Site-Specific and Asymmetric Hydrolysis of Prochiral 2-Phenyl-1,3-propanediol Diacetate by a Bacterial Esterase from an Isolated Strain

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Bacillus cereus 809A and Burkholderia sp. 711C were isolated from soil. These strains demonstrate hydrolysis activity towards prochiral 2-phenyl-1,3-propanediol diacetate and accumulated the corresponding chiral monoacetates into the reaction mixture. When 2-phenyl 1,3-propanediol diacetate was used as a substrate, the produced monoacetates with Burkholderia sp. 711C were obtained in a racemic form but that produced by Bacillus cereus 809A showed an excess of the (S)-form. The resting cell reaction revealed that for Bacillus cereus 809A, there was an enrichment of one of the enantiomers of the monoacetate such that the enantiomeric excess (e.e.) of the (S)-form was over 95%. The purified enzyme from Bacillus cereus 809A hydrolyzed diacetate to monoacetate, and the e.e. value of the (S)-form increased by prolonged reaction in a way similar to the resting cell reaction. From N-terminal amino acids, this esterase is conserved in some strains of Bacillus for which the genomic sequences have been reported.

Key words: esterase; asymmetric hydrolysis; 2-phenyl-1,3-propanediol diacetate; Bacillus cereus

The chiral pool synthetic method was developed to produce useful chiral compounds efficiently.1) Optically pure 2-substituted-1,3-propanediol derivatives are useful chiral building blocks as one of the minimum pool required for the synthesis of biologically active compounds.2,3) Since the strict substrate specificities of enzymes is a distinct advantage for chiral pool synthesis, microbial enzymes are often used to obtain optically pure substances. The stereospecific hydrolysis of racemic esters by esterase is an efficient strategy for optical resolution,4) but the maximum yield of the targeted optically pure compound is below 50%. In contrast, there are two reported methods of lipase-based asymmetric synthesis of 2-substituted-1,3-propanediol monoacetate that give high yields (more than 50%). One is esterification of 2-substituted-1,3-propanediol with vinyl acetate5,6) while the other is hydrolysis of prochiral 2-substituted-1,3-propanediol diacetate.7)

This study was an attempt to produce optically pure 2-substituted-1,3-propanediol monoacetate as a useful chiral building block. We used the prochiral propanediol derivatives, 2-phenyl-1,3-propanediol diacetate (PPdAc) and 2-methyl-1,3-propanediol diacetate to screen for novel microbial esterases in cells. Some strains showed activities for producing monoacetates from both diacetates, but 2-methyl-1,3-propanediol diacetate was not suitable for producing optically pure substances, because all strains used produced monoacetate with low optical purity (data not shown). However, when PPdAc was used as the substrate, strain 809A, which was screened in this study, produced monoacetate of the (S)-form in excess in both resting and whole cell reactions (Fig. 1). There are no reports of approaches that employ resting or whole cells to prepare these compounds cheaply. It would however be a useful method for the production of optically pure compounds, because commercial esterases and lipases are expensive. Furthermore, it is interesting to note that we found that the optical purity of 2-phenyl-1,3-propanediol monoacetate (PPmAc) increased with reaction time. In order to understand the hydrolysis process in detail, we purified a novel esterase from the crude extract of strain 809A and characterized some of its enzymatic properties.

Materials and Methods

Materials. PPdAc and PPmAc were synthesized by and were gifts from Dr. Ohira and Dr. Kuboki (Depart-

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Abbreviations: PPdAc, 2-phenyl-1,3-propanediol diacetate; PPmAc, 2-phenyl-1,3-propanediol monoacetate; PPol, 2-phenyl-1,3-propanediol; e.e., enantiomeric excess; DEAE, diethyl aminoethyl
Enzymatic Hydrolysis of Prochiral Propanediol Derivatives

Fig. 1. Summary of This Study for Producing (S)-2-Phenyl-1,3-propanediol Monoacetate from Prochiral-2-phenyl-1,3-propanediol Diacetate Using a Bacterial Esterase.

reaction mixture containing 10 mM PPdAc, 50 mM formation of PPmAc by hydrolysis of PPdAc in a 1-ml the results of HPLC analysis: e.e. (rate was 0.5 ml/min. The value of enantiomeric excess was hexane and methanol (95:5 vol/vol) and the flow conditions were diluted adequately, spread on solid Luria-Bertani medium,8) and cultivated for 2 to 5 d at 28 °C. The single colonies obtained were picked out and inoculated into LB liquid medium for 2 d. The substrate was dissolved in ethanol and then added to the medium to a final concentration of 10 mM. After 24 h, the products in the supernatant were extracted with ethyl acetate and analyzed by Gas-Liquid Chromatography (GLC), as described below.

Resting cell reaction. Cells were harvested by centrifugation at 10,000 × g for 10 min and washed once with 50 mM potassium phosphate buffer (pH 7.0) at 4 °C. The cells were then resuspended in the same buffer. Resting cell reactions were performed at 28 °C with shaking for 8 h in the reaction mixture, which contained 10 mM PPMAc and 50 mM potassium phosphate buffer (pH 7.0). The final cell density in the reaction mixture was adjusted to an absorbance value at 610 nm of 5.0.

Analytical measurements. The residual substrates and hydrolyzed products extracted with ethyl acetate were analyzed by GLC with a non-polarity capillary DB-1 column (0.25 mm × 30 m, J&W Scientific, Folsom, CA) using a flame ionization detector. The injector temperature was 250 °C and the column temperature was raised 2 °C/min from 80 to 110 °C. The mobile phase was nitrogen flowing at 1.2 ml/min. The optical purity of the PPMAc was analyzed by HPLC with a Chiralcel OD column (4.6 × 250 mm, Daicel Chemical Industries, Tokyo) with detection at 254 nm. The mobile phase was hexane and methanol (95:5 vol/vol) and the flow rate was 0.5 ml/min. The value of enantiomeric excess (e.e.) was determined by the following equation using the results of HPLC analysis: e.e. % of (S) = ((S)-isomer − (R)-isomer)/((S)-isomer + (R)-isomer) × 100.

The enzyme activity was estimated based on the formation of PPMAc by hydrolysis of PPdAc in a 1-ml reaction mixture containing 10 mM PPdAc, 50 mM potassium phosphate (pH 7.0), and an appropriate amount of enzyme. This reaction was performed at 30 °C for 30 min. After the reaction, 1 ml of ethyl acetate was added to the mixture to extract the remaining substrate and products. One unit was defined as the amount of enzyme needed for the formation of 1 μmol PPMAc from PPdAc per min at 30 °C. Protein was measured by the Lowry method,9) with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 10% polyacrylamide slab gels by the method of Laemmli.10) The gels were stained for protein with Coomassie brilliant blue R-250. The molecular weight of the enzyme was estimated by elution time from a G-3000SW HPLC column (Tosoh, Tokyo) with detection at 280 nm. MW-Marker (Oriental Yeast, Osaka, Japan) was used as the molecular weight standard. Amino terminal sequence analysis of the purified enzyme, which was blotted onto PVDF membrane, was performed using the Edman methodology on a 467A Protein Sequencer (Perkin Elmer Japan, Yokohama, Japan).

Preparation of cell extract. Isolated strain 809A was grown at 28 °C for 24 h on LB medium. After cultivation, the cells were harvested by centrifugation (10,000 × g, 10 min, 4 °C) and washed twice with 10 mM Tris–HCl buffer (pH 8.0). The pellet was resuspended in buffer and the suspension was treated with an ultrasonicator (9 kHz) for 10 min, with care taken to keep the temperature below 5 °C. The lysate was then centrifuged at 12,000 × g for 20 min. The resulting supernatant was used as the cell extract.

Protein purification. The enzyme, which catalyzed the hydrolysis of PPdAc to PPMAc, was purified from the cell extract as follows: The cell extract was applied to a DEAE-Sepharose FF (GE Healthcare Bio-Science, Little Chalfont, Buckinghamshire, UK) column (5.0 × 20 cm). After the column was washed with 10 mM Tris–HCl buffer (pH 8.0), the enzyme was eluted with a gradient of increasing NaCl concentrations (0 to 0.4 M). The active fractions were collected and dialyzed against 50 mM potassium phosphate buffer (pH 7.0). Solid NaCl was added to dialyzed active solution to a concentration
of 2 M, and the solution applied to a Butyl-Sepharose FF (GE Healthcare Bio-Science) column (2.6 × 20 cm) equilibrated with the same buffer containing 2 M NaCl. After the column was washed with the same buffer, elution was performed with a gradient of decreasing NaCl concentrations (2.0 to 0 M) and increasing ethylene glycol concentrations (0 to 20%). The active fractions were collected and dialyzed against 10 mM Tris–HCl buffer (pH 8.0). The dialyzed solution was applied to a Resource Q prepacked column (1 ml) (GE Healthcare Bio-Science) equilibrated with 10 mM Tris–HCl buffer (pH 8.0). After the column was washed with the same buffer, the enzyme was eluted with a gradient of increasing NaCl concentrations (0 to 0.6 M). The active fractions were collected and dialyzed against 10 mM Tris–HCl (pH 8.0) and stored at −20 or −80 °C until use.

Results and Discussion

Accumulation of PPmAc by hydrolysis of PPdAc by the isolated strains

About 500 isolated strains were cultivated on LB medium at 28 °C for 24 h. After cultivation, PPdAc was added to the culture and incubated with the cells for 24 h. The reaction mixture was extracted with ethyl acetate in order to measure the accumulation of the corresponding monoacetate, as described in “Materials and Methods.” In most of the samples (about 70%) no products were detected in the ethyl acetate extract, but we found four strains that accumulated PPmAc in the culture. These strains were examined further with regard to hydrolysis of PPdAc with increasing reaction time (data not shown). In the case of two of the strains, further hydrolysis to 2-phenyl-1,3-propanediol (PPol) occurred, but the other two strains, named 711C and 809A, accumulated PPmAc in the medium at high yield.

Identification of the isolated strains

711C was found to be a gram-negative aerobic strain that was rod-shaped, formed light yellow colonies, and was positive for oxidase and catalase. The 16S ribosomal DNA sequence identified this organism as a strain of Burkholderia cepacia. 809A is a gram-positive aerobic endospore forming rod-shaped strain that is negative for catalase. The 16S ribosomal DNA sequence identified this organism as a strain of Bacillus cereus. From the above results, strains 711C and 809A were identified as Burkholderia sp. 711C and Bacillus cereus 809A respectively.

Resting-cell reaction

Activity towards PPdAc was found in cells of strains 711C and 809A but not in the culture supernatants. In order to improve the hydrolysis rate of the substrate and the yield of product, resting-cell reactions were investigated. The cells of both strains hydrolyzed PPdAc to PPmAc at high yield, but measurement of the optical purity of the PPmAc produced by HPLC showed that the material produced by strain 711C was racemic at all time points (data not shown), whereas the optical purity of that produced by strain 809A increased with reaction time (Fig. 2). For strain 809A, the value of e.e. after all the PPdAc was hydrolyzed was more than 99% by HPLC. The specific optical rotation of the PPmAc produced was measured. It gave a value of [α]25 = −58.8° (c 1.2, CHCl3), demonstrating it to be the (S) form. This value corresponds well with the previously reported value of −59.6°.11

In order to understand the hydrolysis mechanisms, the enzyme was purified from strain 809A.

Purification and properties of the enzyme

The enzyme that hydrolyzed PPdAc to PPmAc was purified from a cell-free extract of strain 809A, as described in “Materials and Methods.” The crude extract was purified by column chromatography. One milligram of purified enzyme was obtained from 55 g of cells (wet cell weight), which was from 5-liter culture. The results of the purification are summarized in Table 1. Purified enzyme migrated as a single band, and the relative molecular weight of the purified enzyme was estimated to be about 38,000 by SDS–PAGE (Fig. 3A), and to be about 70,000 by gel-filtration (Fig. 3B). These results suggest that this enzyme is a dimer of two identical subunits. The pH- and temperature-optimum for the activity of enzyme were estimated from the hydrolysis activities. The enzyme showed highest activity towards PPdAc between pH 7 and 9 (Fig. 4A), and was most stable within a pH range of 6 to 10 in 30 min incubations at 30 °C (Fig. 4B).
stability of this enzyme was clearly lower at pH levels lower than 6 and higher than 10. The enzyme showed highest activity at 60°C (Fig. 4C), but it was unstable after prolonged incubation at this temperature (Fig. 4D). pH-dependent non-enzymatic hydrolysis of PPdAc was observed at pH 10 and higher, but was not observed at any temperature under the experimental conditions we examined.

Inhibition of the enzyme by certain metal ions and other reagents was investigated. The activity was inhibited strongly by Ag⁺ (100%), and partially by Cu²⁺ (50%) at a final concentration of 1 mM. Chelating agents such as ethylenediaminetetraacetic acid did not inhibit the activity. The enzyme activity was inhibited by phenyl hydrazine (63%) and phenyl methyl sulfonyl fluoride (80%). These results suggest that a serine residue in the enzyme might play an important role in activity.12)

Hydrolysis of PPdAc by the purified enzyme

A time course study of the hydrolysis of PPdAc and the optical purity of the product was set up using purified enzyme (Fig. 5). The hydrolysis reaction proceeded until PPdAc was completely consumed, and PPmAc was found in the reaction mixture. The optical purity of PPmAc increased with reaction time, as observed in the reaction with resting cells. In order to understand this observation, the stereoisomers of PPmAc were measured quantitatively by HPLC in a reaction with homogeneous, purified enzyme. It is interesting that the value of e.e. (%) was almost constant (about 60% e.e.) while sufficient PPdAc remained in the mixture. After PPdAc was consumed, PPmAc was further hydrolyzed to PPol and the excess of (S)-PPmAc increased. Based on these results, we suggest the following means of hydrolysis of PPdAc by this enzyme: First, it catalyzes pro(R)-specific hydrolysis of PPdAc, and the (S)-form accumulates preferentially in the reaction mixture. The ratio of the (S) to (R)-form products was approximately 4 to 1 (60% e.e.). This value corresponds well to the simulation described by Chen et al.13) That is, the enantiomeric ratio or E value is read as around 4 as calculated from the production rate of each enantiomer, and this value gives an e.e. (%) of about 60 when the substrate conversion rate is 60%. Secondly, the PPmAc produced is further hydrolyzed slowly to PPol, and the amount of the (S)-form increases even though the yield of PPmAc decreases. These results suggest that the stereospecificity of the purified enzyme is not high for PPmAc isomers. A reaction such as that just described changes the ratio of the remaining isomers. Under these conditions, the maximum yield of optically pure (S)-PPmAc (about 55%) was obtained after 2 h, after which the yield of (S)-PPmAc decreased as it was further hydrolyzed to PPol.

N-Terminal amino acid sequence

The enzyme was blotted onto PVDF membrane and the N-terminal amino acid sequence was determined with a protein sequencer, as described in “Materials and
Methods." The N-terminal amino acid sequence obtained was MKTRVNPELLQGLEMFPDLDLRPE. The sequence was used to search genomes of *Bacillus* species by BLAST. This search showed that the sequence had similarities with some *B. cereus* esterases (Fig. 6), but had highest similarity to esterases in *B. thuringiensis*. These results suggest that this esterase has been conserved widely among *Bacillus* species, but it is not known whether these species have an esterase with hydrolysis activity, site-specificity, and asymmetric specificity towards PPdAc.

The resting cell reaction exhibited a high specific hydrolysis rate for PPdAc. Furthermore, we have established a method of producing PPmAc cheaply with high optical purity. However, the maximum yield of PPmAc with high optical purity (90% *e.e.*) was about 55% under the conditions imposed in this study, because the optically pure material obtained was produced by further hydrolysis of the PPmAc produced earlier in the reaction. In order to improve the yield of optically pure PPmAc, further investigations are necessary to determine the reaction conditions. These include temperature, pH, and addition of agents. These attempts are better suited to the enzyme reaction rather than to the resting cell reaction. In order to synthesize chiral reagents by chemo-enzymatic methods, recombinant techniques have been employed in order to express and purify useful microbial enzymes. These recombinant proteins have been purified on an affinity resin, Ni-NTA, in a single step, through the addition of a hexa-histidine tag to the N-terminus. Furthermore, recent genetic approaches have progressed dramatically. Molecular breeding has been applied to a variety of enzymes to improve substrate specificity, catalytic efficiency, stability, pH optima, and function by mutation of genes. In order to improve the stereospecificity of enzymes,
molecular breeding has been applied to other lipases or esterases. In these cases, the modified enzymes had improved stereospecificity. Furthermore, some enzymes had increased catalytic activity and stability in the presence of an organic solvent. We are currently trying to make recombinant esterase from strain 809A in E. coli, which should allow easy purification of this enzyme and efficient synthesis of optically active propanediol derivatives.

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