Directed Evolution of a Novel *N*-Carbamylase/D-Hydantoinase Fusion Enzyme for Functional Expression with Enhanced Stability

Geun Joong Kim, Young Hoon Cheon, Hak-Sung Kim

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Kusung-dong, Yusung-gu, Taejon 305-701, Korea; telephone: 82-42-869-2616; fax: 82-42-869-2610; e-mail: hskim@sorak.kaist.ac.kr

Received 8 August 1999; accepted 6 November 1999

Abstract: Bifunctional enzymes find a wide application as a monitoring facility and a potential biocatalyst in molecular biology and biotechnology. Recombination of natural enzymes to a bifunctional fusion offers valuable tools, but the functional and structural instability of artificial fusion enzymes remains to be solved. Based on structural traits of microbial D-hydantoinase, we attempted to construct a bifunctional *N*-carbamylase/D-hydantoinase fusion enzyme that would be useful for the synthesis of nonnatural D-amino acids in a concerted fashion. The bifunctional ability of D-hydantoinase, as a fusion partner, was noticeable, but the resulting fusion enzyme was subjected to serious proteolysis in vivo, as generally encountered in the expression of large the multidomain polypeptide in *E. coli.* In an effort to improve the structural instability imposed by artificial linear fusion, directed evolution of the fusion enzyme was performed using DNA shuffling with a consensus primer to maintain a crucial domain for the enzyme activity. The evolved fusion enzyme, F11, was selected after repeated rounds, and this enzyme was found to show sixfold increased performance in the production of D-amino acid compared with the parent fusion enzyme, which was mainly due to the enhanced structural stability of the evolved fusion enzyme. This result is an example showing that directed evolution of the linearly fused polypeptide may broaden the opportunity to generate a fusion enzyme with greater potential. © 2000 John Wiley & Sons, Inc. *Biotechnol Bioeng* **68**: 211–217, 2000.

Keywords: linear fusion; bifunctional enzyme; D-hydantoinase; *N*-carbamylase; directed evolution; DNA shuffling

INTRODUCTION

Different ecological niches have provided a multitude of microorganisms that are well adapted to their environments through the evolutionary process, and thus an abundant enzyme pool is available for screening enzymes with the desired property (Dalboge and Lange, 1998). Natural evolution, especially in a static environment, has restricted the enzymes to function within the narrow limits of existing conditions and has forced them to act mainly on a specified substrate. In the field of molecular biology and biotechnology, however, enzymes possessing two or more combined activities, along with appropriate stability, have found a wide application (Nixon et al., 1998). Although natural diversity of the enzymes has provided some candidates that have evolved to possess bifunctional activity, most fusion enzymes have resulted from the in vitro fusion of individual enzymes based on evolutionary traits and a well-defined structure (Ay et al., 1998; Doi and Yanagawa, 1999). Artificial fusion enzymes, simply generated by either end-to-end fusion or tethering with a linker of whole genes encoding intact functional proteins, have shown comparable performance with an individual enzyme in a concerted fashion (Gilbert et al., 1998; Li et al., 1996). However, in general, functional and structural instability of artificial fusion enzymes has limited its practical use (Ay et al., 1998; Chowdhury et al., 1998).

Nonnatural D-amino acids are used extensively in the pharmaceutical field; these include antimicrobial and antiviral agents, artificial sweeteners, pesticides, and pyrethroids. With great commercial demand for various D-amino acids, enzymatic and chemoenzymatic routes have been developed (Yamada and Shimizu, 1988). Of these, a fully enzymatic process employing two sequential enzymes, D-hydantoinase and *N*-carbamylase, is a typical case requiring combined enzyme activity (Grifantini et al., 1998; Kim and Kim, 1995; Runser et al., 1990). In this process, hydantoin derivative is hydrolyzed by D-hydantoinase, and the resulting *N*-carbamyl-D-amino acid is further converted to the final D-amino acid by *N*-carbamylase (Fig. 1). Thus, functional fusion of two enzymes would offer several advantages over individual enzymes with respect to reaction kinetics and enzyme production.

In this study, we attempt to construct a bifunctional fusion enzyme composed of *N*-carbamylase from *Agrobacterium radiobacter* NRRL (B11291) (Grifantini et al., 1996) and D-hydantoinase from *Bacillus stearothermophilus* SD1 (Kim et al., 1997). The artificial fusion was conducted by

Correspondence to: H.-S. Kim

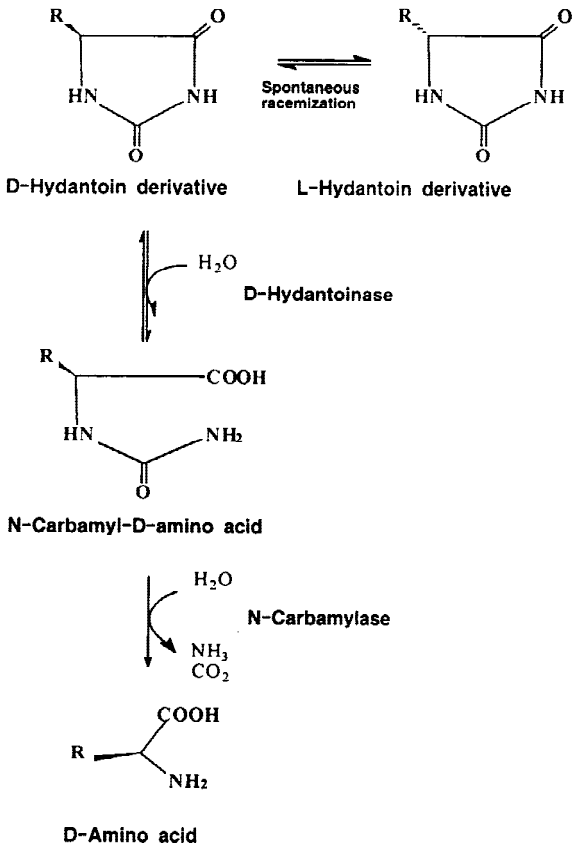

Figure 1. A typical reaction scheme requiring two sequential enzymes (D-hydantoinase and *N*-carbamylase) for the synthesis of nonnatural D-amino acid from 5-monosubstituted hydantoin.

end-to-end fusion of intact *N*-carbamylase to the *N*-terminal region of D-hydantoinase. The resulting fusion enzyme has a distinct bifunctional activity, but the extensive proteolysis caused by structural instability remained to be solved. In an effort to improve the structural stability of the fusion enzyme, we conducted directed evolution using DNA shuffling (Giver et al., 1998; Stemmer, 1994) with a consensus primer to maintain the essential residues for the structural scaffold. After three rounds of screening and evolution, the evolved fusion enzyme (F11) was selected, and its improved stability was evaluated in the production of D-amino acids. Details are reported herein.

MATERIALS AND METHODS

Construction of Fusion Enzyme

For the construction of the fusion enzyme, the *Agrobacterium N*-carbamylase gene (Grifantini et al., 1996) was amplified from chromosomal DNA with two primers, CAN (5′-CAAAG GTCCATGGCACGTCAGATGATA-3′) and CAC (5′-AAGGGATCCTTATCAGAATTCCGCGATCAG-3′), as N- and C-terminal primers, respectively. The resulting fragment was inserted into the *Nco*I and *Bam*HI sites of pTrc99A, yielding the plasmid pTC. The naturally occurring *N*-carbamylase gene has a unique restriction site, *Eco*RI, in its C-terminus, neighboring the stop codon TAA. Two primers, SDN (5′-TAGAATTCATGACAAAAATTATAAAAAATC-3′) and SDC (5′-TACTGCAGT TAAATGGTTAATTCCTCGCTC-3′), spanning the genes encoding the D-hydantoinase from *B. stearothermophilus* SD1, were used. Restriction sites, *Eco*RI and *Pst*I, were introduced into the N-and C-terminal primers, respectively. The amplified D-hydantoinase gene (1.4 kb) was inserted to replace the sequence between *Eco*RI and *Pst*I sites in plasmid pTC, yielding plasmid pTCH (Fig. 2). The resulting fusion gene (2.3 kb) encoding the fusion enzyme (CAB-HYD) was confirmed by DNA sequencing.

Expression and Purification of Fusion Enzymes

Expression of the fusion enzyme (CAB-HYD) was conducted in *E. coli* JM109 under the control of P_{trc} promoter and purified using an antibody raised against the purified D-hydantoinase from *B. stearothermophilus* SD1 (Kim et al., 1997). *E. coli* cells were cultivated in 200 mL of LB broth containing ampicillin (25 μg/mL) at 30°C, and IPTG (isopropyl-β-D-thiogalactopy ranoside) (0.2 m*M*) was added for induction when the $OD_{600\ nm}$ reached about 0.7 to 0.8. After 2-h cultivation, cells were collected by centrifugation, and then resuspended in 5 mL of 20 m*M* Tris-HCl (pH 7.8) buffer containing 0.1 m*M* manganese chloride, 0.1% PMSF (phenymethyl sulfonylfluoride), 0.1% Triton X-100, and 1 m*M* DTT (Dithiothreitol). The fusion protein was purified using the following protocol: Suspended cells were freeze-thawed two times and disrupted by sonication, and the cell lysate was centrifuged at 15,000 rpm for 1 h. The supernatant was incubated overnight with immunoglobulin G (IgG)-immobilized Sepharose 4B in a nitrogen gas atmosphere. After washing with 20 m*M* Tris-HCl (pH 7.8) containing 0.25 m*M* NaCl, immunoabsorbed proteins were eluted from the column with 50 m*M* carbonate buffer containing 2 *M* NaCl. Active enzyme fractions were dialyzed against the buffer (20 m*M* Tris-HCl, pH 7.8) and used for further analyses.

DNA Shuffling and Sequence Analysis

The fusion gene encoding CAB-HYD was amplified from plasmid pTCH using two primers, CAN and SDC. In vitro

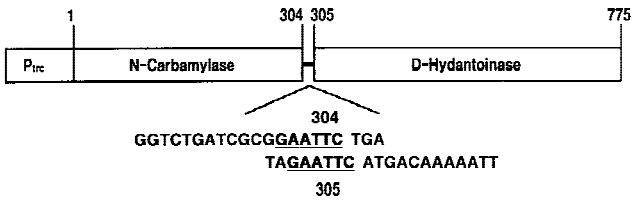

Figure 2. Structural organization of the gene encoding *N*-carbamylase/D-hydantoinase fusion enzyme. Whole amino acid residues of both open reading frames were conserved, and the nucleotide sequence of the junction region is shown in bold.

evolution of the resulting gene (2.3 kb) was performed as described by Stemmer (1994) with some modifications. DNA fragments (50 to 200 bp), digested by DNaseI, were reassembled by PCR (94°C, 1 min; 51°C, 1 min; 72°C, 40 sec + 2 sec/cycle; total 50 cycles) with a consensus primer, HCO (5′-GATCCCCACACGCATTTAGAT-3′). The DNA concentrations were estimated, and then DNaseI-digested fragments and consensus primer were mixed at about 75:1 for a concentration of 40 to 60 ng/μL. The reassembled DNA fragments were amplified by PCR (94°C, 1 min; 51°C, 1 min; 72°C, 2 min; total 35 cycles) with two primers, SDC and CAN. The second and third rounds of shuffling were identical to the first round. To expand the mutation space, five and six of the positive clones were isolated from the first and second round and used for the next round. The evolved gene was sequenced with appropriate primers by the use of an automated DNA sequencer (ABI, Model 377).

Library Construction and Screening

After PCR with reassembled reaction products, amplified DNA fragments (2.3 kb) were purified from agarose gel and then digested with *Nco*I and *Pst*I. The resulting genes were cloned into the plasmid pTrc99A, and transformed into *E. coli* JM109 by electroporation. Transformants were plated on Luria–Bertani (LB) agar plates (50 μg/mL ampicillin). After 1 day, colonies were picked with sterile toothpicks and placed into 96-well plates containing 250 μL of LB medium (50 μg/mL ampicillin). Plates were incubated at 37°C for 12 to 15 h, and then 12 μL of each well was transferred into a new plate containing fresh media. The master plates were preserved, and the new plates were grown for an additional 4 h, and then induced with 0.5 m*M* IPTG. After 5 to 6 h, induced cells from each well were harvested. Cells were resuspended in 100 m*M* phosphate buffer (pH 7.0) and preincubated at 45°C for 6 h. After incubation, activity of the fusion enzyme and total conversion of hydroxyphenylhydantoin (HPH) to D-hydroxyphenylglycine (D-HPG) were determined.

Measurement of Fusion Enzyme Stability

The stability of the fusion enzyme in vivo was determined with some modifications, as described by Frank et al. (1996). *E. coli* cells were cultivated in LB medium at 37°C and induced with 0.5 m*M* IPTG when the $OD_{600\ nm}$ reached about 0.4 to 0.5. After 2-h cultivation, chloramphenicol (100 μg/mL) was added to the culture medium to block further protein synthesis, and aliquots (0.5 mL) were withdrawn at the indicated times. Cells were collected by centrifugation, and a portion of the resulting pellet that corresponds to about 0.1 mL of culture medium was resuspended in SDS sample buffer. Crude extract was analyzed on sodium dodecylsulfate–polyacrylamide electrophoresis (SDS-PAGE), and the fusion protein band was analyzed with a gel scanner. The remaining pellet was used to determine the activity of fusion enzyme. For the stability of fusion enzyme under operational conditions, the fusion enzyme was incubated in 100 m*M* phosphate buffer (pH 7.2) containing 1 m*M* DTT at 45°C, and activities of D-hydantoinase and *N*-carbamylase were assayed as a function of time.

Enzyme Assay and Conversion Test

The activities of *N*-carbamylase and D-hydantoinase were determined at 45°C for 30 min with constant shaking. In the case of D-hydantoinase activity, HPH was used at a final concentration of 15 m*M* as a substrate in a 100 m*M* Tris-HCl (pH 8.0). For the *N*-carbamylase activity, 15 m*M* of *N*-carbamyl-D-hydroxyphenylglycine (NCHPG) in 100 m*M* potassium phosphate buffer (pH 7.0) containing 1 m*M* DTT was used as a substrate. The conversion experiments of HPH and phenylhydantoin (PH) to D-HPG and D-phenylglycine (D-PG) were performed in a total volume of 10 mL that contained 20 m*M* substrate, 100 m*M* phosphate buffer (pH 7.2), and 1 m*M* DTT in a nitrogen atmosphere. Induced cells (50 mg) were added into the reaction vial. The reaction products were analyzed using high-performance liquid chromatography (HPLC) as described earlier (Kim and Kim, 1995).

RESULTS

Construction and Expression of Bifunctional CAB-HYD Fusion Enzyme

Our previous results showing that the N-terminus of D-hydantoinase is dispensable for enzyme activity and tolerable to a β-galactosidase fragment fusion provided the possibility that the hybrid enzymes might be generated via the linear fusion of a protein at the N-terminus of D-hydantoinase, expecting to create a bifunctional fusion enzyme (Kim et al., 1997; Kim and Kim, 1998a). Based on the above finding, we constructed an artificial fusion enzyme composed of the *N*-carbamylase and D-hydantoinase that is useful for the production of D-amino acids in a concerted fashion. *E. coli* JM109 was transformed with the recombinant plasmid derived from pTrc99A containing the fusion gene. In the experiment to express the fusion enzyme, it was observed that the fusion protein gradually disappeared, especially when induction time was longer than 2 h. To determine whether fusion protein is indeed susceptible to proteolysis in vivo, we tested the stability of the fusion enzyme in vivo after addition of chloramphenicol to block further protein synthesis (Frank et al., 1996). As can be seen in Figure 3, the fusion protein band became faint with incubation time, mainly due to extensive proteolysis. The activity of the wild-type fusion enzyme was also shown to decrease with incubation time (see Fig. 5).

As an alternative strategy to reduce proteolysis in vivo, we tested the expression of the fusion enzyme under the control of P_{BAD} promoter in a protease-deficient strain, *E. coli* BL21. Expression level of the fusion enzyme was increased to some extent compared with that using pTrc99A,

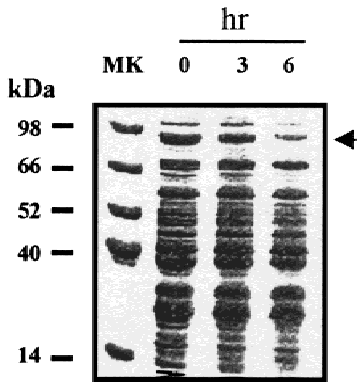

Figure 3. Stability of the wild-type fusion enzyme in vivo. After addition of the chloramphenicol to the culture medium to block the protein synthesis, aliquots were withdrawn at the indicated times, and proteins were analyzed on SDS-PAGE, as described in the text. Arrow indicates the expressed fusion enzyme.

but most (>95%) of the intact fusion enzymes were detected in the insoluble fraction (data not shown).

We investigated different induction conditions with pTCH/JM109 to obtain the fusion enzyme as a soluble form for further experimentation, and found that the specific activity corresponding to the intact fusion enzyme was maximized when cells were induced with 0.2 m*M* IPTG at 30°C for 2 h. The protein band corresponding to the CAB-HYD fusion enzyme was observed between 85 and 88 kDa, which is consistent with the expected size of the fusion enzyme (86 kDa) calculated from the fusion gene. Analysis of the expressed enzyme by SDS-PAGE showed that at least 40% to 55% of the intact fusion enzyme was detected in the crude extract as a soluble form, and it was estimated to be 2% to 3% of the total protein.

The antibody raised against free D-hydantoinase was observed to recognize the CAB-HYD fusion enzyme and was used for purification. The fusion enzyme eluted from the immunoaffinity column appeared at the position of 86 kDa, corresponding to intact fusion protein. The fusion enzyme catalyzed the conversion of HPH to D-HPG via NCHPG, which clearly indicates the bifunctional activity of the fusion enzyme. Aside from the major band of the intact fusion enzyme, minor polypeptides of smaller size resulting from proteolysis were also observed. These polypeptides were removed using an ion-exchange column and found to have no effect on the bifunctional activity of the fusion enzyme. The activities of the CAB-HYD fusion enzyme toward NCHPG and HPH were estimated to be 1.1 and 5.1 U/mg protein, respectively.

In Vitro Evolution of the Fusion Enzyme

As mentioned earlier, the expressed wild-type fusion enzyme (CAB-HYD) was subjected to serious proteolysis in vivo, and this seems to be due to low structural stability of the fusion enzyme. We attempted to improve the structural stability of the CAB-HYD by directed evolution using DNA shuffling. From a practical standpoint, the evolved fusion enzyme should have enough stability to retain the bifunctional activity for a prolonged period of time. We expected that the fusion enzyme evolved to resist proteolysis in vivo would be well correlated with its structural stability, which was supported by in vivo stability test of fusion enzyme, as shown in Figure 3. In this context, we first screened positive clones showing distinct activities of both enzymes after induction with 0.5 m*M* IPTG at 37°C for 5 to 6 h, and further confirmed the stability of the selected clones by total production of D-HPG from HPH for a predetermined period of time, as described in the experimental protocol.

When seeing the diverse nature of enzymes that have evolved to adapt the well-organized enzyme scaffold, a specific enzyme family requires cofactors or metal ions for activity. Hydantoinase family enzymes are also known to have a strong dependency on metal ions such as Mn^{2+} or Zn^{2+} (Syldatk et al., 1999) and, recently, the metal binding site (DXHXHXD) was identified (Kim and Kim, 1998a,b). These residues are essential for maintaining structural integrity and need to be conserved for diminishing the inactive clones, especially in the first round of shuffling, during which point mutation is dominant and resultant positive clones with improved stability are rarely detected. In this sense, a specific primer was added to increase positive clone frequency. From the initial shuffled pool (7000 clones), 5 positive clones showing distinct enzyme activity were isolated. To expand the mutation space, a mixture of five genes was used for the second round, and 57 positive clones were picked from the library of the second round (5500 clones). Among them, the 6 best clones were selected and used for the third round. After the third round of selection, a mutant F11, which exhibited highest stability and total conversion, was screened. The evolved mutant F11 retained its bifunctional activity over 15 h at 45°C. More rounds proved to be nonproductive due to the severe inhibition of cell growth by the evolved fusion enzyme.

Sequencing of the gene encoding F11 revealed 19 point mutations mainly distributed in the HYD domain. Two and six silent mutations were found in the CAB and HYD domains, and two and nine mutations causing the amino acid changes occurred in the CAB and HYD domains, respectively (Table I). No mutation causing a frameshift mutation was found. From the analysis of mutated residues, we found an interesting mutation, F304S, located at the junction of two enzyme domains. The resulting sequence of the junction region was changed from Gly–Leu–Ile–Ala–Glu–Phe to Gly–Leu–Ile–Ala–Glu–Ser. In addition, two mutations, G536S and G645R, resulted in the replacement of hydrophobic residues with hydrophilic residues. The possibility of reducing structural constraints was provided by two substitutions, P684L and P690L, and the other five mutations were conservative. Currently, specific assignment of the changed residues is difficult because no structural data are available. It seems that the cooperative effect exerted by these mutations confers favorable multidomain folding, leading to improved stability of the fusion enzyme.

Table I. Mutation residues in the mutant F11.

Residue[a]	Enzyme	
	CAB–HYD	F11
213	Ser	Cys
304	Phe	Ser
369	Leu	Phe
427	His	Tyr
453	Leu	Phe
475	Leu	Val
536	Gly	Ser
645	Gly	Arg
644	Pro	Leu
684	Pro	Leu
690	Val	Ala

[a]Two mutation residues, 213 and 304, are found in the CAB domain and the other residues are in the HYD domain.

Structural Stability of the Evolved Fusion Enzyme F11

Extract from induced cells expressing the evolved fusion enzyme F11 was analyzed by SDS-PAGE. As shown in Figure 4, the protein band of the evolved F11 showed a similar level to that of wild-type enzyme, and it remained almost intact for a prolonged incubation, even after addition of chloramphenicol. This finding indicates that the F11 has an improved stability compared with the parent fusion enzyme and maintains its structural integrity in vivo. In the case of the parent fusion enzyme, serious proteolysis occurred under the same conditions as shown in Figure 3. The F11 mutant was also found in the insoluble fraction (75% to 80%), and it was associated with bifunctional activity. This result therefore suggests that the aggregation tendency of the fusion enzyme can be partly attributed to the reduction of proteolysis in vivo as described elsewhere (Gilbert et al., 1998).

The mutant F11 was evaluated for its stability under operational conditions (45°C, pH 7.0) by tracing both activities as a function of time, which correlates well with the stability of the bifunctional fusion enzyme. As shown in Figure 5, the mutant F11 retained its *N*-carbamylase activity above 50% for about 18 h, whereas D-hydantoinase activity was maintained at over 85% of initial activity. On the other hand, the parent fusion enzyme (CAB-HYD) lost half of its *N*-carbamylase and D-hydantoinase activity in 5 and 7 h, respectively, under the same conditions. The slight increase in both activities at the beginning of the incubation might result from the solubilization of the fusion enzyme from the insoluble fraction. The disparity in enzyme stability is closely linked with the nascent property of *N*-carbamylase. It has been reported that *N*-carbamylase is susceptible to inactivation under oxidizing conditions and is highly sensitive to hydrogen peroxide (Grifantini et al., 1996; Kim and Kim, 1995).

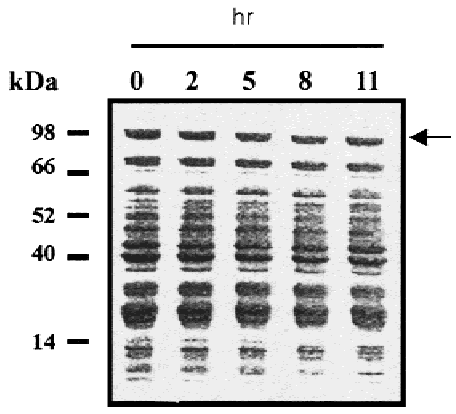

Figure 4. Stability of the evolved fusion enzyme F11 in vivo. The experimental procedure was the same as in Figure 3.

Conversion Reaction Using the Evolved Fusion Enzyme

To test the possibility of using the evolved fusion enzyme in the production of D-amino acids, we conducted a small-scale conversion. A predetermined amount of cells (50 mg) expressing the mutant enzyme was added to the reaction mixture (10 mL) containing 20 m*M* HPH or PH as substrates, and concentrations of substrate and product were determined (Fig. 6). Production of the final product (D-HPG or D-PG) continued to increase as the reaction proceeded, and almost complete conversion (>97%) was achieved within 5 h. Reaction intermediate (NCHPG or NCPG) reached a maximum level in about 1 h and then gradually decreased. For a clear comparison with the parent fusion enzyme, we expressed the parent fusion enzyme (CAB-HYD) under the same conditions (0.2 m*M* IPTG at 30°C for 2 h), and adjusted the protein level to be equivalent to that of F11, as shown in Figure 6. In this case, the reaction was severely retarded within 7 h, and the amount of final product was less than 16% of that using the evolved F11. From these results, it is reasonable to assume that the high performance level of F11 was mainly due to improved stability rather

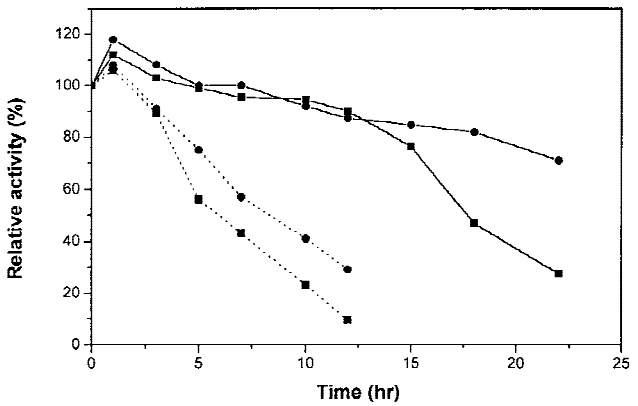

Figure 5. Stabilities of the wild-type and evolved fusion enzymes under operational conditions. (●) D-hydantoinase; (■) *N*-carbamoylase. Dotted lines indicate the wild-type fusion enzyme, and solid lines the evolved fusion enzyme F11.

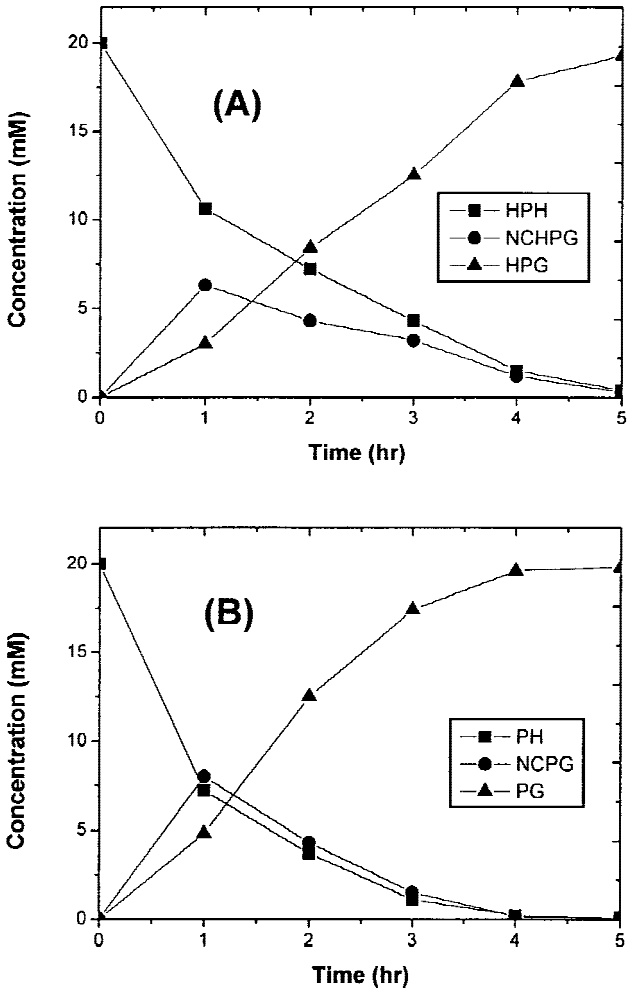

Figure 6. Conversion profiles of hydroxyphenylhydantoin (A) and phenylhydantoin (B) using the evolved fusion enzyme F11. Concentration of each substrate was 20 m*M*, and other experimental conditions were the same as described in the text. HPG and PG represent the corresponding final products, D-hydroxyphenylglycine and D-phenylglycine, respectively. NCHPG and NCPG indicate the intermediates of the sequential reaction.

than the increased catalytic activity. In fact, the mutant F11 showed an approximately 1.7-fold increase in *N*-carbamylase activity (1.9 U/mg protein), but a slight decrease (about 15%) in D-hydantoinase activity (4.4 U/mg protein), when compared with the parent fusion enzyme (CAB-HYD).

DISCUSSION

Artificial fusion of an individual enzyme broadens the application range of natural enzymes. A rational combination of individual enzymes gives a monitoring facility for cell signals, localization, expression, and ligand interaction (Nixon et al., 1998). The use of combined sequential enzymes employed in biotechnology offers better performance over separate enzymes as reported in the synthesis of oligosaccharide (Gilbert et al., 1998) and ethylene (Li et al., 1996). However, despite the guaranteed usefulness of fusion proteins, many cases have had limited success, mainly because of the functional and structural instability of artificial fusion enzymes. Concerning efficient folding and then production of fusion proteins, a recent study argued that production of hybrid proteins in *E. coli* is inherently problematic because the prokaryotic folding mechanism is post-translational, and leads to intramolecular misfolding of concurrently folding domains (Netzer and Hartl, 1997). Although similar phenomena were also observed in our study, diverse mutants displayed some interesting features, suggesting that the folding of hybrid enzyme in vivo can be considerably modulated by the folding ability of the fusion protein itself. This result provides a strong possibility of bypassing the genetic barrier that has restricted the efficient generation of fusion enzymes in the prokaryotic system. In this context, engineering of the distorted or unfolded enzyme imposed by inherent trait in vivo could pave the way to overcome the limited use of potential enzymes (Doi and Yanagawa, 1999; Joo et al., 1999; Lin et al., 1999).

To ensure the experimental observation that even minor alteration in the large fusion protein can lead to significant changes in the property of a fusion protein, we fused *N*-carbamylase to the N-terminal of the D-hydantoinase from *Bacillus thermocatenulatus* GH2 (Kim and Kim, 1998a) using a procedure identical to that of D-hydantoinase from *Bacillus stearothermophilus* SD1. The D-hydantoinase (HYD1) from *B. thermocatenulatus* GH2 was found to have a remarkable homology to that of *B. stearothermophilus* SD1. Only 17 amino acid residues do not match when the conservative substitutions were considered. The CAB-HYD1 fusion enzyme was clearly expressed as a major band even under the expression condition that the CAB-HYD was subjected to proteolysis. Activity of CAB-HYD1 was more dominant in the insoluble fraction than that of CAB-HYD, and it was well associated with the bifunctional activity. Additional different properties in the expression level and structural stability also suggested that the striking difference between the two enzymes seems to come from the different primary structure of the hydantoinase domain (unpublished results). This line of evidence, along with our practical demonstration, supports the idea that linearly fused protein with functional and structural instability can evolve into a stable fusion enzyme using DNA shuffling.

In the first trial of shuffling, the positive clones expressing the fusion enzymes with improved stability were rarely detected. In addition, the degenerated clones were analyzed to commonly contain the modification at residues H362 and H364, which were previously identified as the metal binding sites (Kim and Kim, 1998a,b). In an attempt to obtain more positive clones in the first round, a specific primer was added to conserve the metal binding sites of D-hydantoinase during the gene reassembly. In the second and third rounds, five to six positive clones were combined and, consequently, generation of positive clones was significantly increased. It seems that the mutagenic effect was dominant in the first round, and recombination was most effective in the second and third rounds. The rate of positive clone discov-

ery is known to be much higher in recombination than in mutation. After the third round, a mutant F11 having the highest structural integrity was isolated, but no striking difference in the expression level and specific activity between the wild-type and evolved enzymes was observed. Although the specific role of each residue was not assigned, it is thus likely that substituted residues in F11 facilitate or assist the appropriate folding of each domain, as suggested elsewhere (Brinkmann et al., 1992; Crameri et al., 1996). It is expected that mutational analyses of the isolated mutant would address the specific region responsible for the exerted phenotype of the evolved fusion enzyme.

By necessity, along with stabilization, improved catalytic activities of both fusion partners represent a favorable direction in the evolution of the fusion enzyme. A recent report has provided experimental evidence that generation of a rigid enzyme with high catalytic activity is feasible by directed evolution (Giver et al., 1998). We attempted to continue further rounds of shuffling, but positive clones having higher catalytic activity were not readily detected, because the toxic effect on the host growth proved to be inevitable as the degradation of dihydropyrimidine (Chien et al., 1998) by the fusion enzyme became more extensive. The catalytic efficacy of both fusion partners could be further improved when appropriate expression and selection systems are provided.

References

Ay J, Gotz F, Borriss R, Heinemann U. 1998. Structure and function of the *Bacillus* hybrid enzyme GluXyn-1: native-like jellyroll fold preserved after insertion of autonomous globular domain. Proc Natl Acad Sci USA 95:6613–6618.

Brinkmann U, Buchner J, Pastan I. 1992. Independent domain folding of *Pseudomonas* exotoxin and single-chain immunotoxins: influence of interdomain connections. Proc Natl Acad Sci USA 89:3075–3079.

Chien HR, Jih YL, Yang WY, Hsu WH. 1998. Identification of the open reading frame for the *Pseudomonas putida* D-hydantoinase gene and expression of the gene in *Escherichia coli*. Biochim Biophys Acta 1395:68–77.

Chowdhury PS, Vasmatzis G, Beers R, Lee B, Pastan I. 1998. Improved stability and yield of a Fv-toxin fusion protein by computer design and protein engineering of the Fv. J Mol Biol 281:917–928.

Clark EDB. 1998. Refolding of recombinant proteins. Curr Opin Biotechnol 9:157–163.

Crameri A, Whitehorn EA, Tate E, Stemmer WPC. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nature Biotechnol 14:315–319.

Dalboge H, Lange L. 1998. Using molecular techniques to identify new microbial biocatalysts. Trends Biotechnol 16:265–272.

Doi N, Yanagawa H. 1999. Design of generic biosensors based on green fluorescent proteins with allosteric sites by directed evolution. FEBS Lett 453:305–307.

Frank EG, Gonzalez M, Ennis DG, Levine AS, Woodgate R. 1996. In vivo stability of the Umu mutagenesis proteins: a major role for RecA. J Bacteriol 178:3550–3556.

Gilbert M, Bayer R, Cunningham AM, DeFrees S, Gao Y, Watson DC, Young NM, Wakarchuk WW. 1998. The synthesis of sialylated oligosaccharides using a CMP-Neu5Ac synthetase/sialyltransferase fusion. Nature Biotechnol 16:769–772.

Giver L, Gershenson A, Freskgard PO, Arnold FH. 1998. Directed evolution of a thermostable esterase. Proc Natl Acad Sci USA 95: 12809–12813.

Grifantini R, Galli G, Carpani G, Pratesi C, Frascotti G, Grandi G. 1998. Efficient conversion of 5-substituted hydantoins to D-alpha-amino acids using recombinant *Escherichia coli* strains. Microbiology 144: 947–954.

Grifantini R, Pratesi C, Galli G, Grandi G. 1996. Topological mapping of the cysteine residues of *N*-carbamyl-D-amino-acid amidohydrolase and their role in enzymatic activity. J Biol Chem 271:9326–9331.

Joo H, Lin Z, Arnold FH. 1999. Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. Nature 399:670–676.

Kim GJ, Kim HS. 1995. Optimization of the enzymatic-synthesis of D-*p*-hydroxyphenylglycine from DL-5-substituted hydantoin using D-hydantoinase and *N*-carbamoylase. Enzyme Microbial Technol 17: 63–67.

Kim GJ, Kim HS. 1998a. C-terminal regions of D-hydantoinases are nonessential for catalysis, but affect the oligomeric structure. Biochem Biophys Res Commun 243:96–100.

Kim GJ, Kim HS. 1998b. Identification of the structural similarity in the functionally related amidohydrolases acting on the cyclic amide ring. Biochem J 330:295–302.

Kim GJ, Park JH, Lee DC, Ro HS, Kim HS. 1997. Primary structure, sequence analysis, and expression of the thermostable D-hydantoinase from *Bacillus stearothermophilus* SD1. Mol Gen Genet 255:152–156.

Li N, Jiang XN, Cai GP, Yang SF. 1996. A novel bifunctional fusion enzyme catalyzing ethylene synthesis via 1-aminocyclopropane1-carboxylic acid. J Biol Chem 271:25738–25741.

Lin Z, Thorsen T, Arnold FH. 1999. Functional expression of horseradish peroxidase in *E. coli* by directed evolution. Biotechnol Prog 15: 467–471.

Netzer WJ, Hartl FU. 1997. Recombination of protein domains facilitated by co-translational folding in eukaryotes. Nature 388:343–349.

Nixon AE, Ostermeier M, Benkovic SJ. 1998. Hybrid enzymes: manipulating enzyme design. Trends Biotechnol 16:258–264.

Runser S, Chinski N, Ohleyer E. 1990. D-*p*-Hydroxyphenylglycine production from D,L-5-*p*-hydroxyphenylhydantoin by *Agrobacterium sp.* Appl Microbiol Biotechnol 33:382–388.

Stemmer WPC. 1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Proc Natl Acad Sci USA 91:10747–10751.

Syldatk C, May O, Altenbuchner J, Mattes R, Siemann M. 1999. Microbial hydantoinases — industrial enzymes from the origin of life? Appl Microbiol Biotechnol 51:293–309.

Yamada H, Shimizu S. 1988. Microbial and enzymatic processes for the production of biologically and chemically useful compounds. Angew Chem Int Ed Engl 27:622–642.