Optimization of a Heterogeneous Reaction System for the Production of Optically Active D-Amino Acids Using Thermostable D-Hydantoinase

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Received 28 November 1997; accepted 8 June 1998

Abstract: A thermostable D-hydantoinase from *Bacillus stearothermophilus* SD-1 was previously mass-produced by batch cultivation of the recombinant *E. coli* harboring the gene encoding the enzyme (Lee et al., 1997). In this work, we attempted to optimize the process for the production of *N*-carbamoyl-D-*p*-hydroxyphenylglycine, which is readily hydrolyzed to D-*p*-hydroxyphenylglycine under acidic conditions, from 5-(4-hydroxyphenyl)hydantoin using the mass-produced D-hydantoinase. In an effort to overcome the low solubility of the substrate, enzyme reaction was carried out in a heterogeneous system consisting of a high substrate concentration up to 300 g/L. In this reaction system, most of substrate is present in suspended particles. Optimal temperature and pH were determined to be 45°C and 8.5, respectively, by taking into account the reaction rate and conversion yield. When the free enzyme was employed as a biocatalyst, enzyme loading higher than 300 unit/g-substrate was required to achieve maximum conversion. Use of whole cell enzyme resulted in maximum conversion even at lower enzyme loadings than the free enzyme, showing 96% conversion yield at 300 g/L substrate. The heterogeneous reaction system used in this work might be applied to the enzymatic production of other valuable compounds from a rarely water-soluble substrate. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **60**: 729–738, 1998.
Keywords: D-hydantoinase; D-*p*-hydroxyphenylglycine; heterogeneous system; D-amino acid

INTRODUCTION

D-Hydantoinase (dihydropyrimidinase, E.C. 3.5.2.2.) catalyzes a stereospecific hydrolysis of dihydropyrimidine and structurally related 5-monosubstituted hydantoins and currently is used as an industrial biocatalyst for the synthesis of D-amino acids. Optically active D-amino acids are widely used in the pharmaceutical field as intermediates for the synthesis of semisynthetic antibiotics, peptide hormones, pyrethroids, and pesticides (Syldatk et al., 1990). Yamada et al. (1978) developed a process in which a racemic mixture of chemically synthesized D- and L-5-monosubstituted hydantoin is used as a starting substrate. Of the two isomers, the D-form substrate is hydrolyzed to the corresponding *N*-carbamoyl-D-amino acid by D-hydantoinase. The resulting *N*-carbamoyl-D-amino acid is chemically converted to the D-amino acid under acidic conditions. The unreacted L-form substrate is spontaneously racemized to the D-form under the reaction condition, and theoretically, a 100% conversion yield is obtained. Recently, much effort has been made to replace the chemical conversion of *N*-carbamoyl-D-amino acid to D-amino acid with an enzymatic one using D-carbamoylase to reduce waste disposal (Ogawa et al., 1994; Runser et al., 1990).

Enzyme-catalyzed processes offer several advantages over the chemical counterparts, but low stability of the enzyme under extreme conditions such as high temperatures, often becomes a barrier to development of enzymatic processes. The use of thermostable enzymes has been regarded as a solution to this problem, and isolation of the enzymes from thermophiles has recently become one of major interest. In this context, we have focused on the screening of thermostable D-hydantoinase-producing microorganisms, and isolated and characterized the enzyme from *Bacillus stearothermophilus* SD-1 (Lee, S. G., et al., 1994, 1995). The gene encoding the enzyme has also been cloned and constitutively overexpressed in *Escherichia coli* XL1-Blue by its native promoter in a soluble form (Lee, D. C., et al., 1996). In order for an industrial enzyme to be practically applicable, a cost-effective production of the enzyme is a prerequisite. Recently, we have successfully mass-produced the thermostable D-hydantoinase by batch culture of the recombinant *E. coli* with a constitutive expression system (Lee et al., 1997).

We have attempted to develop a process for the production of *N*-carbamoyl-D-*p*-hydroxyphenylglycine (NCHPG), which is easily hydrolyzed to D-*p*-hydroxyphenylglycine (HPG) under acidic conditions, from 5-(4-hydroxyphenyl)hydantoin (HPH) at a high substrate concentration by using the mass-produced D-hydantoinase. Among various D-amino acids, D-*p*-hydroxyphenylglycine has high commercial de-

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 CCC 0006-3592/98/060729-10

mand. In the development of the enzymatic process, low solubility of the substrate HPH has been one of the serious problems. The solubility of HPH in water is reported to be about 60–70 m*M* (Takahashi, 1986). To overcome the solubility problem, we tried using various organic solvents, but no positive effect was observed in terms of the reaction rate and conversion yield in the D-hydantoinase-catalyzed reaction. As an alternative approach, we have carried out the enzyme reaction in a heterogeneous reaction system in which most of the substrate is present in suspended particles when the substrate concentration is much higher than its solubility. In this case, the solid substrate dissolves into the reaction mixture, and the solubilized substrate is converted to a highly soluble product, NCHPG, by the enzyme.

Heterogeneous reaction systems containing solid substrate have been widely investigated in chemical reactions (Levenspiel, 1972), and a few examples have been reported in enzymatic reactions (Bailey and Ollis, 1986), including steroid transformation (Constantinides, 1980; Maxon et al., 1966) and hydrolysis of cellulosic materials (Ryu et al., 1982). In a D-hydantoinase-catalyzed reaction, a relatively moderate concentration (<100 g/L) of HPH has been used so far (Deepa et al., 1993; Lee and Lin, 1996; Olivieri et al., 1981), but few studies on the factors affecting the kinetics of the reaction system have been carried out.

In this paper, we reported the optimization of the heterogeneous reaction system for production of NCHPG from HPH at high concentration up to 300 g/L by using free or whole cell D-hydantoinases. Factors affecting the kinetics of the heterogeneous reaction system were also investigated in detail.

MATERIALS AND METHODS

Chemicals

Hydantoin, dithiothreitol, phenylmethanesulphonyl fluoride, and *p*-(dimethylamino)benzaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). 5-(4-Hydroxyphenyl)hydantoin was synthesized through the condensation reaction of phenol, glyoxylate, and urea by the method of Ohashi et al. (1981). All other reagents used were of analytical grade.

Preparation of Free and Whole Cell D-Hydantoinase

Thermostable D-hydantoinase from *B. stearothermophilus* SD-1 (Lee, S. G., et al., 1995) was mass-produced in a 5-L fermentor (Korea Fermentor Co. Ltd.) by batch cultivation of recombinant *E. coli* XL1-Blue harboring the gene encoding the enzyme as described earlier (Lee, D. C., et al., 1997).

For use of whole cell D-hydantoinase, the culture medium was centrifuged at 8000*g* after cultivation. The separated cells were resuspended in an appropriate volume of distilled water containing 1 m*M* $MnCl_2$ and directly used as an enzyme source. When free D-hydantoinase was employed, the separated cells were resuspended in distilled water containing 1 m*M* $MnCl_2$, 1 m*M* dithiothreitol, and 1 m*M* phenylmethanesulphonyl fluoride, and the optical density of the cell suspension was adjusted to about 100 at 600 nm. Cells were disrupted using a homogenizer (Nanojet Model Expo, Nanojet Engineering GmbH, Germany) by passing them twice through the nozzle at the pressure drop of 800 bar. After $MnSO_4$ was added at 20 m*M* to the prepared cell extract, heat treatment at 60°C was carried out for 1–2 h with mild shaking. The pH of the solution was adjusted to 8.5 with 5 *N* NaOH solution, and settled at 4°C over 24 h. The resulting aggregates were discarded by centrifugation at 8000*g*, and the supernatant was used as a free enzyme. The total activity recovery of free enzyme was about 85% on the basis of the enzyme activity of fully permeabilized cells (Lee, D. C., et al., 1997).

Assay of D-Hydantoinase

The activity of whole cell D-hydantoinase was assayed as described in our previous work (Lee, D. C., et al., 1997). The activity of whole cell D-hydantoinase during the reaction in a stirred tank reactor was determined as follows. At intervals, 1 mL of the reaction mixture was withdrawn and allowed to settle for 1 min to separate the substrate particles, and 800 μL of the supernatant was centrifuged at 8000*g*. The precipitated whole cells were resuspended in 800 μL Tris-HCl buffer (pH 8.0) containing 1 m*M* $MnCl_2$, and the activity of the whole cell enzyme was determined with or without treatment for permeabilization of cell membrane as described elsewhere (Lee, D. C., et al., 1997). Leakage of free D-hydantoinase from cells was quantified by measuring the activity of D-hydantoinase in the supernatant.

As for the assay of free enzyme, 50 μL of enzyme solution was added to 0.75 mL of 1% hydantoin solution in 0.1 *M* Tris-HCl (pH 8.0). The reaction mixture was incubated at 55°C with mild shaking for 30 min, and the reaction was stopped by adding 0.25 mL of 10% *p*-(dimethylamino)benzaldehyde in 6 *N* HCl. Protein aggregates were removed by centrifugation, and absorbance of the supernatant was measured at 440 nm to determine the amount of *N*-carbamoylglycine produced. One unit of D-hydantoinase activity is defined as the amount of enzyme required to produce 1 μmol of *N*-carbamoylglycine per min at 55°C.

Enzymatic Reaction in a Bench-Top Scale Reactor

The enzyme reaction was carried out in a 2 L-scale stirred-tank type reactor equipped with a propeller-type impeller. The initial volume of the reaction mixture was 1 L, and distilled water was used as the reaction medium. The concentration of 5-(4-hydroxyphenyl)hydantoin, a starting substrate, ranged from 50 to 300 g/L, and a predetermined volume of enzyme solution or cell suspension was added to the reaction mixture as an enzyme source. One millimolar

$MnCl_2$ was also added for supplementing metal ions to the D-hydantoinase. The temperature and pH were controlled at predetermined values, and nitrogen gas was flushed to avoid oxidation of the phenol ring of HPH during the reaction. For pH control, Ross SURE-FLOW combination electrode (Model 8175, ATI Orion Inc., Boston, MA) designed for use in viscous or colloidal solution was used, and 5.0 *N* NaOH solution was added to control the pH of the reaction mixture by using an autotitrator (Radiometer Copenhagen, France).

During the enzyme reaction, the amount of *N*-carbamoyl-D-*p*-hydroxyphenylglycine produced, a monoprotic acid, is equivalent to that of NaOH added for titration. Therefore, the production of NCHPG was estimated by measuring the amount of NaOH added according to the principles of pH-stat methodology (Brocklehurst, 1992). At intervals, the reaction mixture was withdrawn from the reactor, and the concentration of product was analyzed by using high performance liquid chromatography (Shimadzu, Japan) as reported in our previous paper (Lee, S. G., et al., 1995) to confirm the concentration of NCHPG. The amount of NCHPG analyzed by HPLC was found to correlate well with that calculated from the amount of NaOH added. Changes in the volume of reaction mixture due to addition of NaOH solution were considered in the analysis of the reaction mixture.

Analyses

Invertase activity was assayed with 0.15 *M* sucrose solution in 0.025 *M* sodium acetate buffer (pH 5.0) at 30°C (Mansfeld et al., 1992). The amount of glucose formed in the course of the reaction was determined using the dinitrosalicylic acid method (Bailey, 1988). The protein concentration in solution was determined according to Bradford's method (1976) using bovine serum albumin as a standard. The concentrations of 5-(4-hydroxyphenyl)hydantoin, *N*-carbamoyl-D-*p*-hydroxyphenylglycine, and D-*p*-hydroxyphenylglycine in solution were determined by using HPLC (Shimadzu, Japan) as described in our previous paper (Lee, D. C., et al., 1995).

Infrared (IR) spectroscopy was performed in a BOMEM Michelson MB 102-C15 FT-IR spectrometer (Quebec, Canada) with the samples prepared in solid state. Nuclear magnetic resonance (NMR) spectra was obtained with a Bruker FT 500 MHz NMR spectrometer (Model AMX500, Karlsruhe, Germany) using deuterated dimethylsulfoxide (DMSO) as a solvent. The molecular mass of the modified substrate was determined by matrix-assisted laser desorption ionization mass spectrometry (Kratos Kompact MALDI II, Manchester, UK).

RESULTS

Medium for Enzyme Reaction

Enzyme reactions are generally carried out in a buffer solution to maintain the optimal conditions for catalysis. The substrate concentration used in this work reached up to 300 g/L, and in this case, a base titrant had to be added to control the pH of reaction medium because the amount of NCHPG produced is beyond the capacity of a buffer even though a buffer is used. In this context, we tested the possibility of using distilled water as a reaction medium. The production of NCHPG was traced in both various buffers and distilled water, and the enzymatic reaction in distilled water also gave almost the same profile as in buffer solution (data not shown). In addition, no fluctuation in the pH of the reaction mixture was observed despite the titration with 5 *N* NaOH solution. High ionic strength (0.2 *M* NaCl) revealed a negligible effect on the reaction rate. In this work, all the enzyme reactions were carried out in distilled water.

Effect of Temperature and pH on the Enzyme Reaction

In the D-hydantoinase-catalyzed reaction, operation at high temperature improves productivity, mainly due to the enhanced solubility of hydantoin derivatives. An increase in the racemization rate of L-hydantoin derivative to the D-form is also expected at high temperatures. From the preliminary experiments in test tubes, the maximum reaction rate of the D-hydantoinase was observed at around pH 8.5. Thus we first investigated the effect of temperature on the production of NCHPG at pH 8.5 by using both free and whole cell D-hydantoinases. As shown in Table I, the reaction rate significantly increased with the increasing reaction temperature for both free and whole cell enzymes. However, conversion yield gradually reduced as the reaction temperature increased, and a serious decrease in the conversion was observed at 55°C, and this was found to be due to modification of the substrate. Details on the modification of the substrate will be mentioned later. Use of whole cell enzyme resulted in a slightly higher yield compared with that of free enzyme at a given temperature. In the production process of NCHPG, the price of the substrate (HPH) is cost-limiting, and a conversion yield as high as possible is advantageous. The reaction rate at temperatures below 40°C

Table I. Effect of the pH and temperature on the enzymatic production of *N*-carbamoyl-D-*p*-hydroxyphenylglycine from 5-(4-hydroxyphenyl) hydantoin.[a]

Biocatalyst form	Temperature (°C)	Reaction time (h)	Conversion yield (%)
	35	23	93
Free enzyme	40	9	90
(15,000 unit/L)	45	6	86
	55	2.5	72
Whole cell enzyme	35	30	93
	40	9	91
(17,000 unit/L)	45	5	89
	55	2.5	82

[a]The concentration of substrate was 50 g/L. Detailed experimental conditions are described in Materials and Methods.

was too low, which limits the practical application. From the above results, either 40 or 45°C might be considered as an optimal temperature for the production of NCHPG. When the substrate concentration was higher than 200 g/L, the reaction at 40°C resulted in a long reaction time to reach maximum conversion. Thus, the optimal reaction temperature was determined to be 45°C in this work.

The effect of pH on the production of NCHPG was investigated at 45°C by using free and whole cell enzymes (Fig. 1). In the case of free enzyme reaction, initial reaction rates were almost the same except for that at pH 8.0, and final conversion yields were slightly different (Fig. 1A). At pH 8.8, the conversion yield lowered, and the reaction rate significantly slowed down at pH 8.0. The reduction in the solubility of substrate and the racemization rate of the L-form substrate to the D-form at a lower pH was thought to contribute to the result. When the whole cell enzyme was used as a biocatalyst, the reaction progressed slowly at the beginning in contrast to that by the free enzyme, and the reaction time to reach a maximum conversion was also prolonged regardless of the pH (Fig. 1B). The reaction rate was highest at pH 8.5, and the conversion yield reached about 90% in 7 h. In the case of the whole cell enzyme, the cell membrane might be a transport barrier to the substrate and product, which appears to lead to a slow reaction rate at the beginning of the reaction.

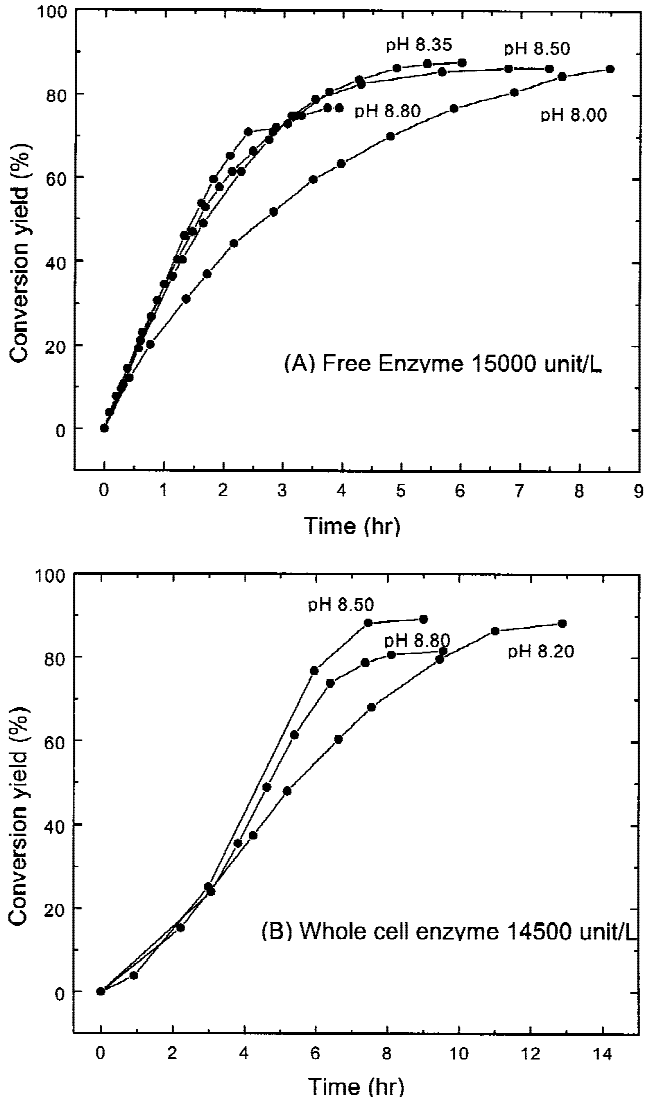

Figure 1. Effect of the pH on the production of *N*-carbamoyl-D-*p*-hydroxyphenylglycine using free (A) and whole cell enzymes (B). The reaction temperature was 45°C.

In order to examine the effect of pH on the reaction at a higher temperature, production of NCHPG was also monitored at 55°C for different pHs. As expected, the conversion yields were below 80%, regardless of the pH for both free and whole cell enzyme reactions (data not shown). In this work, the pH of the reaction mixture was controlled at 8.5 by addition of 5 *N* NaOH solution.

Modification of the Substrate by NaOH Added for Titration

As previously observed, the conversion yield was significantly lowered when the enzyme reactions were carried out at 55°C. Since the enzyme reaction nearly stopped after the conversion reached about 70%, fresh enzyme solution was added to the reaction mixture, but no further reaction proceeded, which led us to speculate that no substrate is available for the enzyme reaction. The reaction mixture was filtered through a 0.2 μm membrane, and the filtrate was analyzed using HPLC. As a result, less than 3.5% of initially added substrate was detected in the reaction mixture. In addition, white colloidal particles were isolated from the cake on the filter membrane, and this compound revealed different physico-chemical properties from the native substrate. The isolated compound was hardly soluble in methanol, meanwhile the authentic substrate is highly soluble, and the retention time in HPLC analysis was longer than that of the authentic substrate. This implies that the substrate might be modified during the enzyme reaction by the NaOH solution added for titration because the NaOH is a sole chemical reagent which causes the modification. To confirm the modification of the substrate, we dropped NCHPG to 10 g/L HPH solution and controlled the pH at 8.5 by adding 5 *N* NaOH solution. As a result, the formation of the white colloidal byproduct was also observed, and the loss of HPH in the solution was quantified by HPLC as a function of time. The amount of the modified substrate was found to increase significantly at 55°C (data not shown). Modification of NCHPG by NaOH was observed to be negligible in the HPLC analysis. The modified substrate obtained by the above procedure was found to be almost identical with that formed during the enzyme reaction based on HPLC, NMR, and IR analyses. Mass spectrometric analysis revealed that the molecular mass of the modified substrate was much higher than that of the authentic substrate, but the precise structure of the modified substrate could not be determined. Modification of the substrate was more significant at higher pHs.

It was interesting to determine the maximum conversion

yield in the D-hydantoinase-catalyzed reaction. As described earlier, the theoretical yield is thought to be 100% because the L-form substrate is racemized to the D-form. To exclude the modification of the substrate by NaOH added for titration, we carried out the enzyme reaction at a low substrate concentration (5 g/L) in a buffer solution without addition of NaOH using two kinds of D-hydantoinase from different sources. As shown in Table II, the maximum conversion yield was about 96% for both enzymes. The pH drop at the end of reaction was about 0.4.

Stability of the Enzyme

The stability of the enzyme under reaction conditions was investigated in the absence of substrate in the reactor with agitation. The free enzyme was fairly stable, showing the deactivation constant of 7.6×10^{-6} s^{-1} (Fig. 2A). In the case of the whole cell enzyme, its activity increased with incubation time, probably due to the permeabilization of the cell membrane, and reached a maximum after 15 h (Fig. 2B). Thus, the stability of the whole cell enzyme was determined by using permeabilized cells. Cells were permeabilized by treatment with 1% Triton X-100 for 1 h at 55°C as previously described (Lee, S. G., et al., 1997). As a result, the whole cell enzyme also exhibited good stability as free enzyme (Fig. 2B). Leakage of D-hydantoinase from cells was negligible, regardless of the storage period of harvested cells at 4°C over a period of 15 days.

Production of NCHPG Using Free D-Hydantoinase

Production of NCHPG from HPH was carried out using free D-hydantoinase at different substrate concentrations in a stirred tank reactor (Fig. 3). In the heterogeneous reaction system, the substrate concentration could be increased up to 400 g/L, but the reaction rate was significantly reduced at concentrations above 300 g/L, resulting in a conversion of less than 70%. The maximum substrate concentration used in this work was 300 g/L.

The reaction rate increased with increasing enzyme loading, and interestingly the conversion yield was enhanced at higher substrate concentrations. When the substrate concentration was 50 g/L, about 88% conversion was obtained (Fig. 3A). In contrast, when the substrate concentration was increased to 300 g/L, the conversion yield was 95%, approaching the maximum value observed in Table II (Fig. 3B).

When the loadings of free enzyme were below a certain level, the conversion yield did not approach the maximum value. For example, the conversion reached about 80% in 20 h when the enzyme loading was 70,000 unit/L for 300 g/L substrate, and no further increase in the conversion was observed even for a prolonged reaction time. The conversion increased only when fresh enzyme solution was added to the reaction mixture, which indicates that the ceasing of the enzyme reaction is due to complete deactivation of the enzyme. From this observation, it is suggested that there exists a certain enzyme loading per unit mass of substrate required to achieve a maximum conversion. The conversion yields obtained at various ratios of enzyme to substrate were plotted in Fig. 4. The conversion yield increased with in-

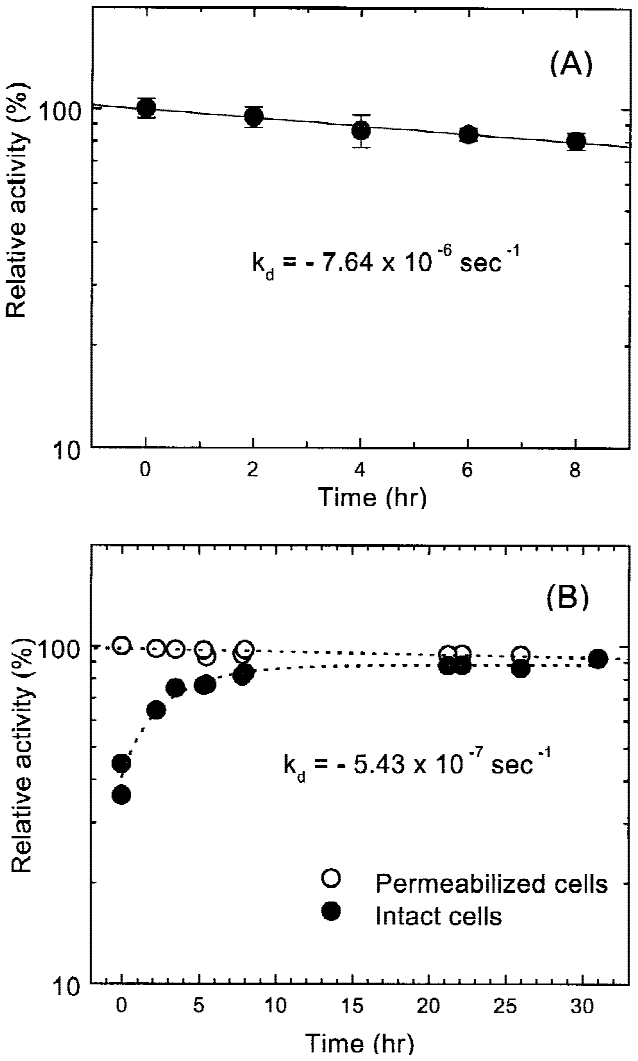

Figure 2. Stability of the enzyme in 0.025 *M* Tris-HCl buffer (pH 8.0) containing 1 m*M* Mn^{2+} at 45°C in the reactor with stirring. (A) Free enzyme; (B) whole cell enzyme.

Table II. Conversion yields obtained in 0.1 *M* diethanolamine (pH 8.70) without titration.[a]

Source of D-hydantoinase	Temperature (°C)	Conversion yield (%)
B. stearothermophilus SD-1	45	96.3 ± 0.5
	55	93.9 ± 0.9
B. thermocatenulatus GH-2[b]	45	96.1 ± 0.8
	55	94.1 ± 1.1

[a]Concentration of substrate was 5 g/L, and D-hydantoinase of *B. thermocatenulatus* GH-2 isolated in our laboratory was also used for comparison.

[b]D-Hydantoinase-producing strain isolated in our laboratory. Refer to Park et al. (1997).

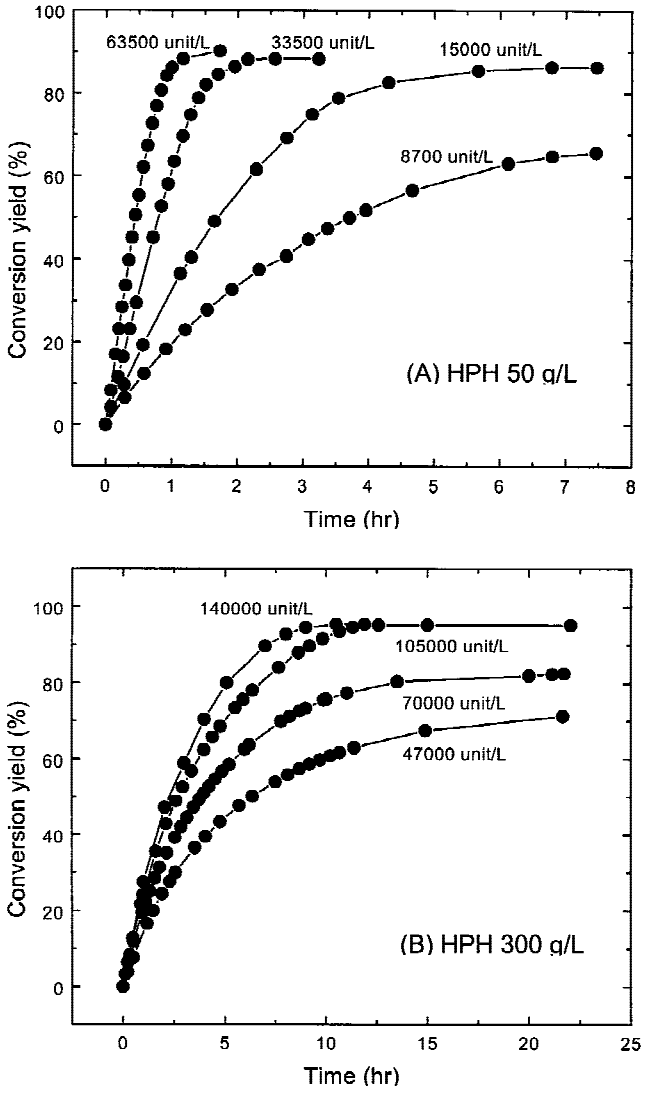

Figure 3. Production of *N*-carbamoyl-D-*p*-hydroxyphenylglycine at different loadings of free enzyme in the heterogeneous reaction system. The substrate concentrations were (A) 50 and (B) 300 g/L. Temperature and pH were 45°C and 8.5, respectively.

creasing enzyme loading, and maximum conversion was obtained at enzyme loadings higher than about 300 unit/g-substrate.

Stability of Free Enzyme in Heterogeneous Reaction System

A previous test on the stability of the enzyme revealed that the activity of free enzyme was maintained in the absence of the substrate particles (Fig. 2A). However, the results shown in Figs. 3 and 4 strongly suggest that the stability of free enzyme would be much different in a heterogeneous reaction system containing a high concentration of substrate particles. In other words, substrate particles might play a role in inactivation of the free enzyme during the reaction. It was reported that the surface tension at the interface causes inactivation of the enzyme (Deeble and Lee, 1985; Kim et al., 1982; Reese and Ryu, 1980). Thus, free D-hydantoinase might also be inactivated by the surface tension at the interface between the solid and aqueous phases in the heterogeneous reaction system. The effect of substrate particles on the D-hydantoinase was predicted by using bovine serum albumin (BSA) and invertase because it was difficult to determine the activity of free D-hydantoinase during the reaction. The solution of BSA (1 mg/mL) was incubated with different substrate concentrations under the same conditions as those for D-hydantoinase, and the concentration of soluble protein was assayed as a function of time. As shown in Fig. 5A, concentration of soluble BSA decreased severely with increasing HPH concentration, and protein aggregates were observed in the reaction mixture. The adsorption of BSA onto substrate particles was found to be negligible. It is likely that the presence of HPH particles caused denaturation of BSA, leading to aggregation of proteins. In the case of invertase, the activity also decreased considerably as the substrate concentration increased even though the incubation conditions were different from those of D-hydantoinase (Fig. 5B).

In order to get some insights into the stability of free D-hydantoinase in the heterogeneous reaction system, the activity of the enzyme during the reaction was calculated from the profiles of product formation. During the enzyme reaction in the heterogeneous system, it can be assumed that the concentration of dissolved substrate remains constant because the solid substrate successively dissolves into the aqueous phase as the enzymatic reaction proceeds. Thus, the reduction of the reaction rate in the early phase of the reaction is directly related with the decrease in the enzyme activity in the reaction mixture. The time course of the change in the enzyme activity at 300 g/L substrate is shown in logarithmic scale in Fig. 6. From this result, it was found that the enzyme lost its activity 10 times faster in the heterogeneous reaction system than in the absence of substrate

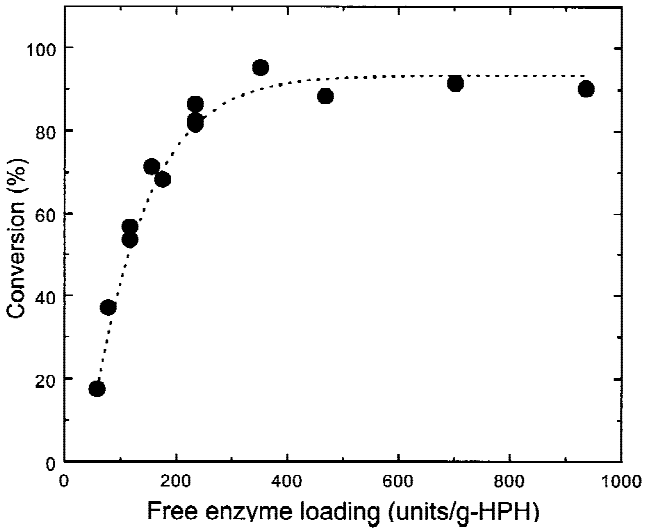

Figure 4. Effect of free enzyme loading per unit mass of substrate on the conversion yield.

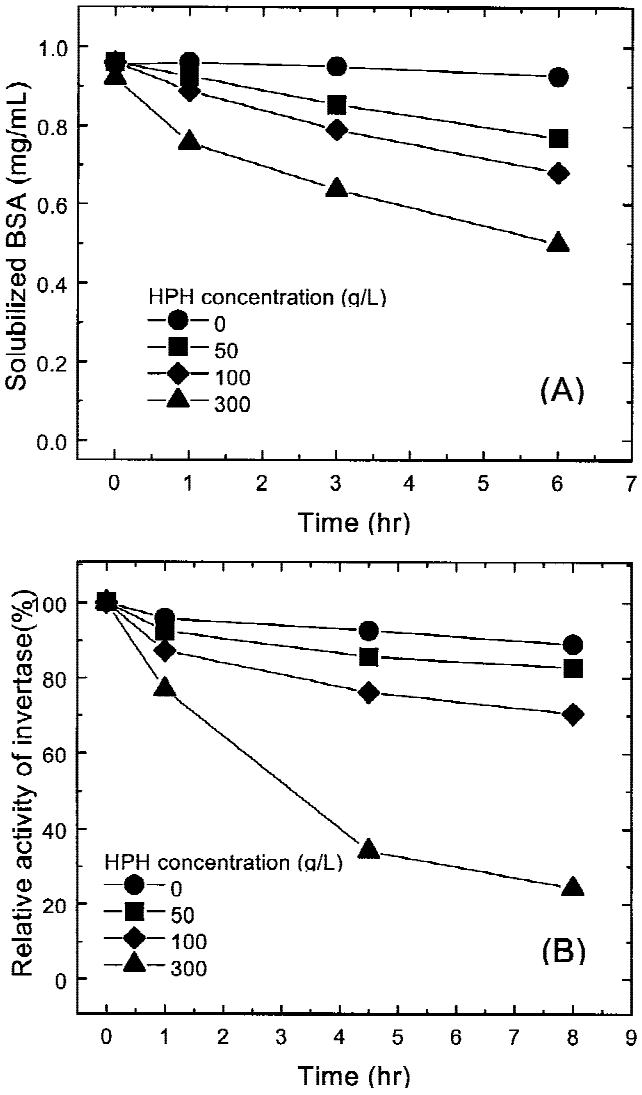

Figure 5. Effect of 5-(4-hydroxyphenyl)hydantoin concentration on (A) bovine serum albumin in 0.05 *M* Tris-HCl buffer (pH 8.5) at 45°C and (B) invertase in 0.025 *M* sodium acetate buffer (pH 5.0) at 30°C.

particles under the same conditions (Fig. 2). This observation also supports that substrate particles have a detrimental effect on the free D-hydantoinase as predicted in Fig. 5. Furthermore, NaOH added for titration also causes inactivation of the enzyme as mentioned later.

Production of NCHPG Using Whole Cell D-Hydantoinase

Production of NCHPG using whole cell D-hydantoinase was also carried out under the same conditions as those with free enzyme. In this case, harvested cells were directly used without any treatment. Figure 7 shows the production profiles at different loadings of whole cell enzyme when the substrate concentrations were 50 and 300 g/L. The reaction rate increased with increasing loading of whole cell enzyme, but the initial reaction rate was relatively low compared with that using free enzyme at a given enzyme loading. As previously described, the activity of the whole cell enzyme gradually increased with reaction time due to permeabilization of the cell membrane, and a sigmoid shape in the production profile was observed. The conversion yield was about 88% for 50 g/L substrate (Fig. 7A), and 96% conversion was obtained at 300 g/L (Fig. 7B).

The reaction time to reach the maximum conversion yield by using whole cell enzyme was comparable to that using free enzyme even though there is a transport barrier across the cell membrane, and this can be attributed to the permeabilization of the cell membrane during the reaction. In addition, the maximum conversion could be obtained even when the loadings of whole cell enzyme were much lower than those of free enzyme. The conversion yields obtained in the reactions were plotted as a function of enzyme to substrate ratio, and the maximum conversions were attained regardless of the loading of whole cell enzyme (data not shown), which is in contrast to the use of free enzyme.

Stability of Whole Cell Enzyme in Heterogeneous Reaction System

In order to investigate the stability of whole cell enzyme in the heterogeneous reaction system, the change in the activity of the whole cell enzyme during reaction was monitored after separating the cells from the reaction mixture as a function of time for both 50 and 300 g/L substrates. Figure 8A shows the change in the activities of permeabilized and intact cells at 50 g/L substrate. The activity of permeabilized cells was stable, retaining over 80% of initial activity for 10 h. When intact cells were tested, the activity increased with time due to the permeabilization of cell membrane, and full activity was obtained in 8 h. In other words, intact cells were fully permeabilized in 8 h. In the case of 300 g/L substrate, inactivation of the whole cell enzyme was more

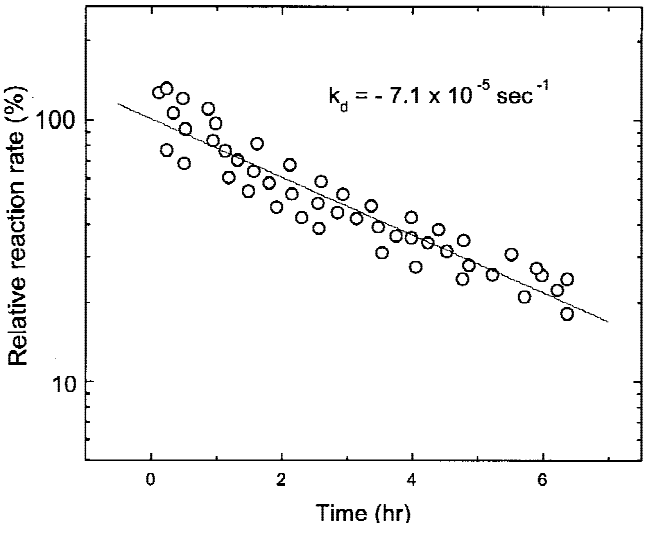

Figure 6. Estimated deactivation rate of free enzyme during the reaction. The activity of the enzyme was calculated from the profile of product formation shown in Fig. 3B.

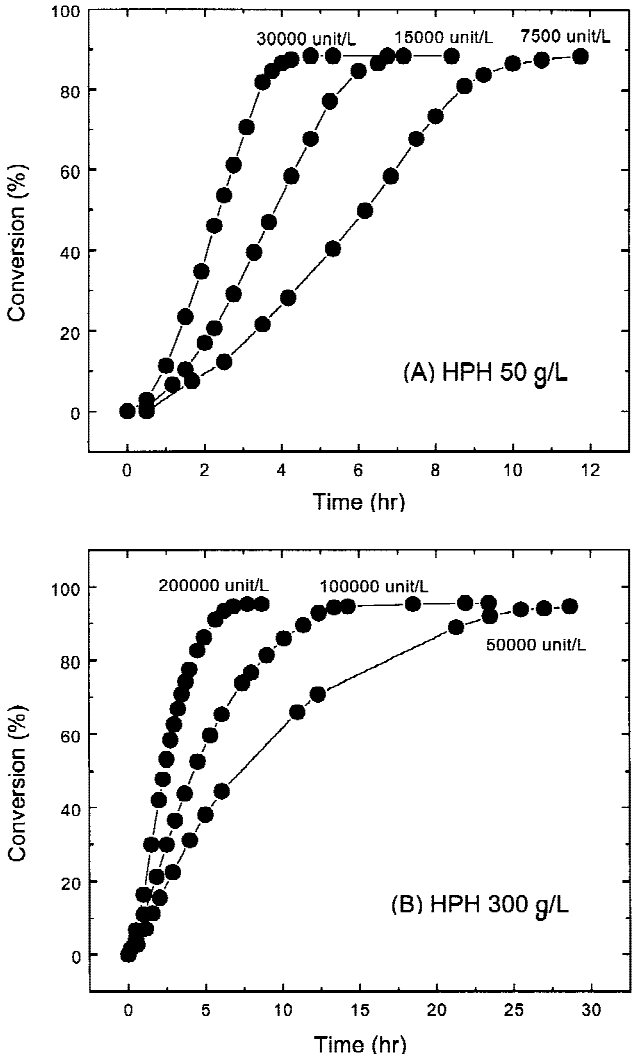

Figure 7. Production of *N*-carbamoyl-D-*p*-hydroxyphenylglycine at different loadings of whole cell enzyme in the heterogeneous reaction system. The substrate concentrations were (A) 50 and (B) 300 g/L. Temperature and pH were maintained at 45°C and 8.5, respectively.

significant than that observed at 50 g/L substrate, and the inactivation became more rapid at higher loadings of the whole cell enzyme (Fig. 8B). These results support that NaOH added for titration also acts as a deactivating reagent. The higher the loading of the whole cell enzyme is at a given substrate concentration, the higher is the addition rate of NaOH, due to the enhanced production rate of NCHPG, which leads to more rapid inactivation of the enzyme. Nevertheless, the whole cell enzyme was more stable than the free enzyme. As for the free enzyme, it was estimated that inactivation of the enzyme by substrate particles and NaOH added for titration accounts for about 90% of the total inactivation in the reaction system, while 10% would be a spontaneous deactivation (Fig. 6).

When the intact cells were incubated in the reaction mixture in the absence of substrate, the whole cells were fully permeabilized in about 15 h (Fig. 2B). On the other hand, about 7 h was required for permeabilization in the presence of 50 g/L substrate (Fig. 8A). The activity of intact cells approached almost the same level as that of permeabilized cells in 3 h when the substrate concentration was 300 g/L (Fig. 8B). However, despite the permeabilization of the cell membrane during the reaction, the change in the integrity of cells was found to be negligible from the microscopic observations, and the enzyme activity in the supernatant after centrifugation of the reaction mixture was not detected.

DISCUSSION

Previously, the thermostable D-hydantoinase from *B. stearothermophilus* SD-1 was mass-produced by batch cultivation of the recombinant *E. coli* harboring the enzyme gene (Lee, S. G., et al., 1997). Here we optimized the heterogeneous reaction system for the production of *N*-carbamoyl-D-*p*-hydroxyphenylglycine, which is readily converted to

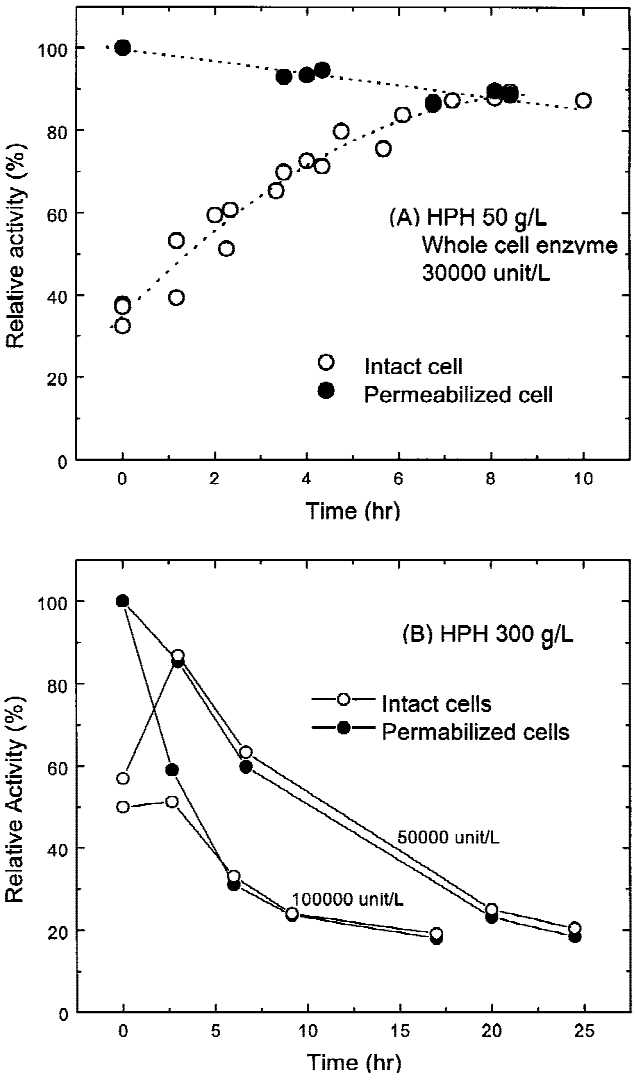

Figure 8. Deactivation of whole cell enzyme during the reaction observed. The substrate concentrations were (A) 50 g/L and (B) 300 g/L. Temperature and pH were 45°C and 8.5, respectively.

D-*p*-hydroxyphenylglycine, from 5-(4-hydroxyphenyl)hydantoin using the mass-produced D-hydantoinase based on the kinetic analysis of the reaction system. In the reaction system, most of the substrate is present in suspended particles, and the substrate concentration could be increased up to 300 g/L.

Enzyme reaction in distilled water also resulted in almost the same profile as in buffer solution, and this can be attributed to the property of the substrate; the hydantoin ring of HPH has a fairly acidic property with a pK_a value of 9.1 at 25°C (Bateman, 1980), which gives a buffering action against intermittent pH elevation by added NaOH solution in the optimal pH range (8–9) of the D-hydantoinase-catalyzed reaction. From a practical standpoint, use of distilled water as a reaction medium is surely more economical on a large scale.

In the D-hydantoinase-catalyzed reactions, operation at high temperatures leads to improved productivity mainly due to the enhanced solubility of the substrate and an increase in the racemization rate of the L-form substrate to the D-form. However, operation at temperatures above 50°C resulted in lower conversion yields, which was due to the modification of the dissolved substrate (HPH) by NaOH added for pH control during the enzyme reaction. It seems that the added NaOH solution might modify the substrate during the mixing time. A continuous process employing a low substrate concentration (<10 g/L) below the solubility limit in a buffer solution might be considered as an alternative to overcome the modification of the substrate, even at temperatures over 50°C, because titration with NaOH is not required. However, in this case, the operational stability of the enzyme, low productivity, and cost of immobilized enzyme might be critical factors in the process development.

As a biocatalyst, three types of the enzyme are available; free, whole cell, and immobilized enzymes. We also tested the immobilized D-hydantoinase by using various support matrices. However, repeated use of the immobilized enzyme was impractical for the heterogeneous reaction system in a stirred tank reactor because attrition of the support matrix with large amounts of substrate particles caused the fouling of the macropores on the surface of the immobilized enzyme. In addition, abrasion of the support matrix was found to be serious. A similar result was also recently reported (Lee and Lin, 1996). Furthermore, cost analysis for the whole process for the production of HPG revealed that the enzyme cost accounts for less than 1% of the total production cost, and this was mainly attributed to mass production of the enzyme using recombinant *E. coli* (Lee, S. G., et al., 1997).

We used free and whole cell enzymes and compared their efficiencies in the heterogeneous reaction system. When the free D-hydantoinase was used in the heterogeneous reaction system, at least 300 units of enzyme per g-substrate was required to achieve a maximum conversion. Enzyme loadings lower than this level resulted in a low conversion, and this was due to severe deactivation of the enzyme in the reaction mixture. The free D-hydantoinase seemed to be inactivated by the surface tension at the interface between solid and aqueous phases as well as by NaOH added for titration. As a result, free enzyme lost its activity 10 times faster in the heterogeneous reaction system than in the absence of substrate particles. Interestingly, the conversion yield increased with increasing substrate concentration up to 300 g/L, even though more significant inactivation of free enzyme was expected. This result can be explained by the fact the dissolved substrate is less modified by NaOH added for titration in the presence of higher concentration of substrate particles. In other words, substrate particles might protect the dissolved substrate from modification by NaOH solution.

In the case of whole cell enzyme, harvested cells can be directly used without any additional steps, but a reduced reaction rate was expected due to the transport barrier for the substrate across the cell membrane. However, the conversion reached a maximum value even at a lower loading of the whole cell enzyme than the free enzyme, showing 96% conversion at 300 g/L substrate, and this is closely related with higher operational stability of the whole cell enzyme. Inactivation of the whole cell enzyme by the surface tension at the interface might be negligible because the enzymes are present inside the cell membrane. As a whole, the whole cell enzyme was found to be less inactivated than the free enzyme in the heterogeneous reaction system, which led to higher conversion even at lower enzyme loadings compared with the free enzyme. As mentioned earlier, there exists a transport barrier for the substrate across the cell membrane when the whole cell enzyme was employed. However, the permeability of the cell membrane increased gradually and reached a maximum value in the course of the enzymatic reaction. The time required to reach the maximum permeability was shortened as the concentration of the substrate particles was increased, and no significant difference in the reaction rate was observed after being fully permeabilized. This result strongly implies that substrate particles also affect the permeabilization of cell membrane as well as the dissolved substrate. Higher conversion yields at higher substrate concentrations were observed, and this would be explained by the fact that the dissolved substrate is less modified by NaOH added for titration in the presence of a higher concentration of substrate particles as mentioned earlier.

From the above observations, it was concluded that whole cell D-hydantoinase can be effectively used in the heterogeneous reaction system for the production of NCHPG from HPH. The amount of whole cell enzyme needed to produce one unit mass of NCHPG was estimated to be half that of the free enzyme. Whole cell enzyme can be simply prepared, and this might be another advantage over the free enzyme system. The heterogeneous reaction system employed in this work can be of practical use for the production of other valuable compound from a rarely water-soluble substrate by using the proper type of enzyme.

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