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Kinetic Analysis of Heterogeneous Enzymatic Process for the Production of D-Amino Acids

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Introduction

Optically active D-amino acids are widely used as intermediates for the synthesis of semi-synthetic antibiotics, peptide hormones, pyrethroids, and pesticides. In the D-hydantoinase catalyzed process, the chemically synthesized D,L-5′-mono-substituted hydantoin is enantioselectively hydrolyzed to the corresponding *N*-carbamoyl-D-amino acid by D-hydantoinase, and this intermediate is further converted to free D-amino acid by *N*-carbamoyl-D-amino acid amidohydrolase (*N*-carbamoylase) or chemical decarbamoylation. 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.

Recently, we optimized a reaction condition using mass-produced D-hydantoinase (Lee et al., 1998) and performed kinetic analysis of D-hydantoinase-catalyzed process for the production of *N*-carbamoyl-D-*p*-hydroxyphenylglycine from D,L-*p*-hydroxyphenylhydantoin at high substrate concentration (Lee et al, 1999). We previously reported the mass production of two enzymes in the recombinant *E. coli* and the efficient enzymatic process using separately expressed whole cell and co-expressed whole cell (Park et al., 2000). Because these two enzymes have a different reaction optima, and stability (Park et al, 2000), kinetic analysis of two sequential enzymes is required to analyze and predict the optimal condition of the process.

In this work, we develop the kinetic model which descibed the heterogeneous reaction system for the production of D-hydroxyphenylglycine from poorly soluble p-hydroxyphenylhydantoin by using recombinant D-hydantoinase and N-carbamoylase. We investigated the effect of racemization of substrate and inhibition of N-carbamoylase at various pH values. Factors affecting the kinetics of the

heterogeneous reaction system were also analyzed on the basis of the kinetic models.

Materials and Methods

Chemicals and Strains

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). The construction of D-Hydantoinase expressing recombinant *E.coli*, XL1 Blue/pHU183 was described elsewhere (Lee et al., 1996). Detailed description of *N*-carbamoylase expressing recombinant *E. coli*, XL1 Blue/pBCAR21 and co-expressed recombinant *E. coli*, XL1 Blue/pHCAR101 have been described elsewhere (Park et al., 2000).

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 liter, and distilled water was used as the reaction medium. The reaction was conducted at 45 °C (Park et al., 2000), and pH of the reaction mixture was controlled at defined values.

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 x 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 eluent was detected at 214 nm.

Parameter Evaluation

Kinetic parameters used in the equations were determined by various methods. The k_{cat} and k_m of enzymes at different pH value are determined by double-reciprocal plot of experimental data. The solubility of *p*-HPH is determined in the distilled water that is adjusted to predetermined pH at 45 °C, and the density of *p*-HPH is estimated by a method described elsewhere (Shugar and Ballinger, 1996). The mass transfer coefficient of *p*-HPH, k_s, was estimated from a simple correlation with critical suspension speed and Schmidt number ($\mu/\rho D$) in the mechanically agitated tank as described

elsewhere (Jadhav and Pangarkar, 1975). The permeability of each compound was used based on the data in the literature (Tranchino and Melle, 1990). The other values that are not determined by experiment are simultaneously estimated by using Marqurdt's method of non-linear regression analysis (Johnson and Faunt, 1992) within 95 % confidence interval. The model equations for the whole cell reaction were numerically integrated using the 4^{th} order Runge-Kutta method. Non-linear regression analysis and integration of model equation are carried out using the language of Boland C^{++}.

Results and Discussion

Simulation of separately expressed whole cell reaction

We performed the kinetic analysis of heterogeneous whole cell reaction using separately expressed D-hydantoinase and *N*-carbamoylase (Fig 1A). As the activities of *N*-carbamoylase were increased, the accumulation of intermediate was reduced to 10.2 mM. However, the initial rate of D-HPG production was slightly increased at pH 7.0, and the production rate of D-HPG was almost the same as increasing of *N*-carbamoylase activities. These results may come from the limitation of substrate racemization at neutral pH (Fig 2A).

The production rate of NC-D-HPG at pH 7.5 was decreased as increasing of loading amounts of *N*-carbamoylase. The intermediate accumulation of NC-D-HPG at pH 7.5 in the aqueous solution was up to 212 mM when the activity of *N*-carbamoylase was 600 U in the reaction solution. As the activities of *N*-carbamoylase were increased, the accumulation of intermediate was reduced to 172 mM. The production rate of D-HPG was increased as increasing of loading amounts of *N*-carbamoylase. However, the reduction of reaction rate as time goes on was observed in all cases. The final product concentration of 3600 U D-hydantoinase and 600 U D-hydantoinase were 103.2 mM and 32 mM, respectively. The intermediate accumulation of NC-D-HPG at pH 8.0 in the aqueous solution was up to 232 mM in 8 hrs when the activity of *N*-carbamoylase was 600 U in the reaction solution (Fig 2B). As the activities of *N*-carbamoylase were increased, the accumulation of intermediate was reduced to 171.3 mM. The production rate of D-HPG was increased as increasing of loading amounts of *N*-carbamoylase. However, the reduction of reaction rate was observed more seriously than the case at pH 7.5. The final product concentration of 3600 U D-hydantoinase and 600 U D-hydantoinase were only 63.8 mM and 21.4 mM, respectively.

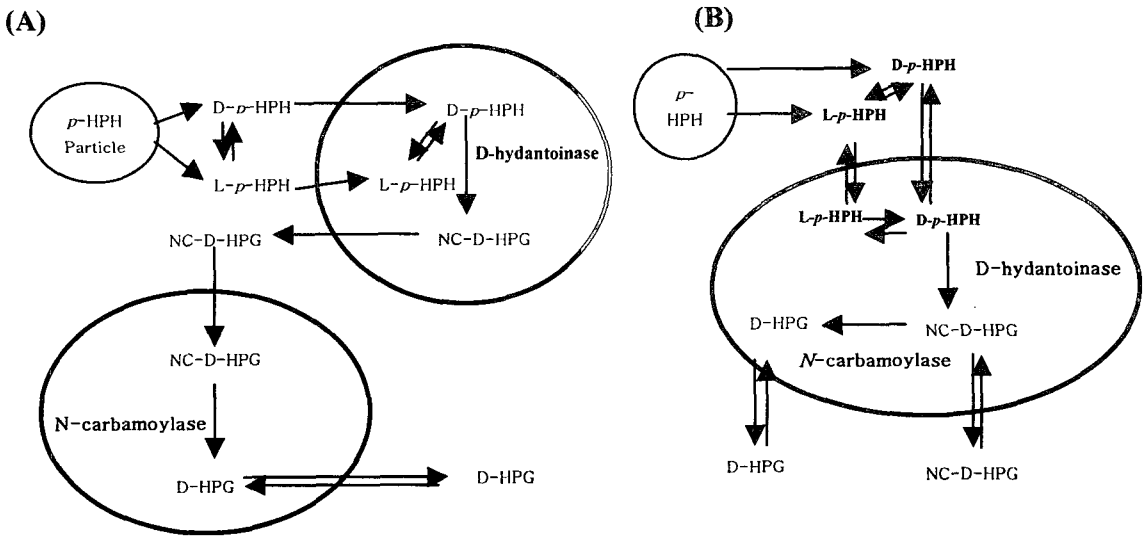

Figure 1. Schematic diagrams of the heterogeneous reaction systems using whole cells with separately expressed (A) and co-expressed (B) D-hydantoinase and *N*-carbamoylase.

As shown in the results, the mathematical models are valid for the prediction of heterogeneous reaction system using whole cell containing D-hydantoinase and *N*-carbamoylase as biocatalysts. The ammonia inhibition of *N*-carbamoylase was investigated the important factor to determine the reaction condition and productivity of final product. The racemization rate of *p*-HPH is also considerable factor to optimize the overall reaction conditions. As pH of reaction mixture goes to neutral condition, the racemization of *p*-HPH was inhibited and activity of D-hydantoinase was relatively low compared with its maximum activity. On the contrary, the reaction rate of D-hydantoinase was increased at alkaline pH, but the production rate of D-HPG was decreased due to the inhibition of *N*-carbamoylase.

Simulation of co-expressed whole cell reaction

We performed the kinetic analysis of heterogeneous whole cell reaction using co-expressed D-hydantoinase and *N*-carbamoylase (Fig. 1B). 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 of each enzyme was measured under standard assay conditions. The ratio of activities per gram cells between D-hydantoinase and *N*-carbamoylase was estimated to be 1:1.2. The amount of D-hydantoinse and *N*-carbamoylase were measured to be 2.04×10^{-6} M and 2.44×10^{-6} M, respectively.

We investigated the reaction properties in terms of product yield of D-HPG and intermediates

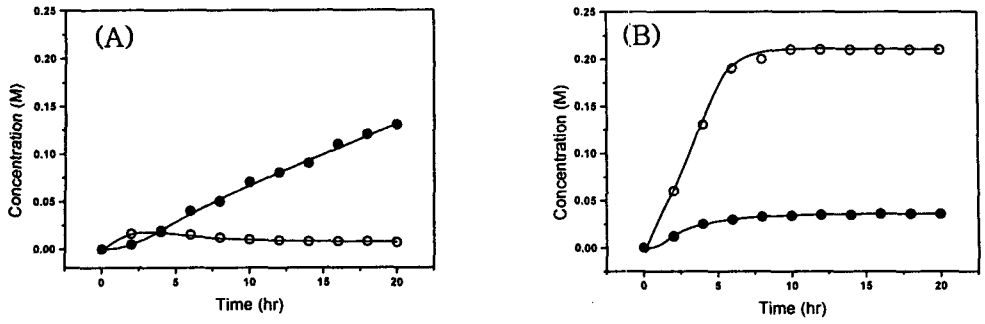

Figure 2. Reaction profiles using separately expressed whole cells at pH 7.0 (A) and pH 8.0 (B). Initial substrate concentration was 238 mM (50 g/L). The total activities of D-hydantoinase and N-carbamoylase in the reaction mixture were the same as 1500 Units/L. Solid lines and symbols represent simulated results and experimental data, respectively. Symbols: NC-D-HPG (●) and D-HPG (○) in the aqueous solution.

accumulation when the whole cell loading was increased in the reaction mixture. As the amounts of co-expressed whole cell were increased, the rate of D-HPG production was increased, and the productivity of D-HPG was increased. The concentration of final product, D-HPG was increased up to 184.9 mM when the concentration of D-hydantoinase and *N*-carbamoylase were 6.12×10^{-6} M and 7.32×10^{-6} M, respectively. The increment of final product concentration was largely increased when the loading amount of enzyme was increased as much as 2 times. However, the product concentration was slightly increased when the loading amounts of enzyme were increased as much as 3 times in the reaction mixture. These results suggested that the limitation of substrate racemization at pH 7.0 is cause by the reduction of production rate. The mathematical models are valid for the prediction of heterogeneous reaction system using co-expressed whole cell containing both of D-hydantoinase and *N*-carbamoylase as biocatalysts. The enzyme loading above 5000 U of D-hydantoinase was not effective for the improvement of productivity of product, D-HPG.

Sensitivity analysis

The optimal solution obtained in model based on optimization is subjected to uncertainty associated with the estimates of the model parameters. The parameter uncertainty may be due to the random error associated with the measurement and the model mismatch. In addition, the effects of measured or unmeasured disturbances may cause the optimal solution to deviate from the normal operating conditions in which the parameter estimates are valid and therefore increase the modeling error.

Sensitivity analysis aims to estimate the change in the optimal solution given an estimated error in the model parameters.

Fig. 3A shows the effect of racemization rate on the profiles of the product, D-HPG production when heterogeneous reaction was performed at pH 7.0. The rate of racemization largely affect on the reaction rate of D-hydantoinase, and the increasing concentration of produced intermediates accelerated the conversion rate of *N*-carbamoylase inside cells.

In the development of enzymatic process, low solubility of the substrate, *p*-HPH has been one of the serious problems. The solubility of *p*-HPH in water is estimated to be 40 mM at pH 7.0 in this work. We investigated the effect of solubility of *p*-HPH on the production of D-HPG in the heterogeneous reaction system (Fig. 3B). The production rate and final productivity of D-HPG was increased with the increasing of solubility. The reaction rate of D-hydantoinase was affected by the mass transfer rate of *p*-HPH below the range of 100 mM. From these results, sufficient solubility of *p*-HPH may improve the process for the production of D-amino acids. The affinity toward substrate of enzymes was considered as a distinguished feature from chemical catalyst. We varied the Michaelis-Menten constant, k_m and investigated the effect of k_m value on the productivity of reaction. The production rate of D-HPG was slightly incresed even when the k_m value of D-hydantoinase was decreased as much as 4 times than original value. The prductivity of D-HPG was also slightly increased as the affinity of D-hydantoinase was increased as much as 4 times. These results indicated that the improvement of

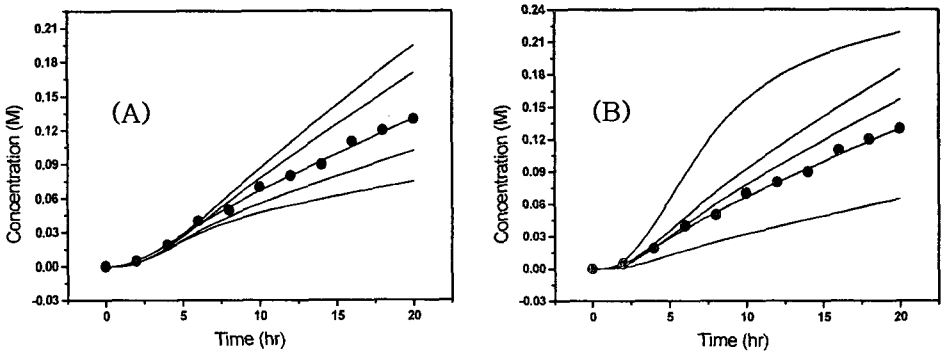

Figure 3. Effect of the spontaneous racemization rate (A) and solubility (B) of substrate on the production of D-HPG at pH 7.0 in the aqueous solution. The total activities of D-hydantoinase and N-carbamoylase were 1500 Units/L and 3000 Units/L, respectively. (A) The constants of spontaneous racemization were 0.5 x 10^{-5}, 1.0 x 10^{-5}, 3.2 x 10^{-5}, 6.4 x 10^{-5} or 9.6 x 10^{-5} cm/sec as indicated. (B) The solubility of substrate was 20, 40, 60, 80, or 100 mM as indicated. Solid lines and symbols represent simulated results and experimental data, respectively

affinity of D-hydantoinase is not significantly contributed the overall productivity of D-HPG.The similar results were observed in the case of *N*-carbamoylase. These results strongly indicate that the critical factors of the productivity and overall reaction rate were physical properties concerned with solubility, substrate transfer rate, racemization rate of substrate, and inhibition of enzyme compared with the other kinetic properties.

Optimal condition

In the case of separately expressed enzyme system, the production rate of D-HPG at pH 7.0 was linear dependent on the production rate of NC-D-HPG because the concentration of NC-D-HPG in the aqueous solution was low and the concentration of intermediate inside cell was not enough for the maximum velocity of *N*-carbamoylase. These phenomena result from the racemization of *p*-HPH is limited the production rate of NC-D-HPG. The enzyme ratio above 1:2 is not necessary for the increasing of the final product, D-HPG in this reaction system.

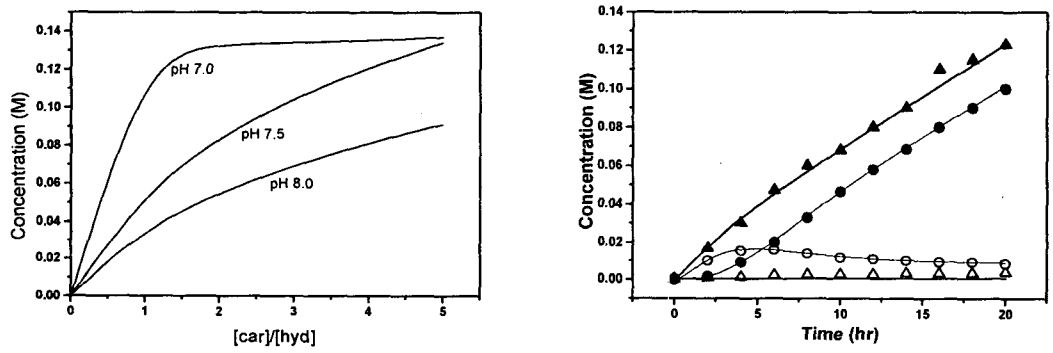

Figure 4. (A) Effect of enzyme loading ratio between D-hydantoinase and N-carbamoylase on the production of D-HPG. (B) Comparison of separately expressed whole cell reaction and co-expressed whole cell reaction at pH 7.0. The total activities of D-hydantoinase and N-carbamoylase were 1200 Units and 1440 Units/L, respectively. Symbols: concentration of NC-D-HPG (○) and D-HPG (●) in the separately expressed whole cell reaction; concntration of NC-D-HPG (△) and D-HPG (▲) in the co-expressed whole cell reaction.

The optimal reaction temperature was determined at 45 °C, and optimal reaction pH was determined at pH 7.0 when we analyzed the reaction system. The optimal ratio of D-hydantoinase and *N*-carbamoylase in the reaction was determined at 1:2 by observation of product yield of D-HPG (Fig 4A).

The reaction system using co-expressed enzymes in an *E.coli* was shown to be more efficient reaction properties. The intermediate accumulation was minimized in the aqueous reaction solution

when we compared with separately expressed enzyme as a catalyst in this work (Fig 4B). These results strongly suggest that mass produced co-expressed enzyme system can be overcome the disadvantages of enzyme reaction compared with chemical process. The more detailed analysis of enzyme reaction in the various bioconversion process was prerequisited for the development of enzymatic process.

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