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Construction and Evaluation of a Novel Bifunctional *N*-Carbamylase–D-Hydantoinase Fusion Enzyme

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A fully enzymatic process employing two sequential enzymes, D-hydantoinase and *N*-carbamylase, is a typical case requiring combined enzyme activity for the production of D-amino acids. To test the possibility of generating a bifunctional fusion enzyme, we constructed a fusion protein via end-to-end fusion of a whole gene that encodes an intact protein at the N terminus of the D-hydantoinase. Firstly, maltose-binding protein (MBP) gene of *E. coli* was fused with D-hydantoinase gene from *Bacillus stearothermophilus* SD1, and the properties of the resulting fusion protein (MBP-HYD) were compared with those of native D-hydantoinase. Gel filtration and kinetic analyses clearly demonstrated that the typical characteristics of D-hydantoinase are maintained even in a fusion state. Based on this result, we constructed an artificial fusion enzyme composed of the whole length of *N*-carbamylase (304 amino acids [aa]) from *Agrobacterim radiobacter* NRRL B11291 and D-hydantoinase (471 aa). The fusion enzyme (CAB-HYD) was functionally expressed with an expected molecular mass of 86 kDa and efficiently converted exogenous hydantoin derivatives to the D-amino acids. A related D-hydantoinase (HYD1) gene from *Bacillus thermocatenulatus* GH2 was also fused with the *N*-carbamylase gene at its N terminus. The resulting enzyme (CAB-HYD1) was bifunctional as expected and showed better performance than the CAB-HYD fusion enzyme. The conversion of hydantoin derivatives to corresponding amino acids by the fusion enzymes was much higher than that by the separately expressed enzymes, and comparable to that by the coexpressed enzymes. Thus, the fusion enzyme might be useful as a potential biocatalyst for the production of nonnatural amino acids.

In the field of molecular biology and biotechnology, enzymes possessing two or more combined activities, along with appropriate stability, have found wide application (25). Although the natural diversity of the enzymes provides 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 well-defined structure (1, 25). Artificial fusion enzymes, simply generated by either end-to-end fusion or by tethering of whole genes encoding intact functional proteins with a linker, have been reported to show noticeable performance in a concerted fashion (6, 21).

Nonnatural D-amino acids are widely used in the pharmaceutical field, with applications such as antimicrobial and antiviral agents, artificial sweeteners, pesticides, and pyrethroids (26, 28, 30). Due to the great commercial demand for various D-amino acids, enzymatic and chemoenzymatic routes have been developed (29). Of these, a fully enzymatic process using two sequential enzymes, D-hydantoinase and *N*-carbamylase, is a typical case requiring combined enzyme activity (8). In this process, hydantoin derivative is hydrolyzed by D-hydantoinase, and the resulting *N*-carbamyl-D-amino acid is further converted to the corresponding D-amino acid by *N*-carbamylase. Therefore, functional fusion of two enzymes was expected to have several advantages over individual enzymes with respect to reaction kinetics and enzyme production, as well as novel properties and reactivity. As practical cases of the multistep sequential reaction, the performance of the hybrid or fusion enzymes sometimes was better than that achieved by successive action of individual enzymes, expanding the potential use of natural enzymes (6, 21, 25).

Previously, we cloned and expressed two hydantoinase genes from *Bacillus stearothermophilus* SD1 (17) and *Bacillus thermocatenulatus* GH2 (15). We also identified some conserved domains possessing essential amino acid residues by comparative analyses of functionally related enzymes (16). These results provided some evidence that supports the presence of a cyclic amidohydrolase family including hydantoinase, dihydropyrimidinase, allantoinase, and dihydroorotase. Further study of the deletion mutants derived from two D-hydantoinases suggested that the N terminus of D-hydantoinase is not essential for maintaining the enzyme structure and is dispensable for enzyme activity (15, 17). From these observations, we made a hypothesis that the hybrid enzymes might be generated via the linear fusion of a protein at the N terminus of D-hydantoinase, resulting in a bifunctional fusion enzyme.

Here we report the generation of fusion enzymes by end-to-end fusion of a whole gene that encodes an intact protein to the N terminus of the D-hydantoinase from *B. stearothermophilus* SD1 or to that of *B. thermocatenulatus* GH2. Based on the distinct fusion ability of D-hydantoinase observed in maltose-binding protein (MBP) fusion, we constructed a bifunctional fusion enzyme composed of *N*-carbamylase and D-hydantoinase. The performance of the resulting fusion enzymes was evaluated and compared with the performances of the coexpressed and separately expressed enzymes. Details are reported herein.

MATERIALS AND METHODS

Construction of fusion enzymes. Restriction enzymes, the pMAL-c2X vector, and amylose resin were obtained from New England Biolabs. Primers SDN (5′-TAGAATTCATGACAAAAATTATAAAAAATC-3′) and SDC (5′-TACT GCAGTTAAATGGTTAATTCCTCGCTC-3′) and primers GHN (5′-TAGAA TTCATGACAAAATTGATAAAAAATG-3′) and GHC (5′-TACTGCAGTTA

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GGACATTTTCACCACATCT-3′), spanning the genes encoding the D-hydantoinase from *B. stearothermophilus* SD1 (17) and *B. thermocatenulatus* GH2 (15), respectively, were used. Restriction sites *Eco*RI and *Pst*I were introduced into the N- and C-terminal primers, respectively. The amplified DNA fragments (1.4 kb) encoding the D-hydantoinases were cloned into the *Eco*RI/*Pst*I site of pMAL-c2X, and the resulting plasmids were transformed into *Escherichia coli* JM109.

For the construction of *N*-carbamylase–D-hydantoinase fusion enzymes, the *Agrobacterium N*-carbamylase gene (7) was amplified from chromosomal DNA with two primers, CAN (5′-CAAAGGTCCATGGCACGTCAGATGATA-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 plasmid pTC. The naturally occurring *N*-carbamylase gene has a unique *Eco*RI restriction site in its C terminus (7), neighboring the stop codon TAA. The amplified D-hydantoinase genes from *B. stearothermophilus* SD1 and *B. thermocatenulatus* GH2 were inserted to replace the sequence between the *Eco*RI and *Pst*I sites in plasmid pTC, yielding plasmids pTCH and pTCH1, respectively. The resulting fusion genes (2.3 kb) encoding the fusion enzymes, CAB-HYD and CAB-HYD1, were confirmed by DNA sequencing. HYD denotes the D-hydantoinase from *B. stearothermophilus* SD1, while HYD1 denotes that from *B. thermocatenulatus* GH2.

Coexpression of *N*-carbamylase and D-hydantoinase in a single host. For the comparison with the fusion enzyme, coexpression of *N*-carbamylase and D-hydantoinase in a single host was conducted under the control of Ptrc. The amplified D-hydantoinase gene used in MBP fusion was cloned into the *Eco*RI and *Pst*I sites in plasmid pTrc 99A to generate pTH. A DNA fragment containing the inserted gene with Ptrc was digested with *Nar*I and *Hind*III, blunt ended, and cloned into the *Eco*RV site of pACYC184 (New England Biolabs) to yield plasmid pYH. For the coexpression, *E. coli* JM109 was transformed by electrotransformation in 10% glycerol with two plasmids, pTC and pYH. Cells were maintained and induced in Luria-Bertani (LB) medium containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml) at 30°C.

Expression and purification of fusion enzymes. Expression of the fusion enzymes MBP-HYD and MBP-HYD1 in *E. coli* JM109 was achieved through by isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) induction at 37°C. The fusion enzymes were purified and cleaved with factor Xa according to the general procedure of the supplier (New England Biolabs). Plasmids pMAL-c2X contains a sequence coding for the recognition site (Ile-Glu-Gly-Arg) of a specific protease, factor Xa, allowing the fused protein to be cleaved from MBP. For comparison, the D-hydantoinases were further purified from the fusion enzyme by treating with factor Xa, followed by loading onto amylose resin.

The fusion enzymes CAB-HYD and CAB-HYD1 were expressed in *E. coli* JM109 under the control of Ptrc and purified using an antibody raised against the purified D-hydantoinase from *B. stearothermophilus* SD1 (19). *E. coli* cells were cultivated in 200 ml of LB broth containing ampicillin (25 μg/ml) at 30°C, and IPTG (0.2 mM) was added for induction when the optical density at 600 nm (OD_{600}) reached about 0.7 to 0.8. After 2 h of cultivation, cells were collected by centrifugation and then resuspended in 5 ml of 20 mM Tris-HCl (pH 7.8) buffer containing 0.1 mM manganese chloride, 0.1% phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100, and 1 mM dithiothreitol (DTT). Both fusion proteins were purified using the following protocol. Suspended cells were freeze-thawed twice and disrupted by sonication, and the cell lysate was centrifuged at 27,000 × *g* for 1 h. The supernatant was incubated overnight with immunoglobulin G (IgG)-immobilized Sepharose 4B (19) under a nitrogen gas atmosphere. After a wash with 20 mM Tris-HCl (pH 7.8) containing 0.25 mM NaCl, immunoabsorbed proteins were eluted from the column with 50 mM carbonate buffer containing 2 M NaCl. Active enzyme fractions were dialyzed against the buffer (20 mM Tris-HCl [pH 7.8]) and used for further analyses.

Oligomeric structure analysis. The oligomeric structures of enzymes were determined in a gel filtration column (Superose-12 HR10/30) mounted onto a fast protein liquid chromatography system (Pharmacia). The flow rate of the mobile phase containing 20 mM Tris-HCl and 150 mM NaCl was 0.3 ml/min. The column was calibrated using the native protein markers (Pharmacia), and a molecular mass standard curve was established using the semilog method based on data obtained from the elution profile of protein markers (Pharmacia).

Enzyme assay and conversion test. The activities of *N*-carbamylase and D-hydantoinase were determined at 45°C for 30 min with constant shaking after addition of whole cells or purified enzymes. In the case of D-hydantoinase activity, either hydantoin or hydroxyphenylhydantoin (HPH) was used at a final concentration of 15 mM as a substrate in 100 mM Tris-HCl (pH 8.0). For the *N*-carbamylase activity, 15 mM of *N*-carbamyl-hydroxyphenylglycine (NCHPG) in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT was used as a substrate.

The conversion experiments were performed in a total volume of 10 ml containing 15 mM or 20 mM hydantoin derivatives, 100 mM phosphate buffer (pH 7.2), and 1 mM DTT under a nitrogen atmosphere. Induced whole cells (95 mg) were harvested and added into the reaction vial. The reaction products were analyzed using high-performance liquid chromatography (HPLC) (14). The expression levels of the recombinant enzymes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by scanning of gels with a densitometer (Bio-Rad). Protein concentration was measured by using a protein assay solution (Bio-Rad). Kinetic constants of the enzyme were also determined as described previously (14).

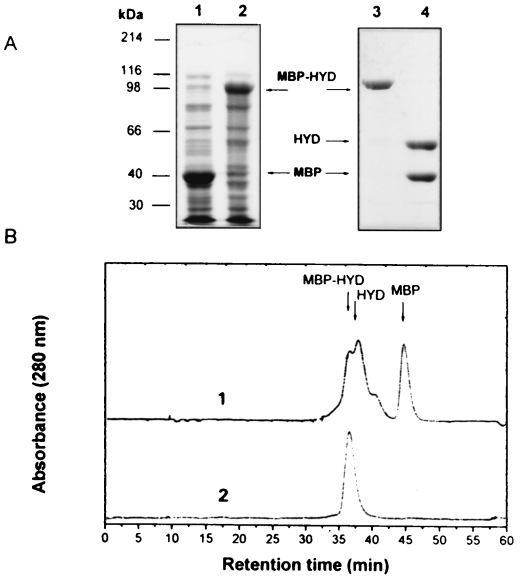

FIG. 1. Expression and gel filtration analysis of the MBP-HYD fusion protein. (A) Crude extracts of cells expressing the MBP-HYD fusion protein and the purified MBP-HYD fusion protein were analyzed on SDS–10% PAGE. Lane 1, crude extract of cells expressing control MBP; lane 2, crude extract of cells expressing MBP-HYD; lane 3, the purified MBP-HYD fusion protein; lane 4, free MBP and D-hydantoinase released from the fusion protein by factor Xa digestion. (B) A protein mixture (MBP-HYD, MBP, and HYD) and purified MBP-HYD fusion protein were analyzed on a Superose-12 gel filtration column. Curve 1, protein mixture; curve 2, purified MBP-HYD. The native size of each protein was estimated based on the elution profile of standard protein markers as follows: blue dextran, 2,000 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldoase, 158 kDa; Fab fragment, 50 kDa. All experiments were repeated three times, and the shift in elution time was negligible (<0.2 min).

RESULTS

Expression and purification of MBP-HYD fusion protein. As a preliminary experiment, a 1.4-kb *Sac*I fragment including the whole open reading frame (ORF) encoding D-hydantoinase from *B. stearothermophilus* SD1 was inserted into the *Sac*I site of pGEM-7Zf(+) to create an in-frame fusion of the D-hydantoinase gene and *lacZ*. The resulting fusion enzyme was found to be fully functional (data not shown). This fusion enzyme carries a fragment encoding an amino-terminal portion of β-galactosidase linked to the N terminus of the D-hydantoinase gene product. This result provided the possibility that fusion of a whole gene encoding intact protein to the DNA encoding N terminus of D-hydantoinase is feasible. The above presumption was first tested by linear fusion of MBP to the N terminus of D-hydantoinase from *B. stearothermophilus* SD1 as shown in Fig. 1A. Free MBP (43 kDa) was expressed in induced cells harboring the control plasmid. In contrast, the MBP-HYD fusion protein of the expected molecular mass (95 kDa) was correctly expressed. The MBP-HYD fusion protein was purified from the cell extract and treated with a specific protease factor, Xa, resulting in free MBP (43 kDa) and D-hydantoinase (52 kDa) (Fig. 1A).

Characterization of the MBP-HYD fusion protein. The oligomeric state of purified MBP-HYD was investigated by using Superose-12 gel filtration column chromatography. As shown in curve 2 of Fig. 1B, the fusion protein eluted as a symmetrical peak corresponding to a tetrameric structure (330 to 360 kDa),

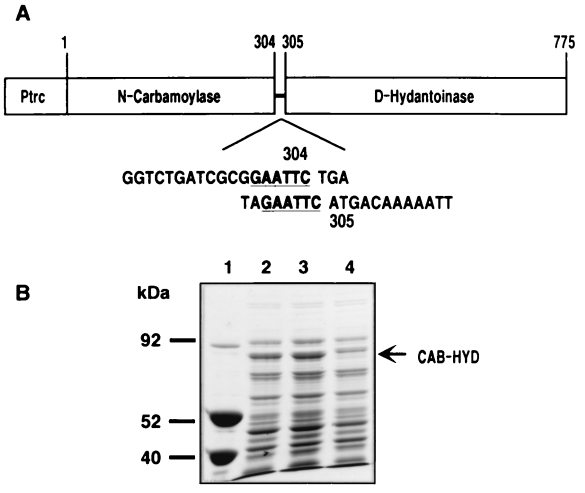

FIG. 2. Construction and functional expression of the CAB-HYD fusion. (A) The organization of the fusion gene encoding the *N*-carbamylase and D-hydantoinase. Whole amino acid residues of both open reading frames were conserved, and the total length of the fusion protein is therefore 775 residues. The nucleotide sequence of the junction region is shown in bold. (B) Crude extract of cells expressing the CAB-HYD fusion enzyme was analyzed on SDS–10% PAGE. Lane 1, control protein marker obtained from the MBP-HYD fusion protein (MBP-HYD, 95 kDa; HYD, 52 kDa; MBP, 43 kDa); lanes 2 and 3, crude extract after induction for 1 and 2 h, respectively; lane 4, crude extract of cells harboring pTrc 99A without the fusion gene.

indicating the acquisition of a distinct oligomeric structure. Free MBP and D-hydantoinase were eluted at the positions corresponding to monomeric (42 to 45 kDa) and tetrameric (180 to 210 kDa) structures, respectively (curve 1 of Fig. 1B). No improper bands resulting from high-molecular-weight aggregates or other oligomeric structures were detected. When the fusion protein was treated with the zero-order length cross-linker EDC (2), a distinct protein band appeared at the tetrameric position on SDS-PAGE (data not shown). These results imply that the oligomeric structure and/or folding of the D-hydantoinase domain is not severely affected even though its N terminus is fused with another protein.

The activity of the MBP-HYD fusion protein toward hydantoin was about 87 μmol/min/mg, and this is quite similar to that of native D-hydantoinase (98 μmol/min/mg). The specific activity of fusion protein (13.2 μmol/min/mg) toward HPH was also similar to that of the native D-hydantoinase (11.8 μmol/min/mg) at 55°C. Free MBP showed no activity for the substrates tested. To examine the effect of fused MBP on the affinity to HPH, the apparent K_m was determined at pH 8.0 for both MBP-HYD and D-hydantoinase, and similar values of 15.3 ± 1.4 and 12.6 ± 1.2 mM were obtained in the presence and absence of fused MBP, respectively. These results indicated that the D-hydantoinase was functionally fused with MBP at its N terminus.

Construction and identification of the bifunctional CAB-HYD fusion enzyme. To make a more valuable bifunctional fusion enzyme, we constructed an artificial fusion enzyme composed of *N*-carbamylase and D-hydantoinase (Fig. 2A). *E. coli* JM109, harboring the recombinant plasmid pTCH, was induced with 0.2 mM IPTG at 30°C, and the crude extract of the cells was analyzed by SDS-PAGE (Fig. 2B). A protein band corresponding to the CAB-HYD fusion enzyme was observed between 85 and 88 kDa at different induction times (Fig. 2B, lanes 2 and 3). This is consistent with the expected size of the fusion enzyme (86 kDa) calculated from the fusion gene.

It has been known that artificial multidomain polypeptides occasionally show a high aggregation tendency when expressed in *E. coli* possessing a posttranslational folding system (6). The CAB-HYD fusion enzyme constructed in this work was one such case. When the expressed enzyme in *E. coli* was fractionated to analyze the enzyme localization, considerable portions were also found in the membrane fraction and cell debris. To estimate the portion of the fusion enzymes expressed as an insoluble or a soluble form, the cell lysate was solubilized in a buffer containing 1 mM EDTA and 1% Triton X-100. As a result, the dominant portion (60 to 65%) of the fusion enzyme was found to be expressed as a soluble form, showing both *N*-carbamylase and D-hydantoinase activities. The expression level of the CAB-HYD fusion enzyme in *E. coli* was estimated to be about 6 to 8%.

The antibody raised against free D-hydantoinase was observed to recognize the CAB-HYD fusion enzyme and was used for the purification of the fusion enzyme. As shown in lane 2 of Fig. 3A, a distinct protein eluted from the immunoaffinity column appeared at position 86 kDa. This enzyme fraction catalyzed the conversion of HPH to D-hydroxyphenylglycine (D-HPG) via NCHPG (Fig. 3B), which clearly indicates the bifunctional activity of the fusion enzyme. Aside from the major band of the intact fusion enzyme, minor polypeptides of smaller sizes were also observed. These polypeptides were removed by using an ion-exchange column, and the bifunctional activity of the fusion enzyme was not changed. The activities of the CAB-HYD fusion enzyme toward HPH and NCHPG were estimated to be 5.1 and 1.3 μmol/min/mg, respectively, which corresponded to about 89 and 37% of the native enzymes under defined conditions. From the gel filtration column chromatography, a major peak corresponding to high molecular aggregates was observed, which implies that independent folding was not maintained in this fusion enzyme. More detailed analyses, such as in vitro refolding and ultracentrifugation, would address the exact nature of this fusion protein.

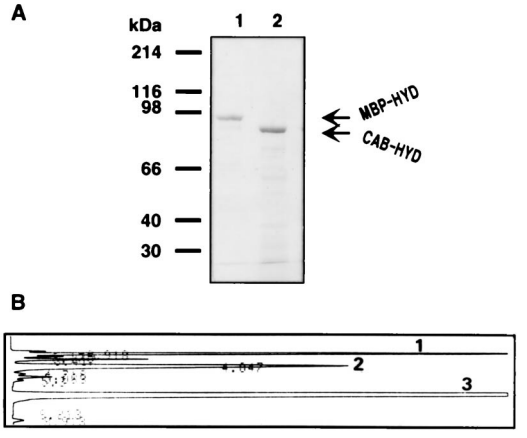

FIG. 3. Affinity purification and bifunctional activity of the CAB-HYD fusion enzyme. (A) The CAB-HYD fusion enzyme was purified using an immunoaffinity column. After elution from the immunoaffinity column, the enzyme was analyzed on SDS–12% PAGE. Lane 1, control protein obtained from the MBP-HYD fusion protein (95 kDa); lane 2, the purified CAB-HYD fusion enzyme. (B) HPLC chromatogram of the reaction products obtained by the action of the purified CAB-HYD fusion enzyme. Peak 1, D-hydroxyphenylglycine; peak 2, *N*-carbamyl-D-hydroxyphenylglycine; peak 3, the starting substrate, HPH. An octyldecylsilane column (CLC-ODS) was used with 10% acetonitrile (pH 3.0) as the mobile phase. The flow rate of the eluent was 1 ml/min. All compounds shown in the chromatogram were detected at 214 nm and identified with authentic samples.

TABLE 1. Conversion of hydantoin derivatives using fusion enzymes[a]

Starting substrate	CAB-HYD result		CAB-HYD1 result	
	Residual substrate[b]	Final product[c]	Residual substrate[b]	Final product[c]
HPH	22	65	<1	97
Phenylhydantoin	<1	>99	<1	>99
Isopropylhydantoin	43	51	38	56
Hydantoin	<1	<2.5	<1	11
Dihydrouracil	<1	13	<1	13

[a] The reaction was performed in 10 ml of potassium phosphate buffer (100 mM; pH 7.2) containing 15 mM hydantoin derivatives, 0.1 mM Mn^{2+}, 0.5 mM DTT, and 0.04% Triton X-100. The reaction was carried out at 45°C for 5 h after the addition of whole-cell enzyme (95 mg). Values were means for two experiments with independently prepared batches.

[b] As percentage of starting substrate.

[c] As percentage of starting substrate, calculated in millimolar concentrations. Hydroxyphenylglycine, phenylglycine, valine, glycine, and alanine are produced from their corresponding hydantoin derivatives, HPH phenylhydantoin, isopropylhydantoin, hydantoin, and dihydrouracil.

Construction of a bifunctional fusion enzyme using D-hydantoinase of *B. thermocatenulatus* GH2. The D-hydantoinase (HYD1) from *B. thermocatenulatus* GH2 studied previously was found to have a remarkable homology to HYD (15). Only 8% of amino acid residues were mismatched vis-à-vis each other, which led us to assume that this enzyme can also be used as a fusion partner for the construction of a bifunctional fusion enzyme. Moreover, the resulting fusion enzyme was expected to exhibit a different property from that of the CAB-HYD fusion enzyme.

MBP-HYD1 was first constructed and purified for analysis using the same procedure as for MBP-HYD. The fusion enzyme was functionally active, exhibiting characteristics similar to those of the native HYD1. Favorable activity toward HPH (34.7 U/mg) was obtained with fusion enzyme MBP-HYD1, which corresponds to about 85% of the activity of native HYD1. MBP-HYD1 retained most of the native property of HYD1 in terms of its oligomeric structure (a tetramer) and apparent K_m for HPH (27.2 ± 1.8 and 25.6 ± 1.2 mM were obtained in the presence and absence of fused MBP, respectively).

When HYD1 was fused with *N*-carbamylase, the bifunctional activity of the fusion enzyme (CAB-HYD1) was also clearly detected when induced with 0.2 mM IPTG at 30°C for 2 h. The activities of the CAB-HYD1 fusion enzyme toward HPH and NCHPG were estimated to be 7.1 and 2.9 μmol/min/mg, respectively, which are 1.4 and 2.2-fold higher than those of CAB-HYD. As a striking difference between CAB-HYD and CAB-HYD1, expression of CAB-HYD1 was dominant in the insoluble fraction (data not shown).

Both CAB-HYD and CAB-HYD1 fusion enzymes also required a reducing agent such as DTT for the *N*-carbamylase activity (7, 8). The fusion enzymes lost the *N*-carbamylase activity more readily than the D-hydantoinase activity. CAB-HYD retained its *N*-carbamylase activity above 50% for about 6 to 7 h at 45°C, while D-hydantoinase activity was maintained at more than 75% of the initial activity. The *N*-carbamylase and D-hydantoinase activities of the CAB-HYD1 fusion enzyme were retained about 78 and 95% of the initial activities, respectively, even after 6 h under the identical conditions. The disparity in enzyme stability seems to be linked with the unstable nature of *N*-carbamylase and independent folding between two domains. It has been reported that *N*-carbamylase is susceptible to inactivation under oxidizing conditions and is highly sensitive to hydrogen peroxide (7, 14).

Conversion of hydantoin derivatives using the fusion enzymes. The ability of the fusion enzyme to convert the hydantoin derivatives to final amino acids was tested in small-scale reactions at pH 7.2. To compare the catalytic activities of the CAB-HYD and CAB-HYD1 fusion enzymes, we expressed both fusion enzymes under defined conditions (0.2 mM IPTG at 30°C for 2 h) and adjusted the protein level to be equivalent to that of CAB-HYD observed in lane 3 of Fig. 2B. The conversion of the various cyclic ureides, including hydantoin derivatives, by both fusion enzymes is shown in Table 1. Hydantoin derivatives with hydrophobic side chains, such as HPH and phenylhydantoin, were completely converted by both fusion enzymes, while an exception was observed in isopropylhydantoin due to the low affinity of D-hydantoinase (19). For cyclic ureides such as hydantoin and dihydrouracil, almost complete conversion to their corresponding *N*-carbamyl-amino acids was observed, but the level of final product was very low. *N*-Carbamylase was reported to have a low activity toward achiral carbamyl substrates (31, 32), and this seems to be the reason for the low conversion yield.

Comparison of the fusion enzyme with the coexpressed and separately expressed enzymes. To further evaluate the performance, the level of final product produced by the fusion enzymes was compared with those produced by the coexpressed and separately expressed enzymes. For the separate expression of two enzymes, *E. coli* strains expressing either D-hydantoinase or *N*-carbamylase were prepared by inserting the corresponding gene into plasmid pTrc 99A. In the case of coexpression, two plasmids, pTC and pYH, encoding the *N*-carbamylase and D-hydantoinase genes, respectively, were transformed into a single host as described in Materials and Methods. For clear comparison, all sets of enzymes were expressed under the control of the identical promoter (Ptrc) and induced with 0.5 mM IPTG at 30°C for 2 h, and the expression levels and activities of each enzyme in different expression systems were examined. As shown in Fig. 4, no significant difference in the expression levels was found for *N*-carbamylase, but the expression level of D-hydantoinase in the coexpression system was about twofold lower than that in the separately expressed enzyme. The CAB-HYD fusion enzyme showed an expression level similar to that of the separately expressed D-hydantoinase

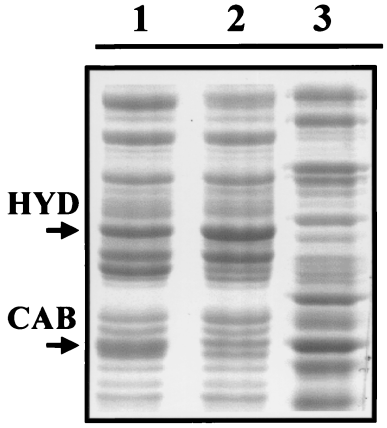

FIG. 4. SDS-PAGE analyses of the coexpressed and separately expressed enzymes. Lane 1, crude extract of *E. coli* cells coexpressing D-hydantoinase and *N*-carbamylase; lane 2, crude extract of *E. coli* cells expressing D-hydantoinase; lane 3, crude extract of *E. coli* cells expressing *N*-carbamylase. Total proteins expressed in each *E. coli* strain were analyzed on SDS–10% PAGE.

TABLE 2. Specific activities of *N*-carbamylase and D-hydantoinase in *E. coli* strains[a]

Plasmids	Sp act (10^{-2} U/mg DCW) of:	
	N-Carbamylase	D-Hydantoinase
pTCH1	0.48	1.18
pTCH	0.46	0.90
pTC/pYH	3.34	0.66
pTH		1.56
pTC	3.62	

[a] The activities were determined in 1 ml of 100 mM potassium phosphate buffer (pH 7.2) containing 20 mM HPH or NCHPG, 0.1 mM Mn^{2+}, 0.5 mM DTT, and 0.04% Triton X-100. pTCH1 denotes the plasmid expressing the CAB-HYD1 fusion enzyme. The reaction was carried out at 45°C with constant shaking (150 rpm).

(see also Fig. 2), but a twofold-lower level than that of separately expressed *N*-carbamylase. These differences in the expression level were well correlated with the differences in the enzyme activities observed in each system, as shown in Table 2.

Since the expression level was dependent on the expression system as mentioned above, direct and precise comparisons of the production rates between the fused and the coexpressed enzymes were difficult. Thus, we carried out conversion experiments under the condition that the total activity of either *N*-carbamylase or D-hydantoinase in the reaction mixture was adjusted to be equivalent. As can be seen in Table 3, when the total activity of *N*-carbamylase was fixed at 0.2 U, the performance by the fusion enzymes (CAB-HYD and CAB-HYD1) was better than that by the coexpressed enzymes. In this case, the low level of D-hydantoinase activity in the coexpressed system seems to result in a low conversion yield. As the D-hydantoinase activity in the coexpressed system was increased to an equivalent level (0.45 U) in the fusion enzyme, the performance by the coexpressed enzyme was comparable to or better than that by the fusion enzymes, probably due to the high level of *N*-carbamylase, leading to low accumulation of intermediates. Obviously, the conversion yield by the fusion enzymes was much higher than that by the separately expressed enzymes.

DISCUSSION

The experimental results shown in this paper clearly demonstrate that D-hydantoinase participates in the generation of a bifunctional fusion enzyme. Fusion of MBP to the N termini of D-hydantoinases did not abolish the activity of the fusion enzyme, and the resulting fusion enzyme was functionally expressed in *E. coli*. As reported so far, many microbial hydantoinases, including eucaryotic counterpart dihydropyrimidinases, have been isolated and purified for characterization (3, 13, 19, 24, 27). In these cases, five or six successive steps were required for purification to homogeneity. We also purified microbial D-hydantoinase using the conventional procedures and observed that the oligomeric structure might be altered during purification. For example, D-hydantoinase from *B. stearothermophilus* SD1 was determined to be a dimer in our previous work (20), but this enzyme was found to be a tetramer when purified from the MBP-HYD fusion protein. This result implies that MBP fusion to the microbial D-hydantoinases facilitates the purification of hydantoinases, expecting that their native structures remain intact. To confirm this possibility, we constructed another fusion enzyme using the D-hydantoinase from *B. thermocatenulatus* GH2 (15) and found that the D-hydantoinase also retains its native properties in a fusion state. Enzymes belonging to a cyclic amidohydrolase family have similar ORF sizes, and the apparent organization of primary and secondary structures is also very similar (4, 9, 11, 16, 18, 22). In this sense, the same strategy can be applied to other related enzymes to construct a fusion protein.

Unnatural D-amino acids are widely used in the synthesis of semisynthetic antibiotics, peptide hormones, and pesticides. These amino acids currently are produced via two sequential reactions mediated by D-hydantoinase and *N*-carbamylase (29, 30). For process development, each enzyme has been separately expressed in different host cells (4, 12, 17, 18) or in a single host (8). In this process, the different expression levels of the two each enzymes, the formation of inclusion bodies, and the transport of restricted substrates through the cell membrane remained to be solved. In an effort to produce D-amino acids in a concerted fashion, we constructed the bifunctional fusion enzyme composed of *N*-carbamylase and D-hydantoinase by an end-to-end fusion method. The resulting fusion enzymes expressed in a single host showed better performance than separately expressed enzymes. It seems that the intermediate produced by the first enzyme is readily available to the second-step enzyme, leading to a faster reaction rate. From the comparison with the coexpressed enzyme, the performance by the fusion enzyme was observed to be comparable, even though precise comparison needs further kinetic analysis. Thus, both fusion enzymes (CAB-HYD and CAB-HYD1) may be used as biocatalysts for the production of D-hydroxyphenylglycine and D-phenylglycine, D-amino acids which are in great demand. However, the low stability of the fused *N*-carbamylase seems to limit the use of the fusion enzyme in a sequential reaction. As an alternative strategy, use of a different carbamylase as a fusion partner or directed evolution of the enzyme using DNA shuffling is expected to bypass this barrier (25). Interestingly, the fusion enzymes (CAB-HYD and CAB-HYD1) also catalyzed the conversion of dihydrouracil to β-alanine, due to a broad substrate range of both enzymes (19, 23, 31, 32). It is expected that the fusion enzyme can be applicable for monitoring the blood level of dihydropyrimidines, an indicative metabolite accumulated in the dihydropyriminuria that is caused by a deficiency of dihydropyrimidinase (5, 10).

We have demonstrated the novel ability of microbial D-hydantoinase to generate a bifunctional fusion enzyme. Moreover, the functional expression of the bifunctional fusion protein was readily detected on an activity-staining plate (17). The results described here raise possibilities that D-hydantoinase may be effectively used as a novel fusion partner to construct a fusion protein that is useful for the synthesis of nonnatural D-amino acids.

TABLE 3. Conversion of HPH by the separately expressed, coexpressed, and fusion enzymes

Enzyme	Total activity (U)[a]		Concn (mM)[b]	
	D-Hydantoinase	*N*-Carbamylase	D-HPG	NCHPG
CAB-HYD1	0.49	0.2	15.4	3.9
CAB-HYD	0.39	0.2	8.2	6.4
Coexpressed	0.04	0.2	0.2	0.6
Coexpressed	0.45	2.3	16.9	0.7
Separately expressed	0.39	0.2	3.5	11.9
Separately expressed	0.49	0.2	3.6	12.5

[a] Activity of each enzyme in the reaction mixture.

[b] The reaction mixture contained 20 mM HPH, 0.1 mM Mn^{2+}, 0.5 mM DTT, and 0.04% Triton X-100 in 10 ml of 100 mM potassium phosphate buffer (pH 7.2). The reaction was performed at 45°C with constant shaking (150 rpm), and product concentrations were determined after 5 h.

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