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Purification and Some Properties of 2-Hydroxychromene-2-Carboxylate Isomerase from Naphthalenesulfonate-Assimilating *Pseudomonas* sp. TA-2

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A 2-hydroxychromene-2-carboxylate isomerase was purified from a cell-free extract of naphthalenesulfonate-assimilating *Pseudomonas* sp. TA-2 to an electrophoretically homogeneous state by successive column chromatography on DEAE-cellulose, DEAE-Toyopearl 650M, Sephadex G-75, Hydroxyapatite, and Mono Q. The enzyme had a molecular mass of 25 and 27 kDa as estimated by SDS-PAGE and Superdex 200, respectively. Its N-terminal 30 amino acid sequence had high homology with the deduced amino acid sequences of the 2HC2CA isomerase of *nahD* (a gene of naphthalene metabolism), *pahD* (a gene of naphthalene and phenanthrene metabolism), and *doxJ* (a gene of dibenzothiophene metabolism). The enzymatic product was a *trans* isomer. The isomerase activity was inhibited in the presence of monoiodoacetate or Hg^{2+}, but not by preincubation with monoiodoacetate or *N*-ethylmaleimide. GSH functioned as a cofactor and activated the enzyme at above 0.15 mM.

Key words: *cis-trans* isomerase, 2-hydroxychromene-2-carboxylate, naphthalene, naphthalenesulfonate, *Pseudomonas*.

Pseudomonas sp. TA-2, which was isolated as a 2-naphthylamine-1-sulfonate (tobias acid)-assimilating bacterium, assimilated 1-naphthalenesulfonate. Salicylate was an intermediate in the metabolic pathway and was further metabolized through the gentisate pathway (*1, 2*).

It has been shown that the metabolic pathway from naphthalenesulfonate to salicylate is similar to an upper naphthalene catabolic pathway, but the metabolism of salicylate is different (*1-4*). In the upper pathway, 1,2-dihydroxynaphthalene, 2-hydroxychromene-2-carboxylate (2HC2CA), *o*-hydroxybenzylidenepyruvate (HBP), and salicylaldehyde are common intermediates (*5-8*) (Fig. 1). A 2HC2CA isomerase was thought to be involved in isomerization of 2HC2CA to HBP (*9*). Recently, Eaton and Chapman took a genetic approach, cloning the 2HC2CA isomerase gene from the naphthalene catabolic plasmid NAH7, and suggested that a 2HC2CA isomerase isomerized 2HC2CA to form *trans-o*-hydroxybenzylidenepyruvate (*t*HBP) (*10*). On the other hand, the pathway for naphthalene metabolism also has similarities to the pathway for dibenzothiophene (*11*). Some dibenzothiophene-degradative genes were shown to have DNA homology with naphthalene-degradative genes (*12, 13*). Furthermore, a 2HC2CA isomerase gene of NAH7 plasmid (naphthalene degradation) was shown to have a high degree of sequence homology with a DNA segment from *dox* (dibenzothiophene degradation) operon of *Pseudomonas* sp. strain C18 (*14*).

Although the genes of 2HC2CA isomerase have been cloned, the properties of the enzyme have not been fully studied. Recently, Kuhm *et al.* reported that a partially purified 2HC2CA isomerase from *Pseudomonas testosteroni* A3 was activated by incubation with the reduced form of glutathione (GSH) (*15*). However, the reaction product was not completely identified, and the involvement of the isomerase(s) in the metabolism was not satisfactorily demonstrated. Because it is important to elucidate the involvement of the isomerase in the metabolic pathway of tobias acid, we have studied a 2HC2CA isomerase from *Pseudomonas* sp. TA-2. Here, we report its purification and characteristics and its similarities to the isomerases of a NAH7 gene and a DOX gene, and provide evidence for the role of GSH in the isomerase reaction.

MATERIALS AND METHODS

Cultivation and Enzyme Production—*Pseudomonas* sp. TA-2 was cultivated in a nutrient medium, a glucose medium, and a tobias acid medium as described in our previous paper (*1*). The cells harvested from 480 ml of a culture in the late logarithmic growth phase were washed twice with 10 mM potassium phosphate buffer (pH 7.0),

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Abbreviations: DTT, dithiothreitol; GSMe, *S*-methylglutathione; 2HC2CA, 2-hydroxychromene-2-carboxylate; HBP, *o*-hydroxybenzylidenepyruvate; MIA, iodoacetic acid; NEM, *N*-ethylmaleimide; *t*HBP, *trans-o*-hydroxybenzylidenepyruvate.

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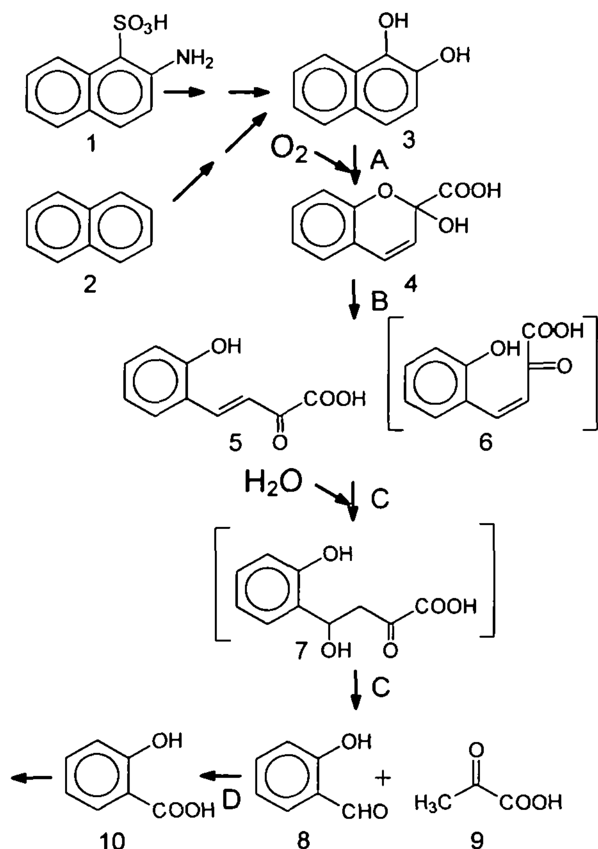

Fig. 1. **Proposed pathways for the metabolism of naphthalenesulfonate and naphthalene to salicylate by *Pseudomonas* sp. TA-2 and NAH 7.** See Refs. *1, 3, 5-10*, and *15*. Chemicals: 1, 2-naphthylamine-1-sulfonate (tobias acid); 2, naphthalene; 3, 1,2-dihydroxynaphthalene; 4, 2-hydroxychromene-2-carboxylate; 5, *trans-o*-hydroxybenzylidenepyruvate; 6, *cis-o*-hydroxybenzylidenepyruvate; 7, 2-oxo-4-hydroxy-4-(2'-hydroxyphenyl)-butyrate; 8, salicylaldehyde; 9, pyruvate; 10, salicylate. Enzymes: A, 1,2-dihydroxynaphthalene dioxygenase; B, 2-hydroxychromene-2-carboxylate isomerase; C, *trans*(*cis*)-*o*-hydroxybenzylidenepyruvate hydratase-aldolase; D, salicylaldehyde dehydrogenase.

resuspended in the same buffer, and then disrupted with a ultrasonic oscillator (Insonator 201M, Kubota). The supernatants obtained by centrifugation were used as cell-free extracts.

Enzyme Assays—2HC2CA isomerase was activated in 10 mM Tris-HCl buffer (pH 7.4) containing 2.5 mM GSH at 10°C for 1-3 h. Its activity was assayed at 30°C for 2 min in a reaction mixture (0.18 ml) containing 0.2 mM 2HC2CA, 2.5 mM GSH, 0.11 unit of *t*HBP hydratase-aldolase, 0.17 M sodium-bicine buffer (pH 8.1), and 4-15 milliunits of the activated 2HC2CA isomerase. The *t*HBP hydratase-aldolase was prepared from a cell-free extract of *Pseudomonas* sp. TA-2, being separated from the 2HC2CA isomerase by Sephadex G-75 column chromatography (Fig. 2). As the enzyme following 2HC2CA isomerase in the naphthalene sulfonate metabolism, it was present in the reaction mixture in sufficient amount to convert the isomerized product of 2HC2CA completely to salicylaldehyde. Although 2HC2CA also isomerized spontaneously to *t*HBP in aqueous solution, the rate of this reaction was too low to appreciably affect the enzyme assay, taking about 5 h to

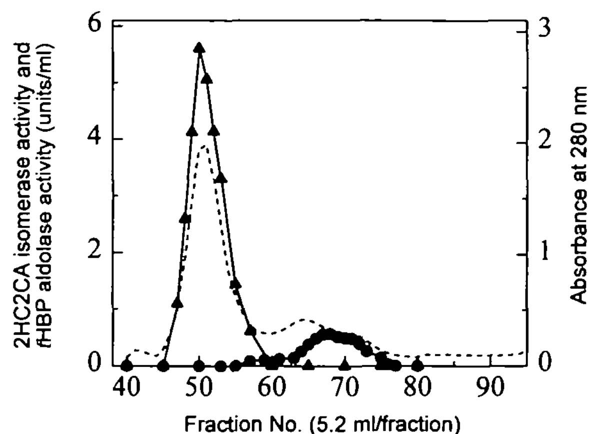

Fig. 2. **Separation of the 2HC2CA isomerase and the *t*HBP hydratase-aldolase on Sephadex G-75 column.** Conditions: column, 2.6×95 cm; buffer, 20 mM Tris-HCl buffer (pH 7.4) containing 200 mM NaCl and 1 mM dithiothreitol (DTT); flow rate, 16 ml/h. Symbols: ▲, *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase activity; ●, 2-hydroxychromene-2-carboxylate isomerase activity; ······, absorbance at 280 nm.

reach 90% completion (*10*). *t*HBP hydratase-aldolase activity was assayed at 30°C for 5 min in a reaction mixture (80 μl) containing 0.26 mM *t*HBP, 0.60 mM 2HC2CA, 81 mM potassium phosphate buffer (pH 7.1), and 0.45-2.1 milliunits of the *t*HBP hydratase-aldolase. In both assays, the reaction mixture was injected directly onto an HPLC apparatus (Shimadzu LC-5A), and the product (salicylaldehyde) was separated from the substrate and quantified by use of an ultraviolet detector (250 nm, Shimadzu SPD-10AV). The analytical conditions were: column, Nucleosil C18 (Macherey-Nagel, 4×50 mm); mobile phase, acetonitrile-water (1:2) containing 10 mM potassium dihydrogen phosphate and 10 mM tetra-*n*-butylammonium hydrogen sulfate; flow rate, 0.5 ml/min. One unit of each enzyme activity was defined as the amount that formed 1 μmol of salicylaldehyde per min at 30°C.

Purification of 2HC2CA Isomerase and tHBP Hydratase-Aldolase—All procedures were done below 4°C. Unless otherwise indicated, 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM dithiothreitol (DTT) was used as the standard throughout the purification.

Step 1. Preparation of cell-free extracts: Bacterial cells in the late logarithmic growth phase were harvested from 6.7 liters of culture in a nutrient medium, washed twice with 10 mM potassium phosphate buffer (pH 7.0), resuspended in the same buffer, then disrupted with an ultrasonic oscillator. The supernatant obtained by centrifugation at 15,000×*g* was used as the cell-free extract. Streptomycin sulfate was added to the cell-free extract to a concentration of 0.7%, and pH was adjusted to 7.4. The precipitate formed was removed by centrifugation (15,000×*g*) and the supernatant was dialyzed against the standard buffer.

Step 2. DEAE-cellulose column chromatography: The dialyzed solution was put on a DEAE-cellulose column (2.6×40 cm) equilibrated with the standard buffer. The column was washed with the buffer and eluted with the buffer containing 0.3 M NaCl. The eluate was dialyzed against the buffer.

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Step 3. DEAE-Toyopearl column chromatography: The dialyzed solution was put on a DEAE-Toyopearl 650M column (2.6×40 cm) equilibrated with the standard buffer. The column was washed with the buffer and eluted with a linear concentration gradient of 0-0.3 M NaCl in the buffer. The enzyme activity was eluted at about 0.2 M NaCl. The eluate was concentrated by ultrafiltration.

Step 4. Gel filtration: The enzyme solution was filtered through a Sephadex G-75 column (2.6×95 cm) equilibrated with the standard buffer containing 0.2 M NaCl, and the active fractions were combined and dialyzed against 1 mM potassium phosphate buffer (pH 7.2). *t*HBP hydratase-aldolase was removed from the isomerase preparation in this step. The fraction containing *t*HBP hydratase-aldolase (5.5 units/ml, 3.5 units/mg) was used for the assay of the 2HC2CA isomerase activity.

Step 5. Hydroxyapatite column chromatography: The dialyzed solution was put on a hydroxyapatite column (1.8×11.5 cm) equilibrated with 1 mM potassium phosphate buffer (pH 7.2) containing 2 mM DTT. The column was washed with the same buffer and eluted with a linear concentration gradient of 1-300 mM phosphate (pH 7.2) containing 2 mM DTT. The enzyme activity was found at about 17 mM phosphate.

Step 6. Mono Q column chromatography: The dialyzed solution was put on a Mono Q column (0.5×5 cm) equilibrated with the standard buffer. The enzyme was eluted with a linear concentration gradient of 0-0.3 M NaCl in the buffer. The enzyme activity was eluted at about 0.2 M NaCl. The eluate was concentrated by ultrafiltration and dialyzed against the buffer without DTT. The enzyme solution was used as the final preparation of 2HC2CA isomerase.

Polyacrylamide Gel Electrophoresis (PAGE) and Isoelectric Focusing—Native PAGE was done with a 7.5% acrylamide slab gel by the method of Gabriel at pH 9.4 (*16*). SDS-PAGE was done in 12.5% acrylamide and 0.1% SDS with a discontinuous Tris-glycine buffer system as described by Laemmli (*17*). Isoelectric focusing (IEF) was done according to the Model 111 Mini IEF Cell instruction manual (Bio-Rad). Protein bands in the gel were stained with Coomassie Brilliant Blue R-250.

N-Terminal Amino Acid Sequence of 2HC2CA Isomerase—The purified enzyme (1.1 nmol) was electrophoresed by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. Its N-terminal amino acid sequence was analyzed by automated Edman degradation using an Applied Biosystem model 471A gas-phase sequencer. Homology searches were done using the protein sequence databases obtained from GenBank, PIR, and SwissProt.

Preparation of 2HC2CA—1,2-Dihydroxynaphthalene dioxygenase from *Pseudomonas* sp. TA-2 was partially purified from the cell-free extract of cells grown in a nutrient medium by removal of nucleic acids with streptomycin sulfate (0.7%) and DEAE-cellulose column chromatography. All purification procedures were performed under nitrogen gas because of the extreme lability of the oxygenase in the presence of oxygen. The oxygenase (specific activity, 3.0 units/mg) was then used to prepare 2HC2CA. The enzyme was activated in 10 mM potassium-phosphate buffer (pH 7.1) containing 2 mM DTT, 10% ethanol, and 1 mM nickel dichloride at 4°C for 30 min, and 180 units of 1,2-dihydroxynaphthalene dioxygenase was dissolved in 1.4 liters of water containing 1 mM 1,2-dihydroxynaphthalene. After incubation at 30°C for 2 min, the solution was adjusted to pH 2 with 1 N HCl and 500 g of NaCl was added. The products (2HC2CA) was then extracted with 0.7 liters of ethyl acetate, and the extract was dried on a rotary evaporator at 40°C. NMR spectra were recorded at 270 MHz for ^1H and 67.9 MHz for ^{13}C in D_2O (JEOL JNM EX-270 spectrometer) to confirm the identity of the product. The data indicated the structure of 2HC2CA as shown by Eaton and Chapman (data not shown) (*10*). The preparation of 2HC2CA was dissolved in 20 ml of ethyl acetate and stored in a freezer at −80°C.

Preparation of the Enzymatic Product for ^1H NMR—2HC2CA isomerase (1.67 units) was incubated in 10 mM Tris-HCl buffer (pH 7.4) containing 2.5 mM GSH at 10°C for 1-3 h. Twenty microliters of this preparation was dissolved in 77 μl of bicine buffer (100 mM, pH 8.1) containing 10 mM 2HC2CA and 2.6 mM GSH. After incubation at 30°C for 2 min, 70 μl of the reaction mixture was subjected to HPLC: column, Nucleosil C18 (Macherey-Nagel, 4×50 mm); mobile phase, acetonitrile-water (1:10) containing 1 mM potassium dihydrogen phosphate and 1 mM tetra-*n*-butylammonium hydrogen sulfate; flow rate, 1 ml/min. The product (retention time, 20.2 min) detected at 350 nm by the spectrophotometer was fractionated in a tube containing 20 μl potassium phosphate buffer (1 M, pH 7.3) and frozen immediately in liquid nitrogen. After repetition of this procedure 17 times, the combined preparations were lyophilized and dissolved in 0.5 ml of the d_6-DMSO. The ^1H NMR spectrum was recorded at 300 MHz (JEOL AL-300 spectrometer).

Effects of Various Reagents on the Isomerase Activity—The isomerase activity was assayed under the standard assay conditions, except that SH reagents, chelators, or metal ions were added to the reaction mixture at the start of reaction. The initial concentrations of the reagents and GSH in the reaction mixture were 2 and 5 mM, respectively. In separate experiments, the enzyme was preincubated with these reagents before the activation. For reagents other than SH reagents, 4 μl of the enzyme (5.4 milliunits) was mixed with 35 μl of the reagent (2.3 mM), dissolved in 100 mM Tris-HCl buffer (pH 7.4), and the mixtures were incubated at 30°C for 30 min. Then the activity was assayed by the standard method. For monoiodoacetic acid (MIA) and *N*-ethylmaleimide (NEM), which reacted with GSH, 180 μl of the enzyme (0.45 unit) was mixed with 20 μl of the reagent (50 mM) dissolved in 500 mM Tris-HCl buffer (pH 7.4). After incubation at 30°C for 30 min, the enzyme solution was filtered immediately through a Sephadex G-25 column (0.6×8.5 cm) equilibrated with 100 mM Tris-HCl buffer (pH 7.4) and the active fractions were combined. The residual activity was measured by the standard method.

RESULTS

2HC2CA Isomerase in Cell-Free Extracts—*Pseudomonas* sp. TA-2 was cultured in the nutrient medium or the mineral salt medium containing 2-amino-1-naphthalenesulfonate (tobias acid) (0.2%) or glucose (0.5%). The 2HC2CA isomerase activities of these cell-free extracts were examined. The activity of the extract from the cells grown in glucose was low (0.18 unit/mg protein), but the activities of

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the extracts from the cells grown in the nutrient medium or the tobias acid medium were high and almost equal (0.97 and 0.99 unit/mg protein, respectively). Therefore, *Pseudomonas* sp. TA-2 was cultured in the nutrient medium for the purification of the enzyme.

Purification and Molecular Weight of 2HC2CA Isomerase from Pseudomonas sp. TA-2—The isomerase was purified to homogeneity from the cell-free extract of *Pseudomonas* sp. TA-2 as described under "MATERIALS AND METHODS." Table I shows a summary of the purification procedure. The Sephadex G-75 was excellent for separating the isomerase and *t*HBP hydratase-aldolase. The specific activity of 2HC2CA isomerase was 62 units per mg of protein, and the enzyme was purified about 380-fold, with a yield of 0.3% of total activity. The final preparation appeared to be homogeneous on both native PAGE and SDS-PAGE (Fig. 3). The molecular mass of the enzyme was estimated to be about 25 kDa by SDS-PAGE (Fig. 3). To estimate the molecular mass under non-denatured conditions, gel filtration was done on Superdex 200. The molecular mass was estimated to be 27 kDa (data not shown). These findings suggest that the enzyme is a monomeric protein.

N-Terminal Amino Acid Sequence of 2HC2CA Isomerase—The N-terminal 30 amino acid sequence of the enzyme was identified. The N-terminal amino acid sequence of the enzyme from *Pseudomonas* sp. TA-2 was aligned in comparison with that of 2HC2CA isomerases from NAH7 plasmid (*nahD*) (*14*), *Pseudomonas aeruginosa* strain PaK1 (*pahE*) (*18*), *Pseudomonas putida* strain OUS82 (*pahD*) (*19, 20*), and *Pseudomonas* sp. strain C18 (*doxJ*) (*13*) (Fig. 4). The N-terminal sequence was found to have 80, 87, 77, and 83% homology with those of the deduced amino acid sequences of *nahD, pahE, pahD,* and *doxJ*, respectively.

Conversion of 2HC2CA to tHBP by the Enzyme—Some researchers (*9, 21*) have reported that the enzymatic product was *cis*-HBP, whereas Eaton and Chapman (*10*) and Kuhm *et al.* (*15*) suggested that *t*HBP was formed. However, none of these studies clearly demonstrated the isomeric form of the enzymatic product, because they used spectrophotometric methods to analyze the product in spite of the difficulty of distinguishing *cis* and *trans* isomers spectrophotometrically. In this study, we analyzed the enzymatic product by ^{1}H NMR. The substrate and the product were separated immediately by HPLC after the incubation of the enzymatic reaction mixture for 2 min at 30°C, because *t*HBP readily reacts with GSH, which was required for the enzymatic reaction. The product was prepared for ^{1}H NMR analysis as described under "MATERIALS AND METHODS." The ^{1}H NMR spectrum shows typical peaks with the *trans* coupling constant (16.5 Hz) at 6.63 and 7.68 ppm, and those with the *cis* coupling constant (9.9 Hz) at 5.60 ppm and 6.53 ppm for olefinic protons, and the ratio of the integrated peak areas were approximately 4:1, respectively (Fig. 5). Next, we examined the stability of the enzymatic product in aqueous solution. When the fractionated enzymatic product was incubated in aqueous solution, the formation of a 2HC2CA-like compound was observed on HPLC analysis. More than 55% of the enzymatic product was converted to this product spontaneously at pH 7 at room temperature in one day, and the reaction reached equilibrium (data not shown). On the other hand, 2HC2CA also converted to *t*HBP spontaneous-

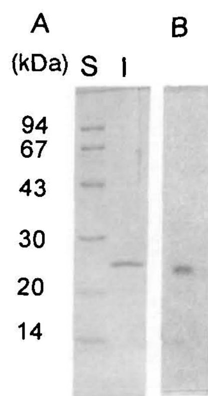

Fig. 3. **PAGE of 2HC2CA isomerase.** (A) SDS-PAGE. Lane I, sample from the Mono-Q column; lane S, molecular mass markers: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). (B) Native-PAGE. Electrophoresis (7.5% gel) was performed at pH 9.4.

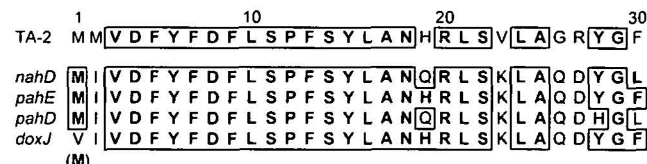

Fig. 4. **N-terminal amino acid sequence alignment of 2HC2CA isomerase and related enzymes.** TA-2, the N-terminal amino acid sequence of 2HC2CA isomerase from *Pseudomonas* sp. TA-2; *nahD*, the deduced sequence of 2HC2CA isomerase encoded by *nahD* (NAH7) from *Pseudomonas putida* PpG1064 (*Pseudomonas putida* PpG7) (*14*); *pahE*, the deduced sequence of 2HC2CA isomerase encoded by *pahE* from *Pseudomonas aeruginosa* strain PaK1 (*18*); *pahD*, the deduced sequence of 2HC2CA isomerase encoded by *pahD* from *Pseudomonas putida* strain OUS82 (*19, 20*); *doxJ*, the deduced sequences of 2HC2CA isomerase encoded by *doxJ* from *Pseudomonas* sp. strain C18 (*13*).

TABLE I. **Purification of 2HC2CA isomerase from *Pseudomonas* sp. TA-2.**

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification	Recovery (%)
Crude extract	568	3,000	18,300	0.164	1.00	100
Streptomycin	697	2,850	13,300	0.214	1.31	95.1
DEAE-Cellulose	234	3,280	6,800	0.483	2.94	109
DEAE-Toyopearl	38.9	1,400	895	1.57	9.56	46.9
Sephadex G75	28.8	264	25.6	10.3	63.0	8.81
Hydroxyapatite	8.2	18.5	1.93	9.57	58.3	0.62
Mono Q	3.0	8.15	0.132	62.0	378	0.27

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ly, and this reaction also reached equilibrium, as elucidated by Eaton and Chapman (*10*). These data are consistent with the interpretation that the enzymatic product was *t*HBP and that 2HC2CA was formed from the enzymatic product; and in this case the peaks with *cis* coupling constant would originate from the olefinic proton of 2HC2CA which was produced during the preparation after the enzymatic reaction. Next, we compared the enzymatic product with *t*HBP converted from 2HC2CA spontaneously. Both their retention times on HPLC and their ultraviolet spectra were identical (spontaneous reaction, R_t=9.51 min, λ_{max}=293 nm; enzymatic reaction, R_t=9.31 min, λ_{max}=295 nm). Therefore, we considered the enzymatic product *t*HBP.

Physicochemical Characteristics of the Enzymes—The effects of temperature on the purified isomerase from *Pseudomonas* sp. TA-2 were examined. After incubation for 10 min, the enzyme was stable at temperature up to 45°C, and 13% of the original activity remained after the incubation at 55°C (Fig. 6B). In the presence of GSH at 2.5 mM, the enzyme was stable up to 50°C, and 68% of the original activity remained after the incubation at 55°C. The effects of pH on stability were also examined. The enzyme was stable in the range of pH 1.5 to 10.0 (at 4°C for 40 h), and the activities increased slightly at the extremes of this range (Fig. 6A). The effects of pH on activity were also examined (Fig. 6C). The *t*HBP hydratase-aldolase activity was sufficient to measure the 2HC2CA isomerase activity at all pHs tested. The optimum pH of the 2HC2CA isomerase was about 8 (Fig. 6C). The isoelectric point (pI) of the enzyme was determined to be 5.0 by isoelectric focusing. Lineweaver-Burk plots gave a K_m value of 53 μM for 2HC2CA (data not shown).

Activation of Isomerase—Since GSH was needed for the activation stage and the reaction stage in the standard assay procedure, we examined the effects of GSH on the activation and the reaction. First, we examined the effect of 2HC2CA on activation as a preliminary experiment. The enzyme was incubated in 93 mM bicine buffer (pH 8.1) containing 0.32 mM 2HC2CA before the enzyme assay. The activity was 29% of that assayed without 2HC2CA pretreatment. Therefore, it was necessary to activate the enzyme without the substrate. Next, we examined the time course of the activation under the several concentrations of GSH (Fig. 7). The activity increased significantly when the enzyme was preincubated with GSH at above 0.15 mM for more than 15 min, and the activation rates depended on the concentration of GSH. The activity after preincubation without GSH was about 10% of that after preincubation with more than 0.156 mM GSH for 60 min, even though the reaction mixture contained 2.5 mM GSH. 2HC2CA appeared to inhibit the activation of the enzyme by GSH in the reaction mixture. This phenomenon was favorable for the measurement of the activity of the enzyme in various states. Figure 6D shows the effects of pH on the activation of the enzyme. The optimal pH for the activation was about 10, and the activations at pH 5 and 7 were 48 and 78%, respectively. Next, GSH-related compounds were used for the activation (Table II). The enzyme was preincubated for 3 h with GSH, GSSG, *S*-methylglutathione (GSMe), or other reductants at 2.5 mM, and this enzyme solution was added to a substrate solution containing *t*HBP hydratase-aldolase and GSH or a substrate solution containing *t*HBP hydratase-aldolase only. After the reaction for 2 min, the reaction mixtures were injected into the HPLC system and

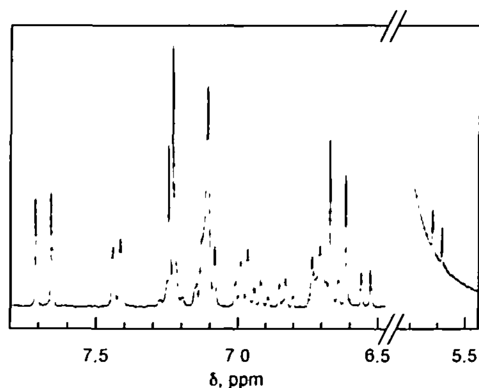

Fig. 5. **NMR spectrum of the enzymatic product in d_6-dimethyl sulfoxide.** The preparation procedures were as described "MATERIALS AND METHODS." The ¹H NMR spectrum was recorded at 300 MHz. ¹H COSY NMR analysis indicated that the peaks around 7.23 ppm were due to impurity.

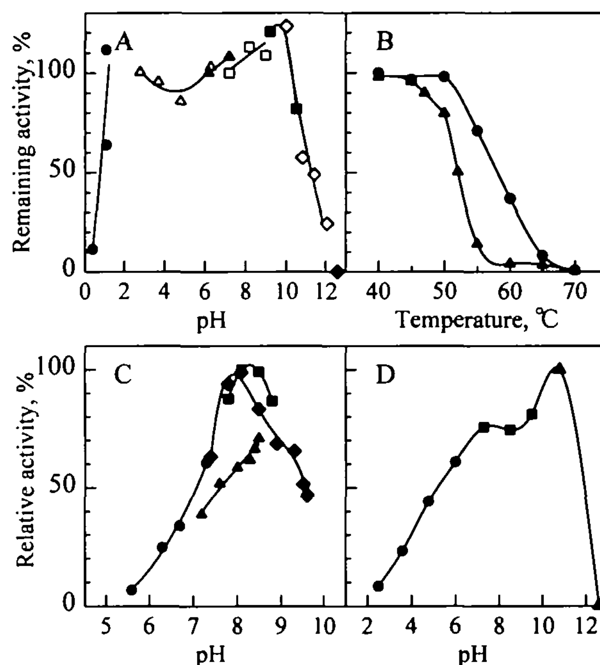

Fig. 6. **Effects of pH and temperature on 2HC2CA isomerase activity, stability, and activation.** A: pH stability: the remaining activity was measured under the standard assay conditions after incubation at various pHs for 40 h at 4°C. The following buffers were used: HCl (●), 250 mM acetate (△), 250 mM phosphate (▲), 250 mM Tris-HCl (□), 250 mM glycine (■), 250 mM CAPS (◇), NaOH (◆). B: Thermal stability: the remaining activity was measured under the standard assay conditions after incubation at various temperatures for 10 min in 100 mM potassium phosphate buffer (pH 7.1) in the absence (▲) or presence (●) of 2.5 mM GSH. C: Optimum pH: the enzyme activity was measured in 170 mM potassium phosphate buffer (●), 170 mM Tris-HCl buffer (▲), 170 mM bicine buffer (■), and 170 mM glycine buffer (◆). A small amount of salicylaldehyde was formed in the blank solutions in 2 min at all pHs. Each blank value was subtracted from the test value. D: Effect of pH on the activation of enzyme: the enzyme was incubated with 2.5 mM GSH in 300 mM acetate buffer (●), 300 mM Tris-HCl buffer (■), and 300 mM CAPS buffer (▲) at 10°C for 4 h. The activity was measured under the standard assay conditions except for the activation of enzyme.

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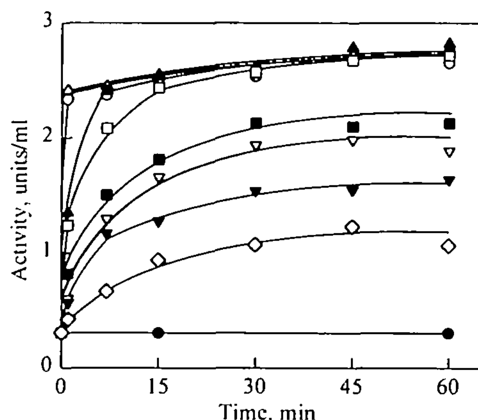

Fig. 7. **Time course of the activation of 2HC2CA isomerase by GSH.** The enzyme was incubated with 0 mM (●), 0.0049 mM (◇), 0.0098 mM (▼), 0.020 mM (▽), 0.039 mM (■), 0.156 mM (□), 0.625 mM (▲), 2.5 mM (△), and 10 mM (○) GSH in 10 mM Tris-HCl buffer (pH 7.8) at 10°C for 0, 1, 7, 15, 30, 45, and 60 min. The activity was measured immediately under the standard assay conditions except for the activation of the enzyme.

TABLE II. **Activation of 2HC2CA isomerase from *Pseudomonas* sp. TA-2 by various reducing agents.**

Reducing agent	Relative activity (%)	
	+GSH in reaction mixture	−GSH in reaction mixture
GSH	100	17[a]
GSSG	55	0.4 (0.4[b])
S-Methyl glutathione	25	0.4
Dithiothreitol	25	0.9 (0.4[c])
2-Mercaptoethanol	9.7	0
Cysteine	11	0.1
Ascorbic acid	5.3	0.2
None	7.4	0.1

The enzyme was incubated with various reducing agents at 2.5 mM in 10 mM Tris-HCl buffer at 10°C for 3 h. The activity was measured without or with GSH (2.5 mM) in the reaction mixture, and is expressed relative to the activity (11 units) measured under the standard assay conditions. [a]GSH (0.4 mM) derived from the enzyme solution was present in the reaction mixture. [b]In the presence of 2.9 mM GSSG in the reaction mixture. [c]In the presence of 2.9 mM DTT in the reaction mixture.

the amount of products was measured. GSSG, DTT, and GSMe, as well as GSH, were effective activator in the presence of GSH in the reaction mixture. In contrast, GSH was the principal factor for the activation in the absence of GSH in the reaction mixture. But this reaction mixture contained 0.4 mM GSH derived from the enzyme solution. To investigate further whether GSH was essential for the enzymatic isomerization, the enzyme was preincubated with GSSG, which is a structural analogue but not a thiol, or DTT, which is a typical thiol, and the activity was measured in the presence of GSSG (2.9 mM) or DTT (2.9 mM) in reaction mixture, respectively. The activities were very low (Table II). GSH was required uniquely for the isomerization of 2HC2CA by the enzyme.

Effects of Various Compounds—The effects of SH reagents, chelators, and metal ions were examined in two estimations. The experimental procedures were as described under "MATERIALS AND METHODS." When the reagents were added at the start of the reaction, the isomerizing activity was inhibited by 99, 65, and 30% by Hg^{2+}, MIA, and Cu^{2+}, respectively, while NEM, *p*-chloromercuribenzoic acid, EDTA, *o*-phenanthroline, 2,2′-bipyridine, KCN, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Sn^{2+}, and Ni^{2+} were ineffective. To investigate inhibitory effects of chelators and SH reagents further, the enzyme was preincubated with 2 mM EDTA, 2 mM *o*-phenanthroline, 2 mM 2,2′-bipyridine, 2 mM KCN, 5 mM MIA, 5 mM NEM, or 5 mM *p*-chloromercuribenzoic acid at 30°C for 30 min before the activation, and then the activity was assayed. More than 80% of the initial activity remained in all cases.

DISCUSSION

In this study, we purified the 2HC2CA isomerase from the cells of *Pseudomonas* sp. TA-2 and characterized it. We first examined the stability of the enzymatic product in the reaction mixture and found that it easily reacted with GSH to give a new product, which was detected by HPLC. For this reason, we measured the isomerase activity by converting the product of its reaction with 2HC2CA in the presence of GSH to salicylaldehyde by use of an excess of *t*HBP hydratase-aldolase, and assaying the salicylaldehyde by HPLC. This method was effective to suppress the formation of byproduct, which was negligible on HPLC analysis. It was also effective for measuring the isomerase activity in cell-free extracts, because the subsequent reaction in the metabolic pathway, catalyzed by salicylaldehyde dehydrogenase, did not proceed and salicylaldehyde was not further converted in the absence of NAD^+ as cofactor.

The activities of the cell-free extracts from the cells grown in tobias acid and nutrient medium were almost equal, and higher than the activity in the extract from the cells grown in glucose. These finding suggested that the amino acids metabolism is involved with the production of tobias acid degradation enzymes in this bacterium. Ensley *et al.* (*22*), Kurkela *et al.* (*23*), and Denome *et al.* (*13*) reported that naphthalene dioxygenase was produced when the bacterium was grown on the medium with tryptophan.

The N-terminal sequence up to residue 30 had high homology with the deduced amino acid sequences of the 2HC2CA isomerase of *nahD* (*14*), *pahE* (*18*), *pahD* (*19, 20*), and *doxJ* (*13*), calculated at 80, 87, 77, and 83% respectively. These results indicate that the naphthalenesulfonate degradation in *Pseudomonas* sp. TA-2 has a close relationship with a naphthalene degradation by the NAH7 plasmid, naphthalene, and phenanthrene degradation by the PAH genes, and dibenzothiophene degradation by the DOX gene. Denome *et al.* reported that DOX proteins converted naphthalene to salicylate and DOX sequence encodes a complete upper naphthalene catabolic pathway similar to NAH (*13*).

Eaton and Chapman suggested that *t*HBP is formed by 2HC2CA isomerase encode on the naphthalene catabolic plasmid NAH7 (*10*). Kuhm *et al.* obtained the 2HC2CA isomerase from *Pseudomonas testosteroni* A3 and examined the enzymatic product by spectrophotometric methods (*15*). However, these data did not demonstrate that the enzymatic product had *trans* form, because it is difficult to distinguish between the *trans* and *cis* isomers spectro-

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photometrically. When, we examined the enzymatic product by ^{1}H NMR, the *trans* form of the enzymatic product was evident from the coupling constant ($J=16.5$) for olefinic hydrogen. Further, we compared the enzymatic product and the spontaneous product, because Eaton and Chapman (*10*) showed that the spontaneous product was *t*HBP. Their retention times on HPLC and their ultraviolet spectra were identical. These data show that the enzymatic product was *t*HBP.

Kuhm *et al.* (*15*) reported the activation of 2HC2CA isomerase from *P. testosteroni* A3 by GSH, GSSG, DTT, and 2-mercaptoethanol. The 2HC2CA isomerase from *Pseudomonas* sp. TA-2 was also activated by these compounds, but the activation levels were diverse. The activation of the TA-2 isomerase by 2-mercaptoethanol and DTT was low compared with the A3 enzyme. Further, the A3 isomerase was less activated at 10 mM GSH than at 2.5 mM, but the TA-2 isomerase was activated to the same degree at both concentrations. The effect of pH on activation by GSH (Fig. 6D), pH optimum (Fig. 6C), and the lack of inhibition by EDTA were similar to those of the isomerase from A3.

Nucleophilic attack by an SH group on a carbon-carbon bond has been proposed for the *cis-trans* isomerization (*15, 24, 25*). This mechanism appears to be applicable to the conversion of 2HC2CA to *t*HBP by this isomerase, because inhibition occurred on addition of Hg^{2+} and MIA, which react with SH group, to the reaction mixture at 2 mM in the presence of 5 mM GSH. Because these reagents also react with GSH, the interpretation of these results was difficult. The fact that GSH was in excess over MIA, however, suggests that MIA might be involved directly in the inhibition of the isomerization. In contrast with this result, the preincubation of enzyme with MIA or NEM caused no appreciable inhibition. This suggests that the enzyme does not contain an SH group that is involved in the enzymatic isomerization. In addition, the deduced amino acid sequences of *doxJ* and *pahE*, which are putative 2HC2CA isomerase genes and appear to be homologous to the isomerase from TA-2, included no cysteine residue. It was presumed that an SH group of the enzyme was not necessary for the enzymatic isomerization. Moreover, GSH appeared to be absolutely required for the enzymatic isomerization of 2HC2CA, though GSSG and GSMe influence the activation. It was conceivable that GSH provided an SH group for the reaction and functioned as a cofactor. Further work is required to elucidate the mechanism of the enzymatic isomerization.

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