). First, DL-hydroxyphenyl hydantoin (DL-HPH) is stereospecifically converted to *N*-carbamoyl-D-*p*-hydroxyphenylglycine (CpHPG) by D-hydantoinase (E.C. 3.5.2.2). Second, either a chemical method (Takahashi et al., 1979; Gokhale et al., 1996) or an enzymatic route enables the subsequent hydrolysis of CpHPG to D-HPG. However, the enzymatic method, mediated by *N*-carbamoyl-D-amino acid amidohydrolase (hereinafter, carbamoylase), appears to be more appealing because the hydrolysis reaction can be conducted under milder conditions.

D-Hydantoinase exists primarily in living organisms (LaPointe et al., 1995; Rai and Taneja, 1998). It is capable of catalyzing the ring opening of dihydropyrimidines, as well as 5-monosubstituted hydantoins, which resembles the reaction catalyzed by dihydropyrimidinase. Consequently, its physiological role in microorganisms as a pyrimidine scavenger has been proposed (Ogawa and Dhimizu, 1997). In contrast, a physiological role of carbamoylase still remains elusive, although it has been found in many microbes (Moller et al., 1988; Ogawa et al., 1994; Louwrier and Knowles, 1996; Ikenaka et al., 1998b). Interestingly, the coexistence of both proteins (D-hydantoinase and carbamoylase) in microorganisms occurs rarely, and few cases have been documented thus far (Olivieri et al., 1979; Moller et al., 1988; Runser et al., 1990).

The production of D-HPG from DL-HPH in single step seems highly likely if the cell containing both D-hydantoinase and carbamoylase is employed for the biotransformation reaction. Indeed, the process for this reaction was developed in 1980s employing *Agrobacterium radiobacter* NRRL B11291 (Olivieri et al., 1981). The reaction scheme by this approach is relatively simple, but it still leaves a large margin for improvement in terms of productivity. In this context, the level of enzyme activity for both enzymes has to be enhanced. Furthermore, an easier way to culture the cells may be beneficial to the process development if considering the efficiency in achieving higher biomass per unit volume per unit time.

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Escherichia coli remains the most robust strain used in the area of applied biotechnology as a result of a comprehensive knowledge of it both in biochemistry and molecular biology. Following this logic, genetic manipulation of *E. coli* to achieve the goal of high D-HPG production seems promising. Continuing our previous work on overexpression of carbamoylase in *E. coli* (Chao et al., 1999), we have further attempted to clone the gene encoding D-hydantoinase from *A. radiobacter* NRRL B11291. By highly expressing both enzymes, one-step conversion of DL-HPH to D-HPG with the recombinant *E. coli* strain is expected. Moreover, the process for D-HPG production using immobilized cells is also evaluated in this study.

Materials and Methods

Materials. DL-HPH was purchased from TCI Co. (Tokyo, Japan). CpHPG was prepared from DL-HPH hydrolyzed by an immobilized D-hydantoinase (Lee and Lin, 1996). Most chemicals used were mainly obtained from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes, T4 DNA ligase, and λ/*Hin*dIII DNA marker were purchased from New England Biolabs, Inc. (Beverly, MA). Polymerase chain reaction (PCR) was performed with Pfu polymerase bought from Promega (Madison, WI).

DNA Manipulation and Bacterial Strains. The *E. coli* strain used throughout this study is DH5α [*deoR endA1 gyrA96 hsdR17*(r_k^- m_k^+) *supE44 thi-1* Δ(*lacZYA-argF169*) *recA1* ϕ*80* Δ*lacZM15*]. The gene encoding D-hydantoinase was cloned by use of PCR with the genomic DNA of *A. radiobacter* NRRL B11291, prepared by Puregene DNA isolation kit (Gentra Systems, Inc.), as the template. The oligonucleotides priming the structure gene of D-hydantoinase were designed on the basis of the DNA sequence in GenBank (accession number X91070). The resulting 1.5-kb *Xho*I-*Hin*dIII fragment of D-hydantoinase gene product, containing its natural promoter, was then ligated into pBluescript II KS+ (Strategen) to give pHDT4. Plasmid pSHDT4 was constructed by ligating a 1.5-kb fragment of the D-hydantoinase gene into the *Xho*I-*Hin*dIII site of pSY544 (S. Yasuda), a derivative of pACYC184. To generate plasmid pKT-HDT4, the DNA fragment containing the D-hydantioniase gene was subcloned into vector pKT254 carrying a ColD origin (Frey et al., 1985) at the *Xho*I-*Hin*dIII site. To enhance plasmid stability, the *parB* gene was recovered from pRA90 (Nycomed Pharma A/S) using *Bam*HI and *Eco*RI. Along with the *parB* gene the carbamoylase gene cleaved from pAH61 (Chao et al., 1999) with *Bam*HI and *Hin*dIII was ligated into pUC118 to yield pAH71.

Media and Culture Conditions. Unless otherwise stated, recombinant *E. coli* strains were grown in Luria–Bertani (LB) medium (Miller, 1972) supplemented with 0.02 M glycerol at 30 °C. Cultivation of *A. radiobacter* NRRL B11291 followed the method previously reported (Olivieri et al., 1981). Antibiotics such as ampicillin (100 μg/mL), kanamycin (50 μg/mL), or streptomycin (10 μg/mL) were used as needed to screen recombinants.

Enzyme Assay. The reaction solution comprised either 10 mM DL-HPH or 20 mM CpHPG and 0.1 M sodium phosphate buffer (pH 7.5). The cells were first permeabilized by toluene, followed by the addition of 10 mg of dry cells to a 5 mL reaction solution. Subsequently, the reaction was carried out at 40 °C. Aliquots of samples were withdrawn and determined for the concentrations of CpHPG and D-HPG. Specific enzyme activities, defined as micromole of product generated per minute per milli-gram of dry cell weight (DCW), for D-hydantoinase and carbamoylase were then calculated.

Immobilization Method. The immobilized cells were prepared by mixing equal volume (7.5 mL) of 1.35 g DCW of recombinant *E. coli* and of 0.3 g of κ-carrageenan at 43 °C. To form a bead shape, the 14-mL cell slurry was pressed through an orifice 1 mm in diameter using a syringe into 0.3 M cold KCl solution for overnight. Bead-type gels 3 mm in diameter were obtained. For hardening treatment, the bead-shape gels were soaked in a solution consisting of 20 mM glutaraldehyde and 70 mM hexamethylenediamine for 15 min.

Production of D-HPG Using Free or Immobilized Cells. The free cells were prepared as described previously (Chao et al., 1999). Production of D-HPG employing free or immobilized cells was carried out in a stirred reactor (30 mL) equipped with a sample port. During the reaction the reactor was purged with nitrogen gas as required to avoid substrate oxidation, and agitation was achieved with a magnetic stir bar. Unless otherwise described, the 10-mL reaction solution in the reactor consisted of 100 mM DL-HPH and 0.1 M sodium phosphate buffer (pH 7.5). The reaction containing the indicated amount of free cells or 1.35 g DCW of immobilized cells was carried out at 40 °C and 200 rpm.

Analytical Methods. Cell density was measured turbidimetrically at 550 nm (OD_{550}). The concentrations of DL-HPH, CpHPG, and D-HPG were determined according to the previous method (Chao et al., 1999).

Results

Cloning of D-Hydantoinase. As indicated in Figure 1a, the D-hydantoinase gene of *A. radiobacter* NRRL B11291 was cloned with primer 1 and primer 5 by use of PCR. Over 30 clones were screened for positive D-hydantoinase by determining enzyme activity. One clone exhibiting D-hydantoinase activity was then scored and designed pHDT4. To confirm the cloned DNA, the sequencing of pHDT4 was carried out, and the resulting nucleotide sequence was found to be identical to that listed in GenBank.

For further analyses of the open reading frame (ORF), three more oligonucleotides (primers 2, 3, and 4 shown in Figure 1a) priming the amino terminus were synthesized. As a consequence of PCR using a different combination of primers, recombinant plasmids containing various ORF of the D-hydantoinase gene were then created (Figures 1a and 1b). With primer 2 and primer 5, the PCR product cloned in pBluescript II SK+ gave plasmid pHDT4-2. The resulting DNA fragment produced by primer 3 and primer 5 in pBluescirpt II KS+, pTrc99A (Pharmacia LKB Biotechnology), and pUHE23-2 (H. Bujard) yielded plasmids pHDT4-3, pHDT4-4, and pHDT4-5, respectively. Furthermore, the PCR product using primer 4 and primer 5 was ligated into pBluescript II KS+, pTrc99A, and pUHE23-2 to generate plasmids pHDT4-6, pHDT4-7, and pHDT4-8, respectively. Among these clones, pHDT4, pHDT4-2, pHDT4-4, pHDT4-5, and pHDT4-7 in strain DH5α displayed a D-hydantoinase activity (Table 1). In contrast to pHDT4-7, pHDT4-8 in strain DH5α showed no D-hydantoinase activity. The result indicates a start codon (ATG) in the primer 4 region because pHDT4-8 loses the ribosome-binding site (RBS) from pHUE23-2 during the plasmid construction with an *Eco*RI digestion (Figure 1b). Plasmid pHDT4-7 regains D-hydantoinase activity as a result of the resumption of RBS in pTrc99A (Figure 1b). As compared to pHDT4-8, pHDT4-5 in strain DH5α became D-hydan-
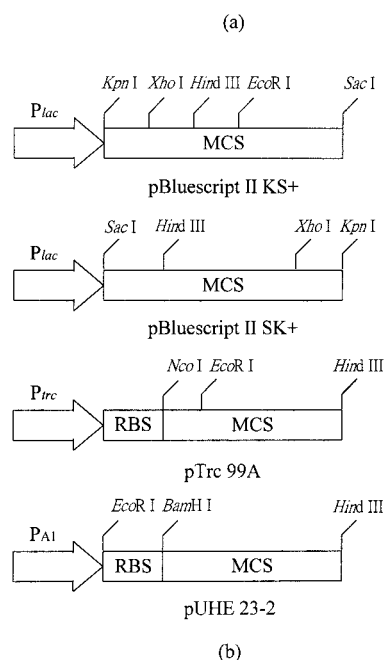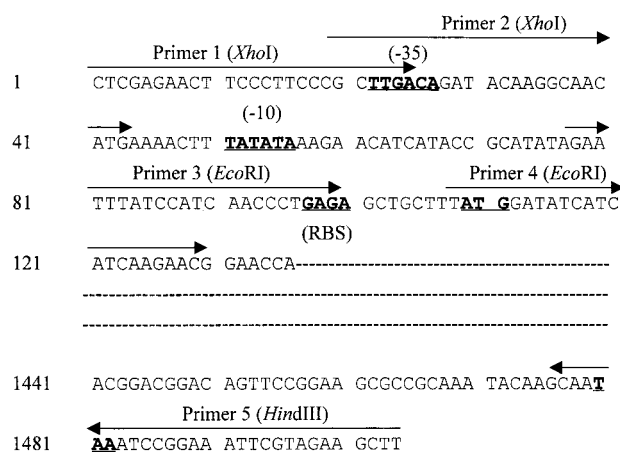
Primer 2 (*Xho*I)
Primer 1 (*Xho*I)   (-35)
1   CTCGAGAACT TCCCTTCCCG C**TTGACA**GAT ACAAGGCAAC

(-10)
41   ATGAAAACTT **TATATA**AAGA ACATCATACC GCATATAGAA
Primer 3 (*Eco*RI)   Primer 4 (*Eco*RI)

81   TTTATCCATC AACCCT**GAGA** GCTGCTTT**AT G**GATATCATC
(RBS)

121 ATCAAGAACG GAACCA--

1441 ACGGACGGAC AGTTCCGGAA GCGCCGCAAA TACAAGCAA**T**
Primer 5 (*Hin*dIII)
1481 **AA**ATCCGGAA ATTCGTAGAA GCTT

(a)

Kpn I   Xho I   Hind III   EcoR I   Sac I
P*lac* ─►   MCS
pBluescript II KS+

Sac I   Hind III   Xho I   Kpn I
P*lac* ─►   MCS
pBluescript II SK+

Nco I   EcoR I   Hind III
P*trc* ─►   RBS   MCS
pTrc 99A

EcoR I   BamH I   Hind III
P*A1* ─►   RBS   MCS
pUHE 23-2

(b)

Figure 1. (a) Nucleotide sequence of the gene encoding D-hydantionase from *A. radiobacter* NRRL B11291. Only amino and carboxyl terminus of the ORF are shown. The extent and direction of the oligonuclotides are indicated with the arrows. As indicated in parentheses, each primer with a restriction site tagged to its 5′ end was synthesized. The sequences homologous to the −35 and −10 region of the promoter are underlined. Putative sites for RBS and start codon are also highlighted. See text for the details. (b) Schematic diagram of the promoter region along with the multiple cloning site (MCS) for the cloning vectors. Abbreviations used: P*lac*, *lac* promoter; P*trc*, *trc* promoter; P*A1*, T7 *A1* promoter. The arrowheads indicate the transcriptional direction.

toinase positive, thereby suggesting the existence of the nature RBS site in primer 3 region, although the RBS in pUHE23-2 was trimmed off during pHDT4-5 construction using an *Eco*RI cleavage. On the other hand, pHDT4-3 and pHDT4-6 in strain DH5α gave no D-hydantoinase activity but did for pHDT4-2, thus indicating the presence of a promoter site which is located between the regions of primer 2 and primer 3.

As deduced from the analyses above, the putative sites for start codon and RBS are highlighted in Figure 1b. Also indicated in Figure 1b, the nucleotides between primer 2 and primer 3 give putative sites for the −10 and −35 region of the D-hydantoinase gene, which resembles the consensus sequences of the σ^{70} promoter in *E. coli*.

Table 1. *E. coli* Strain DH5α Harboring Various Clones for D-Hydantoinase Gene[a]

recombinant plasmid	vector	primers	D-hydantoinase activity
pHDT4	pBluescript II SK+	1,5	+
pHDT4-2	pBluescript II SK+	2,5	+
pHDT4-3	pBluescript II KS+	3,5	−
pHDT4-4	pTrc99A	3,5	+
pHDT4-5	pUHE23-2	3,5	+
pHDT4-6	pBluescript II KS+	4,5	−
pHDT4-7	pTrc99A	4,5	+
pHDT4-8	pUHE23-2	4,5	−

[a] The primers flanking the ORF of D-hydantoinase gene were designed according to the nucleotide sequence analyzed in Figure 1a. PCR products were then cloned into distinct vectors to generate the recombinant plasmids listed in the table. The plus sign denotes D-hydantoinase positive, and the minus sign means D-hydantoinase negative.

Temperature and pH Effects on D-HPG Production. To express both D-hydantoinase and carbamolyase in *E. coli*, plasmid pAH71 was co-transformed with either pSHDT4 or pKT-HDT4 into strain DH5α to yield DH5α/pAH71/pSHDT4 and DH5α/pAH71/pKT-HDT4, respectively. Plasmid pAH71 carrying the carbamoylase gene and *parB* (see Materials and Methods) remained stable up to 100 generations in DH5α. As in the same strain background, D-hydantoinase-harboring plasmids pSHDT4 and pKT-HDT4 stayed stable up to 70 generations. Supplemented with carbon sources in LB medium, recombinant strain DH5α/pAH71/pSHDT4 exhibited a higher activity in the presence of glycerol (Table 2). In contrast, glucose showed a severely negative effect on the enzyme activity, and the reduction level was more than 4 times as compared to that with glycerol solely.

It is very common to initially determine the optimal conditions for biotransformation reactions. As shown in Figure 2a, the enzyme activity positively correlated with temperature for both free and immobilized cells. However, it was surprising to see that cells immobilized by κ-carrageenan tended to be thermally sensitive. In contrast, the immobilized method rendered recombinant cells more resistant to pH variations (Figure 2b). The pH optimum was 7.5 for free cells and ranged from 7.5 to 10 for immobilized cells. To maintain the stability of enzymes for a long period of reaction (Ikenaka et al. 1998c), a reaction at 40 °C and pH 7.5 was then chosen for D-HPG production.

The reaction appears to be a heterogeneous system because the reaction substrate, DL-HPH, has a nature of low solubility in water. Accordingly, agitation efficiency should be carefully examined. As expected, in the case of a reaction system containing immobilized cells, the reaction rate in terms of enzyme activity strongly depended on the rotational speed (Figure 3). It resulted in a more than 2-fold increase in activity when the rotational speed was doubled. The reaction rate peaked at 200 rpm and decreased at speeds exceeding 200 rpm. It is known that high shear stress resulting from high rotational speed tends to cause physical damage to the entrapment gel.

D-HPG Production Using Recombinant *E. coli* Cells. Our interest resides in the one-step production of D-HPG by use of recombinant *E. coli* cells overexpressing both D-hydantoinase and carbamoylase. As depicted in Figure 4a, recombinant strain DH5α/pAH71/pSHDT4 reached a conversion yield of 97% within 8 h, whereas *A. radiobacter* NRRL B11291 attained only 20% after 10 h. Furthermore, the use of DH5α/pAH71/pKT-HDT4 in the reaction showed a faster reaction rate. Within 5 h,

Table 2. Dependence of Enzyme Activity in DH5α/pAH71/pSHDT4 on Carbon Sources[a]

carbon source	specific enzyme activity (μmol/min/mg DCW)
glycerol	0.035
glycerol + glucose	0.008
glucose	0.004

[a] Enzyme activities were measured as described in Materials and Methods. The concentrations of carbon sources supplemented in LB medium was 0.02 M; when glycerol and glucose were used simultaneously the concentration was 0.01 M for each.
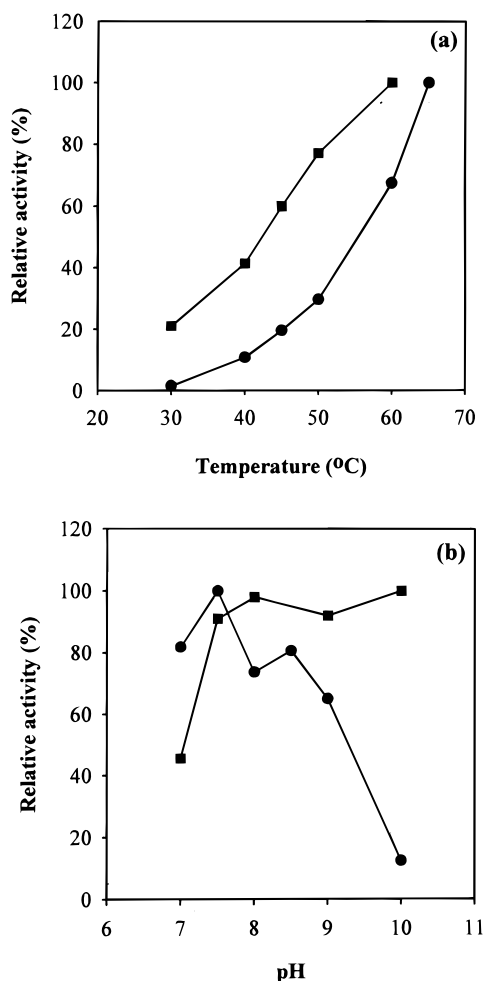

Figure 3. Effect of rotational speed on the activity of DH5α/pAH71/pSHDT4 immbolized with κ-carageenan. The reaction conditions were as described in Materials and Methods but at varied rotational speed.

Figure 2. Effect of temperature (a) and pH (b) on the enzyme activity of DH5α/pAH71/pSHDT4. The methods for cell cultivation and immobilization and reaction conditions were as described in Materials and Methods. For the assessment of temperature effect, the condition of pH 7.0 is used. When the pH effect is examined, the reaction temperature is kept at 40 °C. The buffer used was 0.1 M sodium phosphate (pH 7−9) or 0.1 M boric acid (pH 9−10). Symbols: immobilized cells (solid squares); free cells (solid circles).

Figure 4. Reaction profile of D-HPG production using free or immobilized cells. The reaction conditions were as described in Materials and Methods. (a) A 192 mg DCW was prepared from strains DH5α/pAH71/pSHDT4 (circles), DH5α/pAH71/pKT-HDT4 (triangles), or *A. radiobacter* NRRL B11291 (squares). (b) Immoblized cells were prepared from strain DH5α/pAH71/pKT-HDT4. The concentration of CpHPG and D-HPG is represented by solid and open symbols, respectively.

the strain obtained a conversion yield of 97%. It is worth noting that the reaction intermediate CpHPG accumulates during the first 2-h reaction for recombinant cells. The result may indicate a limiting activity of carbamoylase in the cells. To be more conclusive, both enzyme activities in DH5α/pAH71/pSHDT4 and DH5α/pAH71/pKT-HDT4 were further measured. The resulting activity for carbamoylase was roughly similar for both strains, while DH5α/pAH71/pKT-HDT4 displayed more than twice the D-hydantoinase activity (Table 3). Therefore, the increase in reaction rate by DH5α/pAH71/pKT-HDT4 can be attributed to the elevated level of D-hydantoinase
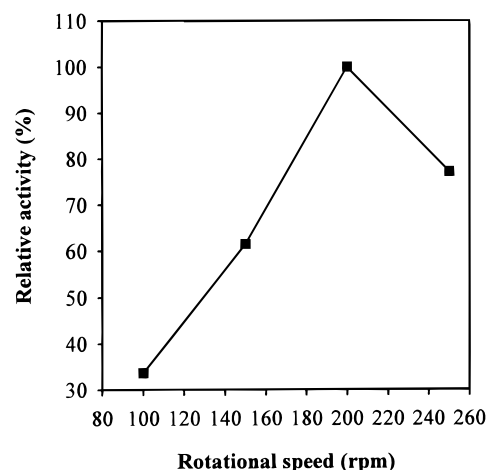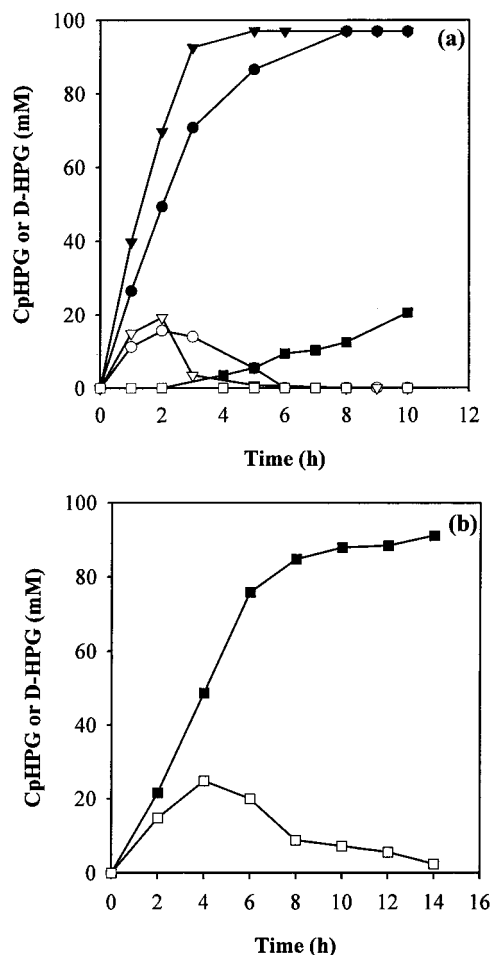
activity in the strain. Overall, the results shown above demonstrate the successful approach for D-HPG production using recombinant *E. coli* strains. The resulting question that thus emerges is whether immobilized cells may serve the same purpose. Figure 4b is a typical profile

Table 3. Specific Enzyme Activity of D-Hydantoinase and Carbamoylase Expressed in DH5α/pAH71/pSHDT4 (A) and DH5α/pAH71/pKT-HDT4 (B)[a]

strain	D-hydantoinase (μmol/min/mg DCW)	carbamoylase (μmol/min/mg DCW)
A	0.035	0.062
B	0.09	0.07

[a] The measurement of enzyme activity was as described in Materials and Methods.

of D-HPG production for the cells immobilized by κ-carrageenan. The reaction curve somehow exhibited a more sluggish trend, and a conversion higher than 90% was obtained after 10 h. This outcome indicates a transport barrier established by immobilized materials.

The biocatalyst load (mg DCW per unit volume) in the reaction system deserves more consideration. To examine this effect, reactions were carried out with varied amounts of DCW prepared from DH5α/pAH71/pKT-HDT4. Under the same reaction condition, the reaction rate, taken from the first 2-h reaction, showed a positive relationship with the cell density (Figure 5). The reaction reached a saturated level at a load of 19.2 mg DCW/L and decreased sharply at a load of 25.6 mg DCW/L. As mentioned earlier, a heterogeneous system presents a case with a mixing problem. It seems possible to improve the reaction efficiency by increasing the agitation rate when dealing with a higher biocatalyst load. By doing so, the reaction rate doubled when the rotational speed was raised from 200 to 400 rpm (Figure 6).

Repeated Use of Free or Immobilized Cells. In the development of a production process, the reusability of cells offers another important operational parameter for reference. To determine the reusability, the cells were allowed to react for 12 h, followed by centrifugation to collect the cells. The cells were then washed with cold saline water (or KCl solution for immobilized cells), and the reaction volume was adjusted to maintain a constant amount of cells used for the rest of reaction run. The procedure was then repeated as needed. Figure 7a shows that the conversion yield of 97% can be obtained for at least five cycles with the free cells. The conversion yield dropped to 70% at the seventh run. However, the reusability of immobilized cells was even worse. The conversion of 93% can only maintained at the first two runs. An abrupt decrease in conversion (55%) occurred at the fourth cycle (Figure 7b).

Discussion

We have successfully cloned the gene encoding D-hydantionase from *A. radiobacter* NRRL B11291 into *E. coli*. The expression level of D-hydantoinase (0.09 μmol/min/mg DCW) in *E. coli* was amplified by 11 times as compared to that in *A. radiobacter* (0.008 μmol/min/mg DCW). Further analyses of the ORF unveiled the putative regions for promoter and RBS site as indicated in Figure 1a. It is intriguing to see that the -35 and -10 region of the ORF are highly homologous to the consensus sequences in *E. coli*, with only a slight difference in the -10 region. However, the space between the -35 and -10 hexanucleotide longer than 17 bases seems very uncommon (Makrides, 1996). An optimal spacer of 8 bases spanning the RBS and start codon was also found in the ORF, but the RBS has a comparatively low similarity to the consensus Shine−Dalgarno site.

The dependence of enzyme activities on carbon sources was not expected, and glucose gave a negative effect (Table 2). Since both the D-hydantoinase and carbam-
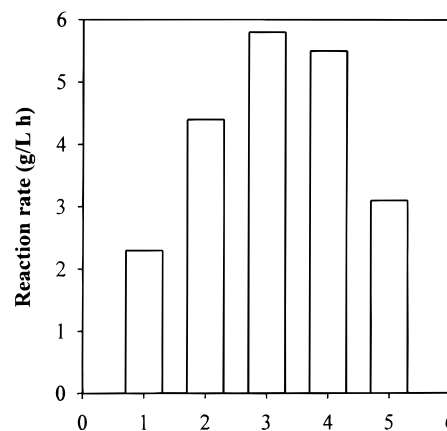

Figure 5. Effect of biocatalyst load on the reaction rate. The cells were prepared from strain DH5α/pAH71/pKT-HDT4. According to the method described in Materials and Methods, a 10-mL reaction volume was used with varied amounts of cell loading. The amounts of cells used in the reaction were as follows: 1, 128 mg DCW; 2, 160 mg DCW; 3, 192 mg DCW; 4, 224 mg DCW; 5, 256 mg DCW.
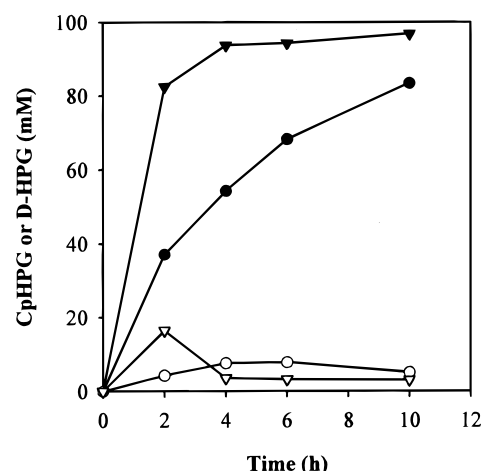

Figure 6. Reaction profile of D-HPG production using free cells at varied rotational speed. A 256 mg DCW of DH5α/pAH71/pKT-HDT4 was used, and the reaction was operated at 400 rpm (triangles) or 200 rpm (circles). The concentration of CpHPG and D-HPG is represented by solid and open symbols, respectively

oylase genes cloned carry their own natural promoter, it is possible that the observed effect may be due to catabolite repression, a well-known phenomena exerted by glucose (Botsford and Harman, 1992). However, searching D-hydantoinase and carbamoylase genes found no sequence homogeneous to the known cAMP receptor protein (CRP)-binding site. Nevertheless, another hint comes from the fact that the accumulation of CpHPG remains roughly comparable for each enzyme assay described in Table 2. In other words, the difference in enzyme activities shown in Table 2 is mainly contributed by the various levels of D-HPG produced, thereby suggesting that carbamoylase is subject to the observed glucose effect. Plasmid pAH71 carrying the carbamoylase gene was constructed by placing the gene right in the transcriptional orientation of the *lac* promoter in pUC118. A fact tells that the carbamoylase activity is largely determined by the *lac* promoter rather than by its own promoter. Accordingly, the *lac* promoter is subject to catabolite repression, and the diminished level of carbamoylase in the presence of glucose is the consequence.

Coupling the reactions of D-hydantoinase and carbamoylase in *E. coli* has been proven an efficient approach
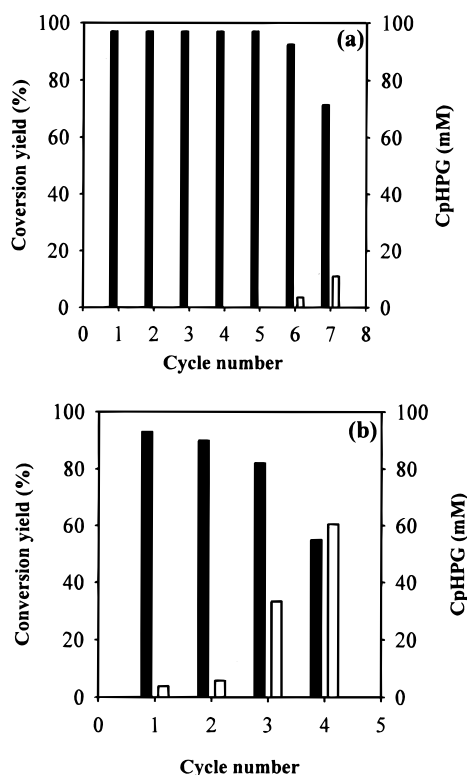
Figure 7. Repeated use of cells for D-HPG production. The cells were prepared from DH5α/pAH71/pKT-HDT4. As described in the text, the reaction was carried out using either free (a) or immobilized cells (b). Symbols: conversion yield (solid bars); CpHPG concentration (open bars).

to D-HPG production in this study. Our results show that recombinant *E. coli* strains display at least 6 times as much productivity (Figure 4a). Furthermore, a conversion yield of more than 90% can be obtained either with free or immobilized cells (Figures 4a and 4b). From the reaction profiles, it could be seen that the reaction intermediate gradually built up during the course. There is evidence that carbamoylase is the step setting the pace of the reaction. Strain DH5α/pAH71/pKT-HDT4 exhibited a 2.57-fold increase in D-hydantoinase activity (E) over strain DH5α/pAH71/pSHDT4, while their carbamoylase levels were roughly at par (Table 3). However, the "reaction flux (F)", defined as micromole of product produced per unit time per unit mass, increased only by 30% by taking data from the first 3-h reaction (Figure 4a). As defined by Kacser and Burns (1973), the so-called flux control coefficient $[(dF/F)/(dE/E)]$ can therefore be calculated as 0.2 for D-hydantoinase, suggesting that the major contribution to the overall reaction is carbamoylase (accounting for 80%). The conclusion of carbamoylase as the rate-determining step is in an agreement with the recent report (Grifantini et al., 1998). They further showed that carbamoylase behaved like a buffering agent in the reaction system. However, in their experimental condition a conversion of 61% was obtained by recombinant *E. coli* strains expressing both D-hydantoinase and carbamoylase.

By fixing the reaction time, the free cells can be recycled six times to obtain a conversion yield of more than 90% (Figure 7a). The reaction intermediate, CpHPG, starts to build up at the sixth cycle. In contrast, the reusability of immobilized cells is less impressive. A conversion higher than 90% can be obtained only for the first two cycles. However, from the start of the run CpHPG begins to accumulate (Figure 7b). Regardless of

free or immobilized cells, the buildup of CpHPG during the process of recycling suggests a more severe loss in carbamoylase activity. Indeed, carbamoylase has been shown to be highly sensitive to oxidizing environments (Grifantini et al., 1996). A recent study on improving the thermostability of carbamoylase by mutagenesis (Ikenaka et al., 1998a) further points out the thermal instability of this protein. It is very common to utilize hardening agents such as glutaraldehyde and hexamethylenediamine to enhance the strength of κ-carrageenan-treated gel (Tosa et al., 1979). We found that treating the gel with glutaraldehyde and hexamethylenediamine for more than 30 min would cause diminishment in enzyme activity. However, the hardening treatment did not improve the reusability of immobilized cells (data not shown). In fact, we also checked the cell leakage by assaying enzyme activity from the supernatant of reaction solution post the end of each run. The results showed no signs of enzyme activity, and therefore, no cells leaked out. Furthermore, we observed that the gel became whiter, and the concentration of CpHPG plus D-HPG exceeded the total amount of substrate used at the third and fourth runs (Figure 7b). The results strongly suggest that the adsorption of DL-HPH on the surface of immobilized cells results in the carryover of the substrate to the next reaction run and the gel turns pale as a result. In conclusion, the poor reusability of immobilized cells is attributed to the fouling on the gel surface. Similarly, past studies also found the low solubility of DL-HPH making the use of immobilized D-hydantoinase very impractical (Lee and Lin, 1996; Lee and Kim, 1998). In this study, we present data showing a close interaction of biocatalyst load and agitation rate (Figures 5 and 6). Apparently, surveying an optimal condition for these two operational parameters appears to be a very practical issue when dealing with this heterogeneous system.

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