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Directed Evolution and Characterization of a Novel D-Pantonohydrolase from *Fusarium moniliforme*

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D-Pantonohydrolase has attracted increasing attention as a biocatalyst for stereospecific production of D-pantoic acid. The *Fusarium moniliforme* D-pantonohydrolase was selected for directed evolution through error-prone Polymerase Chain Reaction (PCR) combined with DNA shuffling for improved activity and pH stability using a convenient two-step high-throughput screening method based on the product formation and pH indicator. After three sequential error-prone PCRs and two rounds of DNA shuffling followed by screening, about 60 positive mutants were produced and a best mutant, Mut H-1287, with improved activity and pH stability was obtained. As compared to wild-type D-pantonohydrolase, Mut H-1287 showed a 10.5-fold higher specific activity; moreover, it could retain 85% of its original activity after incubation under low pH. Gene analysis indicated that the Mut H-1287 had D63H, K118Q, and V241I substitutions. The wild-type and evolved D-pantonohydrolase (Mut H-1287) was purified in three steps. The activities and characteristics of purified wild-type and evolved D-pantonohydrolase were also studied and compared.

KEYWORDS: D-Pantonohydrolase; *Fusarium moniliforme*; D-pantolactone; error-prone PCR; DNA shuffling; characterization

INTRODUCTION

D-Pantoic acid and D-pantolactone are known as important intermediates for the production of calcium D-pantothenate used as a food additive and for various pharmaceutical products (*1*). Conventional production of pantolactone employs chemical methods, which yield an optically inactive racemic mixture, and the mixture must be resolved to obtain the D-(−)-isomers. For the food industry, the natural grade of D-pantolactone of biological resolution makes it more attractive than that of chemical origin. The employment of D-pantonohydrolase is an alternative process for D-pantoic acid and D-pantolactone synthesis (*2*). Strains or enzymes with D-pantonohydrolase activity can catalyze the stereospecific hydrolysis of chemically made DL-pantolactone to generate pantoic acid as a chiral building block further converted to D-pantolactone (**Figure 1**). An enzymatic stereospecific hydrolysis process to produce D-pantoic acid and D-pantolactone is not only economical and friendly to the environment but also simple to operate (*1*, *3*, *4*).

It was reported that several filamentous fungi, including *Fusarium*, *Gibberella*, *Gliocladium*, *Aspergillus*, *Cylindrocarpon*, and *Volutella*, could produce D-pantonohydrolase (*3*). D-Pantonohydrolases from *Fusarium oxysporum* and *Fusarium moniliforme* have been applied in industries (*3*−*6*). The genes

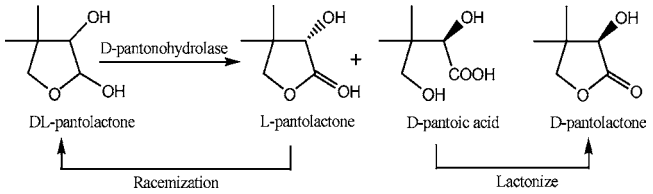

Figure 1. Procedure of ezymatic stereospecific hydrolysis to produce D-pantoic acid and pantolactone.

(GenBank accession no. AB010465 and AY728018) encoding D-pantonohydrolases from *F. oxysporum* AKU 3702 and *F. moniliforme* CGMCC 0536 were also successfully cloned and expressed (*7*, *8*). D-Pantonohydrolase showed an excellent stereoselective activity to D-pantolactone and was called a "supercatalyst", but the activity greatly decreased under low-pH conditions (*9*). The properties of D-pantonohydrolase, especially activity and pH stability, should be improved for practical scale reaction. In this study we discussed a method used for the production of D-pantonohydrolase with higher activity and pH stability.

In recent years, there has been increased realization of the power of biocatalysts for the industrial synthesis of bulk chemicals, pharmaceuticals, agrochemicals, and food ingredients, especially when high activity, stability, and stereoselectivity are required (*10*−*14*). Unfortunately, naturally available enzymes are seldom optimally suited for industrial applications (*15*). Many efforts had been made to modify the properties of

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enzyme. Compared with rational design of proteins, directed molecular evolution of proteins does not require any information about enzyme structure−function relationships (*16*, *17*) and commonly relies on random mutagenesis and recombination followed by screening or selection schemes. The directed evolution of enzymes has become a powerful and effective method for designing enzymes and emerged as an alternative approach to rational design (*18*, *19*). A number of non-recombinative and recombinative mutagenesis methods developed with appropriate screening or selection procedures (*20*−*26*) successfully improved enzyme properties including activity, stability, substrate specificity, and enantioselectivity in artificial environments (*16*, *27*). Because atomic resolution three-dimensional information of D-pantonohydrolase is unavailable, the directed evolution method was adopted to improve the properties of D-pantonohydrolase in this study.

In the present work, the expression of the gene encoding the D-pantonohydrolase from *F. moniliforme* CGMCC 0536 in the periplasmic space of *Escherichia coli* and directed evolution of D-pantonohydrolase were described. The characteristics of the purified wild-type D-pantonohydrolase and evolved enzyme were also studied and compared.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Materials. *E. coli* BLR(DE3)-pLysS [F^- *ompT hsdSB* (r_B^- m_B^-) *gal dcm*.(*srl-recA*)*306*::Tn*10* (DE3) pLysS] containing a compatible chloramphenicol-resistant plasmid that provided a small amount of T7 lysozyme cutting a specific bond in the peptidoglycan layer of the *E. coli* cell wall (*28*) was purchased from Novagen and used as the host for plasmids pET20b-LAC and pET20b-mut-LAC. *E. coli* transformants were grown in Luria−Bertani (LB) medium. pET20b (Novagen), with an N-terminal *pelB* signal sequence and the protein expressed by a foreign gene could be transported into the periplasmic space, was used as the expression vector. The plasmid of pMD18-T-LAC containing the D-pantonohydrolase gene was constructed and stored in our laboratory (*8*). Pyrobest DNA polymerase, restriction enzymes including *Eco*RI and *Sal*I, T4 DNA ligase, and PCR reagents were purchased from TaKaRa Biotechnology (Dalian, China) Co., Ltd. Primers were purchased from Sangon (Shanghai, China). DL-Pantolactone was provided by XinFu Biochemistry Co., Ltd. (Zhejiang, China). All other chemicals were obtained from readily available commercial sources and of analytical grade.

DNA Manipulation and Transformation. DNA manipulation, plasmid isolation, and agarose gel electrophoresis were operated according to the methods of Sambrook et al. (*29*). Transformation of *E. coli* was conducted according to the method of Chung et al. (*30*).

Expression of D-Pantonohydrolase in the Periplasmic Space of *E. coli*. The wild-type gene of D-pantonohydrolase was obtained using proof-reading *Pyrobest* DNA polymerase (Takara, Japan) in the presence of the following primers: P1, 5′-CCGGAATTCGCTAAGCTTCCTTCTACGGC-3′ (underlined part is the *Eco*RI site); and P2, 5′-CCGGTCGACCTAATCATAGAGCTTGGGAC-3′ (underlined part is the *Sal*I site), which were designed according to the sequence resulting from pMD18-T-LAC. After recovery by agarose gel electrophoresis, the PCR products and the vector pET20b were digested with *Eco*RI and *Sal*I and then ligated by T4 DNA ligase. The ligated plasmid pET20b-LAC was transformed into *E. coli* BLR(DE3)pLysS. A single colony of the transformant was selected and transferred into 3 mL of LB broth with chloramphenicol (34 μg/mL), tetracycline (12.5 μg/mL), and ampicillin (100 μg/mL) and then incubated overnight with vigorous shaking (200 rpm) at 30 °C. One part of the culture was used to extract recombined plasmid by alkaline lysis procedure (*29*). The plasmid was digested with *Eco*RI and *Sal*I to identify whether the recombined plasmid had been constructed or not. If the identified single colony was a positive clone, the other culture was transferred to a 500-mL Erlenmeyer flask containing 100 mL of the same fresh medium and further incubated at 30 °C with agitation (200 rpm) until the OD_{600} reached 0.8 and then induced by IPTG (final concentration = 1 mM). The culture was induced and incubated for ≈6 h under the same conditions and then centrifuged (6000*g*, 10 min at 4 °C), and the cell pellets were collected and washed twice using Tris-HCl buffer (pH 7.5) for assaying enzyme activity according to the standard method after purification.

Error-Prone PCR and DNA Shuffling. Random mutations were introduced to gene-encoding pantonohydrolase by error-prone PCR and DNA shuffling. The recombinant plasmid pMD18-T-LAC was used as template DNA in the error-prone PCR. Primers P1 and P2 were used to amplify the full-length gene-encoding D-pantonohydrolase. Error-prone PCR was carried out in a total volume of 100 μL with the following reagents: 20 mM Tris-HCl, pH 9.0, 50 mM KCl, 6.0 mM $MgCl_2$, 0.15 mM $MnCl_2$, 20 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 50 pmol of each primer, 5 ng of template DNA, and 5 units of *Taq* DNA polymerase for 30 cycles of 95 °C for 30 s, 58 °C for 60 s, and 72 °C for 60 s by using a MJ Research thermal cycler (PTC-200). PCR products were visualized on a 1.5% ethidium bromide stained gel. At the same time, the D-pantonohydrolase gene was amplified using Pyrobest DNA polymerase as a control. The products of PCR were purified through agarose gel electrophoresis to remove the original template and avoid the influence of wild-type gene on the construction of the mutant libraries. Moreover, to produce a small mutant library, the mismatch rate was controlled by using the appropriate amount of Mg^{2+} and Mn^{2+}. The genes carrying positive mutation in the procedure of error-prone PCR were selected as the source of DNA for DNA shuffling. The detailed DNA shuffling method was that of Stemmer (*17*). About 5 μg of DNA substrate was digested with 0.025 unit of DNaseI in 50 μL of 0.5 M Tris-HCl, pH 8.0, and 0.1 M $MnCl_2$ for 15 min at 15 °C. After digestion, the products of ≈100 bp were purified by a 2% agarose gel. These fragments were then reassembled using a primerless PCR reaction. The products of self-reassembling PCR was diluted 40 times and used as the templates of high-fidelity PCR. Three-sequencial error-prone PCRs and two-round DNA shufflings were carried out in this experiment. The final mutation rates after error-prone PCR and DNA shuffling were determined by sequencing.

Screening Method. A two-step screening method was designed as follows: a modified plate method was developed for rapid screening. An aliquot (50 or 100 μL) of the culture containing the constructed recombinants was spread onto LB agar plates supplemented with chloramphenicol (34 μg/mL), tetracycline (12.5 μg/mL), ampicillin (100 μg/mL), and bromocresol purple (0.01% w/v). In addition, plates containing both 0.5% (w/v) of D-pantolactone and 0.5 mM IPTG were incubated for 48 h at 30 °C. The pH of the plate medium was 7.0 after sterilization. This modified procedure was based on the product formation of yellow halos as a result of hydrolysis by positive colonies. The pH became lower and lower at the microregions around the positive colonies with the hydrolysis process of the D-pantolactone. About 100 colonies were screened on each plate. After incubation, the yellow halos produced by potential improved variants were identified, and positive clones were selected by the formation of yellow halos and their sizes. Colonies giving rise to larger halos were picked from the corresponding plates. The crude colonies were cultured in 50 mL of LB medium at 30 °C for 12 h and were induced at 30 °C for 8 h and then centrifuged at 5000*g* for 10 min. The cell pellets were collected and washed twice with Tris-HCl buffer, pH 7.5, to assay enzyme activity and pH stability. About 0.5 g of wet cells was harvested from the cultures. The washed cells were suspended in 50 mM Tris-HCl buffer, pH 7.5, and ruptured with sonication for 20 min with ice cooling. The supernatant resulting from centrifugation at 8000*g* for 30 min was used as crude enzyme, and the enzyme activity was determined according to the standard enzyme assay method. At the same time, the same amount of enzyme was incubated under low pH for 30 min, and then the remaining activity was checked according to the standard enzyme assay method. The strains that have both higher D-pantonohydrolase activity and stability under low-pH conditions were selected to determine specific activity. Dozens of strains with the highest activity and stability under low pH were selected as the parents for the next round of directed evolution. After three sequential error-prone PCRs and two rounds of DNA shufflings, the mutants were analyzed in detail.

Wild-Type D-Pantonohydrolase and Evolved Enzyme Purification. D-Pantonohydrolase activity and protein content were determined

in samples from each purification step. Unless otherwise specified all steps were conducted at 4 °C. The purification process employed a Biologic Duo Flow system (Bio-Rad). The washed cells were suspended in 50 mM Tris-HCl buffer, pH 8.0, and ruptured with sonication for 20 min with ice cooling. The supernatant resulting from centrifugation at 48000*g* for 30 min was used as the crude enzyme, and its activity was taken as 100% for the calculation of recovery. The crude enzyme was applied to an anion ion exchange column (High Q IEX, 20 mL, Bio-Rad), equilibrated with 50 mM Tris-HCl buffer, pH 8.0. D-Pantonohydrolase bound to the column was eluted in 50 mL of a NaCl concentration linear gradient (0−0.5 M) at a flow rate of 1 mL/min. Solid ammonium sulfate was added, over ice, into the crude extract to 30% saturation. After centrifugation (10000*g*, 20 min), ammonium sulfate was added to bring the supernatant to 100% saturation. The latter was stored overnight and then centrifuged. The precipitate was redissolved and dialyzed against several changes of 0.05 M Tris-HCl buffer, pH 8.5. Active D-pantonohydrolase fractions were applied to a hydrophobic interaction chromatography column (Methyl HIC, 5 mL, Bio-Rad), equilibrated with 50 mM Tris-HCl buffer [1.5 M $(NH_4)_2SO_4$ in the buffer, pH 8.5]. The enzyme was eluted with a linear gradient of 1.5−0.6 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl buffer, pH 8.0, at a flow rate of 1 mL/min. The active fractions were pooled and dialyzed against 10 mM NaAc buffer (10% glycerol in the buffer, pH 5.4) for 18 h. Then, the enzyme was applied to a cation ion exchange column (High S, IEX, 5 mL, Bio-Rad), equilibrated with 10 mM NaAc buffer (10% glycerol in the buffer, pH 5.4), and eluted with a 50-mL linear NaCl gradient from 0.1 to 0.5 M at a flow rate of 1 mL/min. The active fractions were pooled and used as a purified enzyme preparation. The separated and purified active fractions were concentrated and dialyzed against water, then freeze-dried and stored at −20 °C for further studies.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). The molecular mass under denaturing conditions was determined by SDS-PAGE performed using a Mini-gel system (Bio-Rad). The gels were cast with 0.75 mm spacers (Bio-Rad). The changes in the oligomeric structure and folding state of enzymes were determined on a FPLC system with a Superdex-75 gel filtration column. The flow rate and mobile phase used were 0.5 mL/min and 20 mM Tris-HCl (pH 8.0, 150 mM NaCl), respectively. A molecular mass standard curve was established by plotting the elution profiles of protein markers (Sigma) versus the known molecular masses on a semilog paper. Aliquots of each eluted fraction were subjected to SDS-PAGE and enzyme assay. SDS-PAGE was performed according to Laemmli's discontinuous Tris−glycine buffer system at 10 mA for 2−3 h with a 5% acrylamide stacking gel, pH 6.8, and 12% separating gel, pH 8.8 (*31*). Proteins in the gel were stained with Coomassie brilliant blue R-250 and destained with 0.5 M NaCl aqueous solution.

Enzyme Assay and Protein Assay. The purified enzymes were added to 4 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 154 mM D-pantolactone. After reaction at 30 °C for 60 min, the same volumes of 2 mM EDTA solution in methanol were added to stop the reaction. The supernatant obtained by centrifugation was subjected to HPLC analysis as described below for assaying the D-pantonohydrolase activity. One unit of D-pantonohydrolase activity was defined as the amount of enzymes required to produce 1.0 mM of pantoic acid at pH 7.5 and 30 °C for 60 min (*9*). Protein quantitative analysis was determined according to the Bradford method (*32*) with bovine serum albumin as a standard.

Analytical Methods. Pantolactone and pantoic acid were quantified on a reverse phase column (Zorbax SB C^{18}, HP, America, 250 × 4.6 mm^2, 5 μm) at 215 nm at 25 °C with an eluent of 10% (v/v) acetonotrile containing 18 mM KH_2PO_4 (adjusted to pH 3 with 1 M HCl) at a flow rate of 1 mL/min. The retention times for pantoic acid and pantolactone were 4.0 and 7.0 min, respectively (*33*).

After filtration to remove insoluble materials, the hydrolysate was extracted three times with ethyl acetate (1:1 v/v) to remove unreacted pantolactone. After the pH had been adjusted to 1.0 with 6.0 M HCl, the aqueous layer was heated at 80 °C for 15 min, so that pantoic acid in the solution could be converted to pantolactone. The solution was then extracted with ethyl acetate (1:1 v/v), and pantolactone crystals were obtained after the organic solvent was evaporated. The optical purity was estimated by comparing the $[\alpha]^{20}_D$ value(*c* l% w/v, water) with that of optically pure D-pantolactone (−50.0°) (*2*).

Sequence Analysis of D-Pantonohydrolase Variant Genes. Positive variants were picked at each round of screening for mutant libraries. The selected plasmid, pET20b-mut-LAC, was digested by *Eco*RI and *Sal*I, and the D-pantonohydrolase variant gene was cloned into pBluescript SKIII phagemid at the same site. A single strain containing a mutant D-pantonohydrolase gene was prepared and sequenced on DNA Sequencer 377 (PE). Nucleotide sequences of mutant D-pantonohydrolase genes were aligned with the wild-type gene sequence using the Megalign Program, J. Hein Method, Lasergene software (DNA Star, Inc., Madison,WI) with the standard settings. The amino acid sequences deduced from nucleotide sequences were also compared using the Megalign Program, Lipman−Pearson Protein Alignment, and Lasergene software (DNA Star) with the standard settings.

Effect of pH on the Activities of Wild-Type and Evolved Enzyme. In the asymmetric hydrolysis of DL-pantolactone, control of the pH of the reaction mixture is necessary to keep both enzymatic hydrolysis rate and optical purity of D-pantoic acid high. The D-pantonohydrolase activities could be assayed at the pH ranging from 3.5 to 10.6 for wild-type and mutant enzyme. However, when the pH was >7.5, D-pantolactone would be hydrolyzed to pantoic acid spontaneously during long-term hydrolysis, resulting in low optical purity of the product (*3*, *4*). Thus, to obtain a high optical purity of pantoic acid, in this study, only the pH ranging from 3.5 to 7.5 was discussed. The purified enzymes were dissolved in different buffers to achieve an initial activity for 30 min, after which the relative residual activity was measured to assess the pH stability with standard assay conditions. To investigate the optimal pH and the effect of pH on D-pantonohydrolase activity, buffers including citric acid−Na_2HPO_4, pH 3.4−8.0, Tris-HCl, pH 7.2−9.1, and glycine−sodium hydroxide, pH 8.6−10.6, with concentrations of 100 mM for each were used.

Determinations of Kinetics Parameters. The Michaelis constant K_m values and V_{max} for each enzyme were determined by assaying purified wild-type and evolved enzyme at increasing substrate concentrations ranging from 154 to 615 mM. The temperature, pH, and quantity of the enzyme were kept the same as for the standard enzyme activity assay described above. The values of K_m and V_{max} were calculated according to Lineweaver−Burk plots.

RESULTS

Expression of D-Pantonohydrolase in the Periplasmic Space of *E. coli*. Many proteins require the formation of stable disulfide bonds to fold properly into a native conformation. Without the formation of stable disulfide bonds, these proteins may be degraded or accumulated as inclusion bodies. It is not easy to express the eukaryotic gene in a prokaryotic host. Even if the gene can be expressed, the products are not always soluble and in an active form in the endocellular environment. The environment in the periplasmic space is different from the endocellular situation. Disulfide bonds of protein are usually formed only when the products are exported into the periplasmic space, and it can meet the requirement of refolding. To gain the active D-pantonohydrolase in *E. coli*, we expressed the D-pantonohydrolase into the periplasmic space of *E. coli*. At the same time, pET20b, the vector with signal sequence, was used for the expression. The products expressed in the host could be transported into the periplasmic space of *E. coli* by N-terminal *pelB* signal sequence, and the signal sequence was cut down by the signal identification system of the host after the products expressed entered the periplasmic space, and then the protein refolded to the active form. The activity of D-pantonohydrolase expressed in the periplasmic space of *E. coli* was determined, and the results are shown in **Table 1**, which illustrates that the active D-pantonohydrolase was successfully expressed in the periplasmic space of *E. coli*.

Construction and Screening of Mutant Libraries. The products of error-prone PCR and DNA shuffling (**Figure 2**)

Table 1. D-Pantonohydrolase Activity of Recombinant Cells[a]

strain/plasmid	specific activity (units/mg)
E. coli BLR(DE3)pLysS	0
E. coli BLR(DE3)pLysS/pET20b-LAC	0
E. coli BLR(DE3)pLysS/pET20b-LAC–IPTG	39.5

[a] The host strains and the recombinant strains without induction by IPTG could not show the activity of D-pantonohydrolase. However, the recombinant strains with induction had detected the activity of D-pantonohydrolase.

were purified, digested with *Eco*RI and *Sal*I, and ligated into pET20b to create the recombination library of pET20b-mut-lac. At the same time, the wild-type D-pantonohydrolase gene was also cloned into plasmid pET20b as a control. The ligation mixture was transformed into BLR(DE3)pLysS electrocompetent *E. coli* by electroporation. Transformants were plated on the LB−agar medium supplemented with chloramphenicol, tetracycline, ampicillin, bromocresol purple, D-pantolactone, and IPTG and then incubated for 48 h at 30 °C. The fields around positive colonies with improved properties could become yellow. However, negative mutants could not change the color of the plate with purple background. Moreover, mutants with higher activity and stability under low pH could make the halos bigger, and the time of color changing earlier, which was easily visually inspected. We screened >45000 colonies totally, and 59 clones turned out to be yellow halos, showing the activity of D-pantonohydrolase. Among these clones, seven better ones were retested using HPLC, and the clone with the greatest improvement in activity and pH stability was selected. At the same time, several variants selected were sequenced, and their amino acid substitutions are listed in **Table 2**. The mutation causing a frame shift was not found. After three sequential error-prone PCRs and two rounds of DNA shuffling followed by a screening method, a best mutant, Mut H-1287 (GenBank accession no. DQ334870) with the greatest improvement in activity and pH stability was selected as the evolved D-pantonohydrolase for further studies.

Purification of Wild-Type and Evolved D-Pantonohydrolase and Molecular Properties. The wild-type and evolved D-pantonohydrolases were purified in three steps. The results of the purification are summarized in **Table 3**. Purifications of 48.3- and 52.3-fold were achieved, but only 22.7 and 23.8% of the crude enzyme activities were recovered for the wild-type and evolved D-pantonohydrolases, respectively. The enzymes were stable in storage at 4 °C in 20% glycerol for several weeks. The purified enzymes of wild-type and evolved D-pantonohydrolase both gave only one band on the SDS-PAGE, with a molecular mass of ≈40 kDa (**Figure 3**). From the eluted fractions of gel filtration column chromatography, Mut H-1287 was found to be eluted with an apparent molecular mass between 38 and 45 kDa. No higher molecular mass aggregates or oligomeric structures were found even at a high concentrations of purified enzymes. Therefore, the molecular mass and oligomeric structure of evolved D-pantonohydrolase, Mut H-1287, were identical to those of the wild-type enzyme.

Determination of Activity, Optimal pH, and pH Stability. When identical units of enzymes were used for clear comparison, the optimal pH, pH stability, and effect of pH on the activities of the purified enzymes were examined. After incubation at different pH buffers for 30 min, especially between pH 5.0 and 7.0, the evolved D-pantonohydrolase was found to be more stable than the wild type, whereas the maximum specific activity of evolved D-pantonohydrolase was observed at pH 7.0. Compared to the wild type, the optimal pH of the evolved enzyme, Mut H-1287, shifted from pH 7.5 to 7.0. The best evolved D-pantonohydrolase showed a 10.5-fold higher specific activity as compared to the wild type, and the detailed results are shown in **Figure 4**. The stability of the evolved enzyme under low pH was improved at no expense of D-pantonohydrolase activity. **Figure 5** shows that after incubation at pH 6.0, 85% of the activity of D-pantonohydrolase was retained; however, the wild type retained only 40% activity.

Effect of Temperatures on Wild-Type and Evolved D-Pantonohydrolase Activities. To evaluate the effect of temperature on the enzyme activity, the enzyme reaction was carried out at 30 °C for 60 min. After incubation in a preheated water bath at various temperatures ranging from 20 to 90 °C, the relative residual activities were assayed with standard assay conditions. The activities of enzymes that were assayed under the standard reaction were taken as 100%. Both the wild-type and evolved D-pantonohydrolase activities slowly decreased at 60 °C and dropped significantly at 70 °C. Thus, the enzymes were sensitive to heat. The thermostability of the evolved enzyme, Mut H-1287, was not apparently changed as compared to the wild-type enzyme, and the details are shown in **Figure 6**.

Effect of Metal Ions and Other Reagents on Wild-Type and Evolved D-Pantonohydrolase Activity. The effects of different metal ions and other reagents on the activities of purified wild-type and evolved D-pantonohydrolase were examined by incubating the enzymes in the presence of the reagents at 30 °C for 1 h. The residual activity was assayed according to the standard method. As shown in **Table 4**, the activities of purified wild-type and evolved D-pantonohydrolase were inhibited by several metal ions, such as Cd^{2+}, Hg^{2+}, Cu^{2+}, Ag^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}. The wild-type and evolved D-pantonohydrolases were greatly affected by EDTA, with 94.9 and 94.5% inhibitions, respectively. The results of metal analysis showed that the calcium can activate the wild-type and evolved D-pantonohydrolase. The maximum hydrolysis activities were attained at a 0.5 mM concentration of calcium for both wild-type and evolved D-pantonohydrolase, but a further increase in the concentration did not change the activities significantly.

Determination of the Kinetic Parameters and Stereospecificity. The values of K_m and V_{max} were calculated to be 95 mM and 4.27 mM min^{-1} mg^{-1} for wild type and 83 mM and 5.32 mM min^{-1} mg^{-1} for evolved D-pantonohydrolase, respectively. The values of K_m and V_{max} of D-pantonohydrolase were slightly changed after directed evolution. Purified evolved D-pantonohydrolase was analyzed for substrate specificity and compared with the wild-type enzyme (**Figure 7**). Neither the wild-type nor the evolved D-pantonohydrolase could hydrolyze the L-pantolactone, but for D-pantolactone, both wild-type and evolved D-pantonohydrolase showed high stereospecificity. With regard to the hydrolyzing reaction, no detectable L-pantoic acid compound was formed (**Figure 7**). After directed evolution, the evolved enzyme consistently retained the strict selectivity to D-(−)-isomers, just like the wild-type D-pantonohydrolase, which indicated that the three mutations did not affect the strict D-stereospecificity of the enzyme. However, substitution of the evolved enzyme had caused a slight effect on kinetic parameters during kinetic studies, which may reflect differences in substrate binding.

DISCUSSION

Directed Evolution of D-Pantonohydrolase. D-Pantonohydrolase is useful in the procedure of resolution of racemic

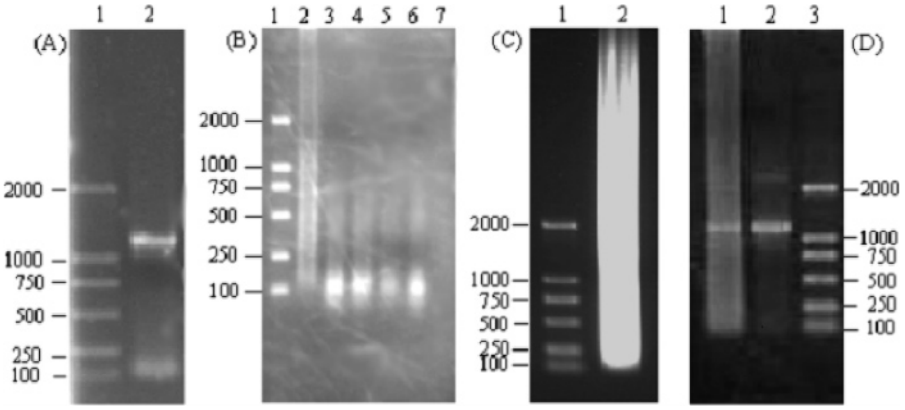

Figure 2. Agarose gel electrophoresis of error-prone PCR and DNA shuffling: (**A**) error-prone PCR products of D-pantonohydrolase gene (lane 1, DNA ladder DL-2000; lane 2, products of error-prone PCR); (**B**) products of DNaseI digestion (lane 1, DNA ladder DL-2000; lanes 2–7, products of DNaseI digestion at different times); (**C**) detection of self-reassembly PCR [lane 1, DNA ladder DL-2000; lane 2, products of self-reassembly PCR (after 52 cycles)]; (**D**) detection of PCR with primers (lanes 1 and 2, products of high-fidelity PCR; lane 3, DNA ladder DL-2000).

Table 2. Base and Amino Acid Substitution of Several Mutants

sample	base site	base	amino acid
Mut A-81	665	T changed to A	silent
	1038	T changed to G	silent
Mut C-437	352	A changed to C	K changed to Q
	1035	T changed to C	silent
	1038	T changed to G	silent
Mut E-861	352	A changed to C	K changed to Q
	721	G changed to A	V changed to I
	1038	T changed to G	silent
Mut G-1559	3	G changed to A	V changed to I
	1038	T changed to G	silent
Mut H-457	721	G changed to A	V changed to I
	1038	T changed to G	silent
Mut H-1287	187	G changed to C	D changed to H
	352	A changed to C	K changed to Q
	721	G changed to A	V changed to I
	1038	T changed to G	silent

Table 3. Purification and Properties of Wild-Type and Evolved D-Pantonohydrolase

	enzyme	protein (mg)	activity (units)	specific activity (units/mg)	recovery (%)	purification (fold)
crude enzyme	wild-type	55	22.12	0.4	100	1
	evolved	55	315.53	5.74	100	1
high Q IEX	wild-type	10.5	13.45	1.28	60.8	3.2
	evolved	11.2	225.34	20.12	71.4	3.5
HIC	wild-type	1.68	6.47	3.85	29.25	9.6
	evolved	1.72	97.75	56.83	30.99	9.9
high S IEX	wild-type	0.26	5.02	19.31	22.7	48.3
	evolved	0.25	75.1	300.4	23.8	52.3

pantolactone to produce D-pantolactone, but the activity and stability of the enzyme under low pH are not satisfactory enough to be applied to industrial processes. Experiments were carried out to enhance the expected properties of D-pantonohydrolase by directed evolution—evolving the enzyme by error-prone PCR and DNA shuffling, followed by screening of mutants with desired properties. In addition, research on the obtained sequence and structure of the evolved enzyme would offer more valuable information for the structure−function relationship of D-pantonohydrolase.

Unlike protein engineering by rational design, directed molecular evolution does not require any knowledge a priori

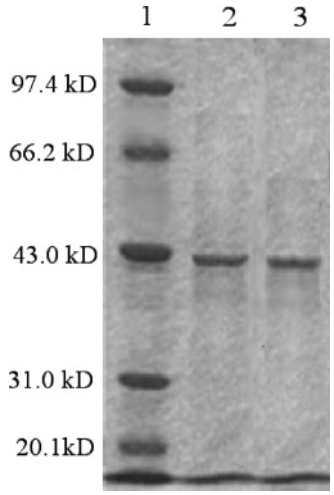

Figure 3. SDS-PAGE analysis of wild-type and evolved D-pantonohydrolase: lane 1, molecular mass marker proteins (rabbit phosphorglase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; rabbit actin, 43.0 kDa; bovine carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 20.1 kDa); lane 2, purified wild-type D-pantonohydrolase; lane 3, purified evolved D-pantonohydrolase.

of protein structure or structure−function relationships. It is well-known that screening efficiency was the hinge of directed evolution (*34*). In our present research, a simple and rapid two-step screening method was constructed, which was sensitive enough to ensure that even a small enhancement to achieve the desired result could be observed. At the same time, when the mutant libraries were selected and the plasmid selected, the screenings were also taken into consideration. The host, *E. coli* BLR(DE3)pLysS, can produce a small amount of T7 lysozyme, cutting a specific bond in the peptidoglycan layer of the *E. coli* cell wall (*28*), and make the cell wall broken, which made it easy for substrates to enter the cells. Moreover, the plasmid pET20b was chosen as the vector to carry the gene gained by error-prone PCR and DNA shuffling procedures. The protein expressed by a foreign gene could be transported into the periplasmic space, which makes the D-pantonohydrolase closer to the substrate. It was helpful to screen the mutants with improved properties; therefore, the host, *E. coli* BLR(DE3)-pLysS, was used in our research combined with plasmid with signal sequence.

Previous research had shown that the temperature had an important influence on the solution of recombinant enzyme in

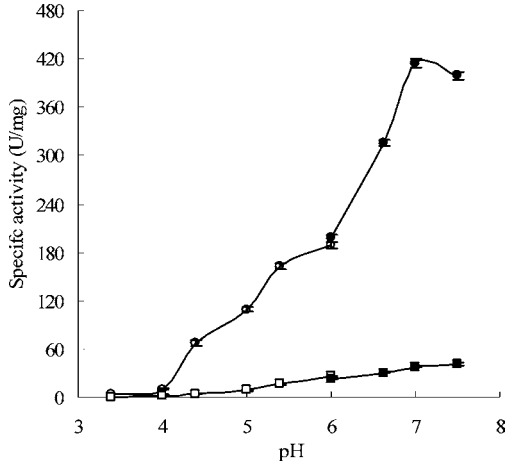

Figure 4. Activity of wild-type and evolved D-pantonohydrolase at different pH values: □, wild-type D-pantonohydrolase in citric acid–Na_2HPO_4 buffer; ■, wild-type D-pantonohydrolase in Tris-HCl buffer; ○, evolved D-pantonohydrolase in citric acid–Na_2HPO_4 buffer; ●, evolved D-pantonohydrolase in Tris-HCl buffer.

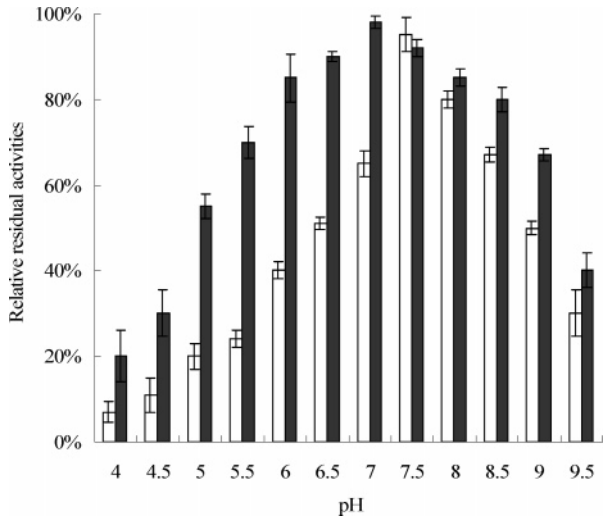

Figure 5. Comparison of relative residual activity between wild-type (□) and evolved D-pantonohydrolase (■) at different pH values. The enzymes were treated in different buffers for 30 min, and then the D-pantonohydrolase activities were determined according to the standard enzyme assay method. The activities of enzymes that assayed under the standard reaction were taken as 100%.

the recombinants. Growth at 37 °C always caused some proteins to accumulate as inclusion bodies, whereas incubation at ≤30 °C led to soluble and active protein (*35*). Growth and induction at 25 or 30 °C might be optimal for expressing soluble active protein. In some cases, prolonged (e.g., overnight) induction at low temperatures might prove to be optimal for the yield of soluble protein. For the reasons mentioned above, to make the expressed target proteins in a soluble, active form. we adopted 30 °C as the cultivation and induction temperature.

An efficient selection or screening procedure, which allows the testing of a large set of variants and minimizing the effort of finding improved ones, is needed to explore sufficient sequence space. The method for screening improved mutants based on the substrate hydrolysis was adopted in this experiment, which combined product formation with activity of D-pantonohydrolase. This principle, product formation combined with activity of enzyme, has been widely used in the directed enzyme evolution (*36*). According to the screening method reported by

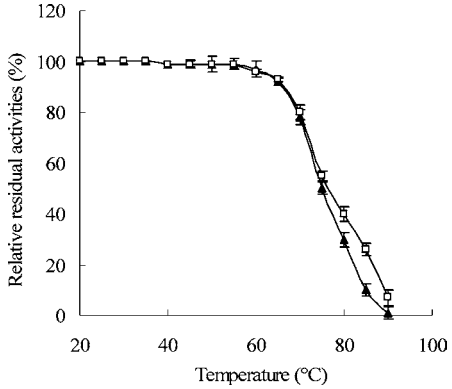

Figure 6. Comparison of relative residual activities between wild-type (▲) and evolved D-pantonohydrolase (□) at different temperatures.

Table 4. Effect of Metal Ions and Some Chemicals on Wild-Type and Evolved D-Pantonohydrolase Activity[a]

		relative activity (%)	
chemical	concn (mM)	wild-type D-pantonohydrolase	evolved D-pantonohydrolase
none		100	100
EDTA-Na_2	1	5.12	5.44
citrate	30	21.0	23.5
$CoCl_2$	1	48.7	47.2
$NiCl_2$	1	53.3	54.6
$FeCl_2$	1	6.3	6.7
$BaCl_2$	1	62.4	70.1
$CaCl_2$	1	118.6	121.0
$MgCl_2$	1	81.2	83.5
$MnSO_4$	1	15.5	14.4
$ZnSO_4$	1	13.38	14.0
$AgNO_3$	1	5.32	5.53
Hg_2Cl_2	1	3.21	3.00
$CuSO_4$	1	5.51	6.21

[a] Enzyme activities were determined in the presence of an additional test chemical substance under the standard assay conditions.

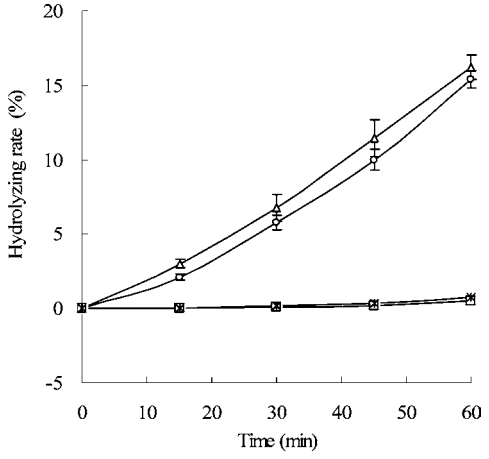

Figure 7. Rates of hydrolysis of different substrates with enzymes: △, D-pantolactone as substrate for wild-type D-pantonohydrolase; ○, D-pantolactone as substrate for evolved D-pantonohydrolase; ∗, L-pantolactone as substrate for wild-type D-pantonohydrolase; □, L-pantolactone as substrate for evolved D-pantonohydrolase.

Bornscheue et al. (*37*), a preliminary test of different indicators revealed that bromocresol purple gave best results. The method constructed in this experiment allowed us to isolate a clone that could present a bigger yellow halo on the screening medium agar plates containing bromocresol purple as the pH indicator.

The earlier and bigger the yellow halos formed, the higher the activity and pH stability of the D-pantonohydrolase. The results showed the feasibility of selecting the enzyme with improved properties based on product formation. The screening results of positive mutants illustrated that the screening method based on product formation and pH indicators was sensitive, reliable, and cost-effective.

Sequence and Structural Analysis of Variants. The presence of the G187C, A352C, and G721A mutations appeared to be the most beneficial, as the Mut C-437 and Mut H-457 variants carrying K118Q and V241I, respectively, exhibited 2.4- and 2.7-fold increases in specific activity, whereas the Mut H-1287 variant carrying D63H, K118Q, and V241I had improved 10.5-fold. Moreover, stability under low pH was also improved, as compared to the wild-type D-pantonohydrolase. This result indicated that D63H, K118Q, and V241I substitutions might benefit to increase the overall hydrolysis rate at low pH through improvement of activity. Although the mutation of position 1038 was found in all variants sequenced, this substitution was a silent mutation. No noticeable difference in activity and pH stability was observed when this substitution was removed. It is suggested that mutation at position 1038 was unlikely to contribute to the improved activity and pH stability, but helpful for expression of D-pantonohydrolase in *E. coli* BLR(DE3)-pLysS. The single mutation separately combined with silent mutation of position 1038 had a positive effect on the activity and pH stability. Therefore, it could be deduced that D63H, K118Q, and V241I perhaps had an influence on the improved activity and pH stability of D-pantonohydrolase. Furthermore, three amino acid substitutions changed the relationships between the amino acids, and the secondary structure of the backbone and/or side-chain interactions further resulted in significant changes in specific activity and low-pH stability.

There are a few reports of mutations that led to the simultaneous improvement of multiple properties of enzymes, such as activity, stability, and substrate tolerance, by directed evolution (*38*, *39*). The reason is that an improvement of any one property is likely to be obtained at the cost of another one, unless multiple constraints are imposed simultaneously (*40*). However, in the case of Mut H-1287, only three mutations (D63H, K118Q, and V241I) resulted in an enhanced activity and pH stability. Invesitgations of the mechanisms of simultaneous improvement of activity and pH stability of enzymes are underway.

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