Characterization of the *ybdT* Gene Product of *Bacillus subtilis*: Novel Fatty Acid β-Hydroxylating Cytochrome P450

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ABSTRACT: We have characterized the gene encoding fatty acid α-hydroxylase, a cytochrome P450 (P450) enzyme, from *Sphingomonas paucimobilis*. A database homology search indicated that the deduced amino acid sequence of this gene product was 44% identical to that of the *ybdT* gene product that is a 48 kDa protein of unknown function from *Bacillus subtilis*. In this study, we cloned the *ybdT* gene and characterized this gene product using a recombinant enzyme to clarify function of the *ybdT* gene product. The carbon monoxide difference spectrum of the recombinant enzyme showed the characteristic one of P450. In the presence of H_2O_2, the recombinant *ybdT* gene product hydroxylated myristic acid to produce β-hydroxymyristic acid and α-hydroxymyristic acid which were determined by high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry. The amount of these products increased with increasing reaction period and amount of H_2O_2 in the reaction mixture. The amount of β-hydroxyl product was slightly higher than that of α-hydroxyl product at all times during the reaction. However, no reaction products were detected at any time or at any concentration of H_2O_2 when heat-inactivated enzyme was used. HPLC analysis with a chiral column showed that the β-hydroxyl product was nearly enantiomerically pure *R*-form. These results suggest that this P450 enzyme is involved in a novel biosynthesis of β-hydroxy fatty acid.

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β-Hydroxy fatty acids are usually found as their CoA derivatives in the fatty acid β-oxidation pathway, where β-hydroxy acyl-CoAs are produced from the corresponding acyl-CoAs by sequential reaction of acyl-CoA dehydrogenase and Δ2-enoyl-CoA hydratase. Gram-negative bacteria have β-hydroxy fatty acids as acyl moieties of cell wall lipids such as lipid A and ornithine lipid (1), whereas gram-positive bacteria such as *Bacillus* produce acylpeptides containing β-hydroxy fatty acid, as antibiotic compounds (2). Whether this acyl moiety is derived from the β-hydroxy intermediate in the β-oxidation pathway or is produced in another metabolic pathway is unclear.

In contrast, α-hydroxy fatty acids are components of sphingolipids of various species. We have studied fatty acid α-hydroxylase from *Sphingomonas paucimobilis*, a bacterium with large amount of sphingoglycolipid containing α-hydroxymyristic acid as an acyl moiety. Fatty acid α-hydroxylase is a unique enzyme which introduces an oxygen atom from H_2O_2 into fatty acid to produce α-hydroxy fatty acid (3). We recently cloned the fatty acid α-hydroxylase gene from this bacterium (4). Sequence analysis of the cloned gene and spectral analysis of the recombinant enzyme indicated that fatty acid α-hydroxylase from *S. paucimobilis* was a cytochrome P450 (P450) enzyme, which is designated as $P450_{SP\alpha}$ here. P450 enzymes have several highly conserved regions in their amino acid sequences associated with their secondary structure (5). $P450_{SP\alpha}$ also has the conserved sequences, especially in helix-K. However, a few sequences of $P450_{SP\alpha}$ are unique as compared to other bacterial P450s: (i) the conserved Thr in helix-I, which is believed to be important for O_2 activation (6), was absent; (ii) the presence of an aromatic region (7); and (iii) the consensus sequence in the heme-binding region, which was modified by insertion of amino acids (4).

Recently, the complete genome sequence of *B. subtilis* (8) was released into the DDBJ/GenBank/EMBL database. By homology search, we found that the deduced amino acid sequence of the *ybdT* gene (accession number, AB006424) product, which is a 48-kDa protein of unknown function, was significantly homologous to that of $P450_{SP\alpha}$. Moreover, the *ybdT* gene product appeared to have sequences highly homologous to those of $P450_{SP\alpha}$ in helix-I, aromatic region, and heme-binding region. Thus, we hypothesized that *ybdT* encodes fatty-acid hydroxylating P450, and in this study, we characterized its gene product.

MATERIALS AND METHODS

Materials. Bacillus subtilis IFO14144 was obtained from the Institute for Fermentation (Osaka, Japan). *Escherichia coli* BL21 and KOD DNA polymerase were purchased from Toyobo Co., Ltd. (Tokyo, Japan). The expression plasmid pGEX-4T-1 and glutathione-Sepharose 4B were purchased from

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Abbreviations: ADAM, 9-anthryldiazomethane; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; P450, cytochrome P450; $P450_{BS\beta}$, fatty acid β-hydroxylating P450 from *Bacillus subtilis*; $P450_{SP\alpha}$, fatty acid α-hydroxylating P450 from *Sphingomonas paucimobilis*; TMS, trimethylsilyl.

Amersham-Pharmacia. 9-Anthryldiazomethane (ADAM) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). 3-[(3-cholamidopropyl)-Dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from Nakarai Tesque (Kyoto, Japan). Diazomethane was synthesized from *N*-nitroso-*N*-methylurea (Sigma Chemical Co., St. Louis, MO). α-Hydroxymyristic acid and β-hydroxymyristic acid were purchased from Sigma Chemical Co. Other reagents was purchased from Wako Pure Chemicals (Osaka, Japan).

Cloning of the ybdT *gene, and expression and purification of a glutathione* S*-transferase-*ybdT *gene product fusion protein. Bacillus subtilis* was cultured in Luria broth at 37°C. Genomic DNA was isolated by standard procedure (9). The *ybdT* gene was amplified by polymerase chain reaction (PCR) using the following primers: 5′-CGGGATCCATGAATGAG-CAGATTCCACATG-3′ (the sense primer including the start codon of *ybdT* gene) and 5′-CCGCTCGAGGACAACAAA-ATGGTATCAGAAG-3′ (the antisense primer, complementary to the sequence downstream from the *ybdT* gene). After amplification, a 1.4 kbp single band was determined by agarose-gel electrophoresis. The amplified DNA fragment was isolated from the agarose gel and digested by *Bam* HI and *Xho* I. The *Bam* HI-*Xho* I fragment was ligated into a *Bam* HI-*Xho* I-digested pGEX4T-1, and the resulting expression plasmid including the *ybdT* gene was transfected into *E. coli* BL21. Sequencing of the cloned polymerase chain reaction fragment indicated that the nucleotide sequence of the isolated gene from *B. subtilis* IFO14144 was completely identical to the reported sequence of *ybdT*. Expression and purification of a glutathione *S*-transferase-*ybdT* gene product fusion protein were performed by a modification of the method previously described (4). Briefly, cells were disrupted with sonication in 0.1 M Tris-HCl (pH 7.5), 20% glycerol, and 1 mM dithiothreitol, and the supernatant was obtained by centrifugation at 100,000 × *g* for 60 min. Glutathione *S*-transferase-*ybdT* gene product fusion protein was bound to glutathione-Sepharose and eluted with 0.1 M Tris-HCl (pH 7.5), 20% glycerol, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM reduced glutathione, and 1 mM dithiothreitol.

Enzyme assay and determination of reaction product. Fatty acid hydroxylation activity was determined by a modification of the method for assaying $P450_{SP\alpha}$ activity (4). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 5.9), 0.2 mM H_2O_2, 60 μM myristic acid, and the enzyme preparation in a total volume of 0.2 mL. Incubation was carried out at 37°C for the indicated periods. The reaction was terminated by the addition of 20 μL of 2 N HCl, and then the products and the substrate were extracted with ethyl acetate. The extract was washed with distilled water, and the ethyl acetate layer was evaporated. For high-performance liquid chromatography (HPLC) analysis, the resulting residue was treated with ADAM. HPLC analysis of ADAM-derivatized fatty acids was performed by a minor modification of the method described previously (10).

For gas chromatography–mass spectrometry (GC–MS), the products were treated with diazomethane and then sequentially treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide. GC–MS was performed essentially as described previously (3). For GC, samples (1 μL) were introduced onto the column which was operating at 250°C. A GC oven temperature program was then applied: the initial temperature of 150°C was held for 4 min, and then the temperature was increased linearly to 270°C by 4°C/min. Helium was used as the carrier gas at a flow rate of 0.5 mL/min. The GC–MS interface temperature was 250°C. Electron-impact MS was performed with an ion source temperature of 250°C, emission current of 0.37 mA, and electron energy of 40 eV.

Analysis of optical configuration of α-hydroxy fatty acids. The optical configuration of the hydroxyl products was determined by HPLC with an OA-3100 column (Sumika, Osaka, Japan) according to the method of Nakagawa *et al*. (1). After the reaction, the hydroxymyristic acids were treated with 3,5-dinitrophenyl isocyanate (Sumika) in the presence of a small amount of dry pyridine at room temperature overnight. The solvents used for the HPLC analysis was methanol containing 50 mM ammonium acetate and 10% distilled water. Flow rate was 0.5 mL/min.

RESULTS AND DISCUSSION

As shown in Figure 1, the amino acid sequence of the *ybdT* gene product indicated as BSβ showed significant homology to that of $P450_{SP\alpha}$ (overall identity, 44%). Helix-I, helix-K to the aromatic region, and the heme-binding region were highly homologous. As described in the introduction, many P450 have the conserved Thr in helix-I. However, both the *ybdT* gene product and $P450_{SP\alpha}$ lack the conserved Thr, suggesting that the *ybdT* gene product does not require O_2 activation for its catalytic activity, as $P450_{SP\alpha}$ does not (4). In helix-K, the Glu-Xaa-Xaa-Arg motif which is conserved in all P450 was also conserved in the *ybdT* gene product. The aromatic region was proposed by Gotoh and Fujii-Kuriyama (7). This region was originally found in eukaryotic P450, but not in bacterial P450 with a few exceptions, one of which was $P450_{SP\alpha}$. The *ybdT* gene product also has an aromatic region similar to that of $P450_{SP\alpha}$ (75% identical). Moreover, the consensus motif of the heme-binding region was modified by insertion of amino acids, similar to $P450_{SP\alpha}$ (4), although the heme-binding Cys was found at position 363. On the basis of these observations, we assumed that the *ybdT* gene product would show similar enzymatic properties to $P450_{SP\alpha}$, and thus characterized the *ybdT* gene product using a recombinant enzyme.

We constructed a fusion gene of glutathione *S*-transferase gene and the *ybdT* gene in pGEX 4T-1, and then the fusion protein was expressed in *E. coli*. The CO difference spectrum of the fusion protein is characteristic of P450, which showed a peak at 446 nm (Fig. 2). Thus, we refer to the *ybdT* gene product as $P450_{BS\beta}$ (fatty acid β-hydroxylating cytochrome P450 from *B. subtilis*) below. Next, we investigated whether the recombinant $P450_{BS\beta}$ enzyme could hydroxylate fatty acid in the presence of H_2O_2 similarly to $P450_{SP\alpha}$. HPLC analysis of products using myristic acid as the substrate

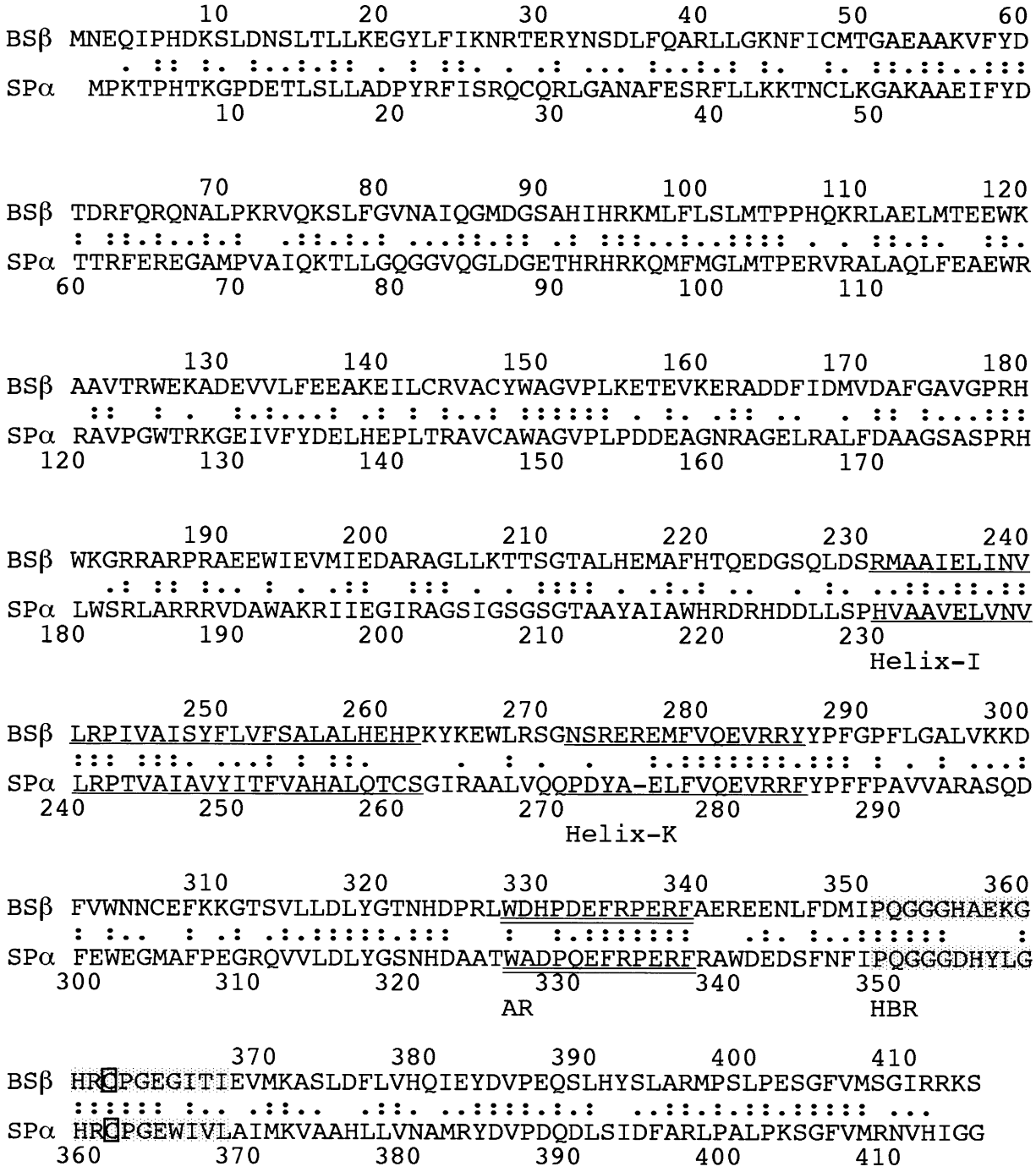

FIG. 1. Comparison of amino acid sequences of fatty acid β-hydroxylating cytochrome P450 from *Bacillus subtilis* ($P450_{BS\beta}$) and fatty acid α-hydroxylating cytochrome P450 from *Sphingomonas paucimobilis* ($P450_{SP\alpha}$). $P450_{BS\beta}$ and $P450_{SP\alpha}$ are indicated as BSβ and SPα, respectively. Helix-I and helix-K are underlined. The aromatic region (AR) is double-underlined. The heme-binding region (HBR) is shaded. Heme-binding cysteines are boxed. Dots and double dots indicate similar amino acids and identical amino acids between $P450_{BS\beta}$ and $P450_{SP\alpha}$, respectively.

showed newly produced peaks at retention times of 12.5 and 15.2 min after reaction (compare Fig. 3A and 3B). The retention times of these peaks corresponded with those of authentic β-hydroxymyristic acid (12.5 min) and α-hydroxymyristic acid (15.2 min), respectively. To further characterize these products, we performed GC–MS analysis for methyl, trimethylsilyl (TMS)-products. One of these products showed a fragmentation pattern of the methyl, TMS-derivative of β-hydroxymyristic acid (Fig. 4A). The ions at *m/z* 175 and 257 are formed by cleavage between C_3 and C_4 and between C_2 and C_3, respectively. The ion at *m/z* 175 is characteristic for the hydroxylation of fatty acid at the C_3-position. The ion at *m/z* 315 (M – 15) is formed by cleavage between CH_3 and Si of the TMS moiety. The fragmentation pattern of the other prod-

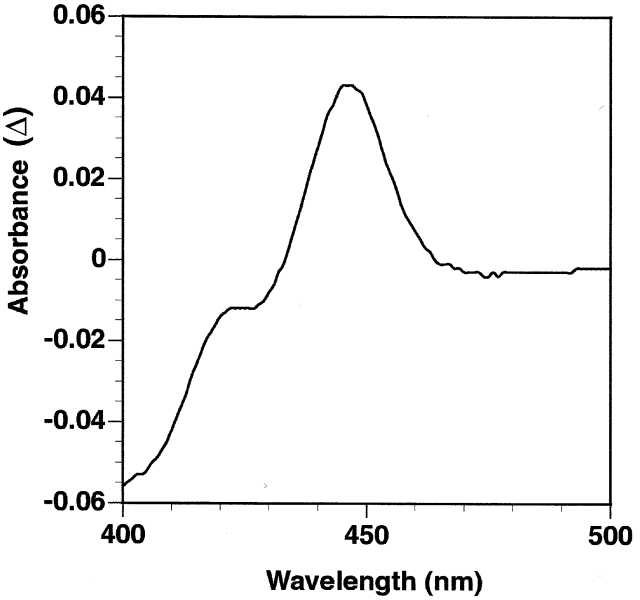

FIG. 2. CO difference spectrum of $P450_{BS\beta}$. $P450_{BS\beta}$ was dissolved in 0.1 M potassium phosphate buffer (pH 7.2) containing 20% glycerol and 1 mM dithiothreitol. The final concentration of protein was 0.28 mg/mL. For abbreviation see Figure 1.

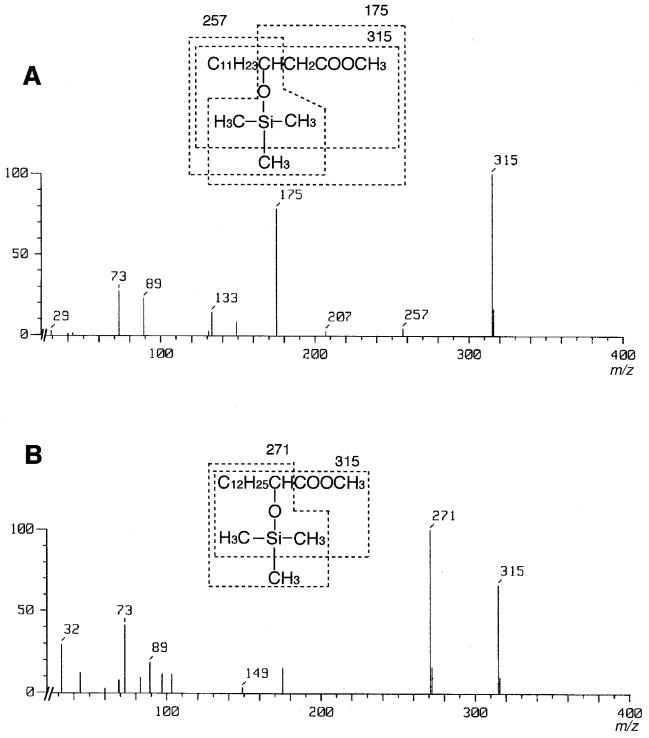

FIG. 4. Determination of methyl, trimethylsilyl (TMS)-derivatives of products by gas chromatography–mass spectrometry. Methyl, TMS-derivatives of two products found in Figure 3 showed fragmentation patterns of β-hydroxymyristic acid (A) and α-hydroxymyristic acid (B).

uct corresponded to that of the methyl, TMS-derivative of α-hydroxymyristic acid (Fig. 4B). The ion at *m/z* 315 also was seen. The ion at *m/z* 271 is the characteristic fragment ion of an α-hydroxy fatty acid that has lost the carboxylmethyl group of *m/z* 330 (M). Furthermore, fragmentation patterns of these products were nearly identical to those of methyl, TMS-derivatives of authentic α-hydroxymyristic acid and β-hydroxymyristic acid (data not shown). Based on these results, we concluded that the reaction products were β-hydroxymyristic acid and α-hydroxymyristic acid.

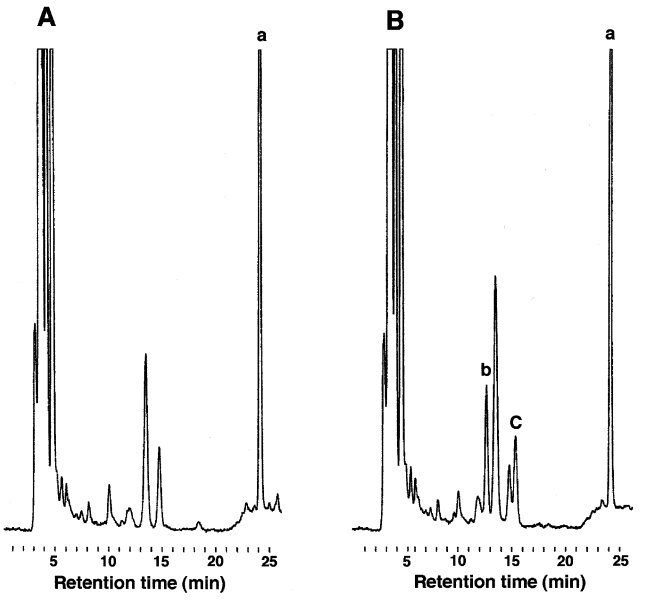

FIG. 3. Identification of products by high-performance liquid chromatography (HPLC). Incubation periods were 0 min (A) and 4 min (B). Peak **a** is that of myristic acid as the substrate. The retention times of peaks **b** and **c**, newly produced after the reaction, corresponded with those of β-hydroxymyristic acid and α-hydroxymyristic acid, respectively.

To determine whether these products were formed enzymatically, we investigated the effects of incubation period, the amount of H_2O_2 added to the reaction mixture, and heat treatment of the enzyme on the amount of products formed. As shown in Figure 5A, the total amount of products increased in a time-dependent manner. The amount of β-hydroxymyristic acid was slightly greater than that of α-hydroxymyristic acid at all sampling times. The amounts of β-hydroxymyristic acid, α-hydroxymyristic acid, and total products increased with the concentration of H_2O_2 in the reaction mixture and approached a plateau at a concentration of 200 μM (Fig. 5B). Other peroxides such as cumene hydroperoxide, *t*-butylhydroperoxide, *t*-butylperoxybenzonate, and *m*-chloroperoxybenzoic acid had no effect on the activity of $P450_{BS\beta}$ (data not shown). Conversely, no product was detected at any time or at any concentration of H_2O_2 when heat-treated enzyme was used. Turnover rate was approximately 300 nmol/min/nmol P450, which was comparable to that of $P450_{SP\alpha}$ (4). These results indicated that formation of these products was due to an enzymatic reaction of $P450_{BS\beta}$.

We demonstrated that $P450_{BS\beta}$ showed fatty acid β-hydroxylation and α-hydroxylation activities and specifically required H_2O_2 for its activity, similar to $P450_{SP\alpha}$ (11). Based on the specific requirement for H_2O_2 and high turnover rate in the pres-

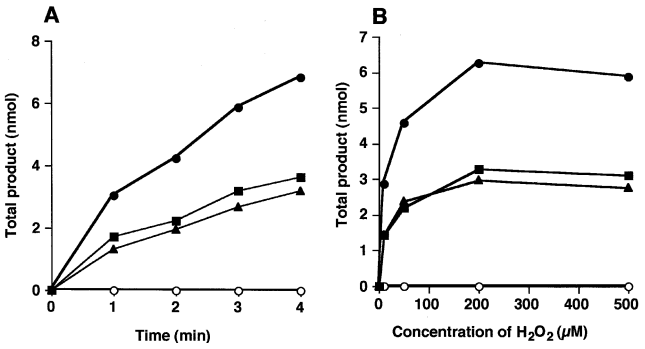

FIG. 5. Changes in the amount of product formed as a result of varying the incubation period (A) and varying the amount of H_2O_2 (B). Amount of the enzyme preparation in the reaction mixture was 1.5 μg (containing 5.7 pmol of P450). (A) Closed and open circles represent the amounts of product formed during the indicated incubation periods when nontreated and heat-treated (100°C, 10 min) enzyme were used, respectively. The concentration of H_2O_2 was 0.2 mM. Closed squares and triangles represent the amounts of β-hydroxyl product and α-hydroxyl product, respectively, when nontreated enzyme was used. (B) The amounts of products formed in the presence of various concentrations of H_2O_2 were measured. The incubation period was 4 min. Symbols are the same as those in (A).

ence of H_2O_2, it may be more appropriate to refer to $P450_{SP\alpha}$ and $P450_{BS\beta}$ as "peroxygenases" (12) rather than "monoxygenases." To our knowledge, this is the first direct evidence that β-hydroxy fatty acid is produced by such a "peroxygenase."

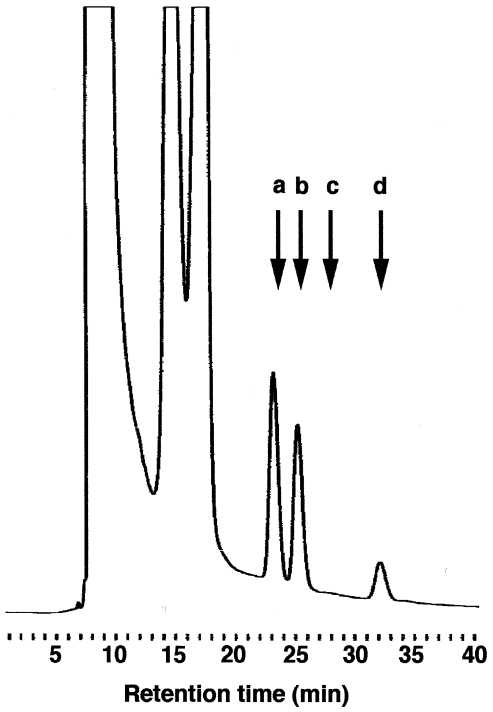

FIG. 6. HPLC analysis of optical configuration of the products. Arrows show the retention times of authentic *R*-enantiomer (**a**, 23.5 min) and *S*-enantiomer (**c**, 28.0 min) of β-hydroxymyristic acid and authentic *R*-enantiomer (**b**, 25.7 min) and *S*-enantiomer (**d**, 32.6 min) of α-hydroxymyristic acid, respectively. For abbreviation see Figure 3.

Configuration analyses indicated that β-hydroxymyristic acid of this acylpeptide was an *R*-enantiomer (D-enantiomer) (13). This configuration is due to favoring the *R*-enantiomer as the fatty acid substrate in acyltransferation step of the acylpeptide biosynthesis (14,15). By HPLC analysis using a chiral column, we observed that β-hydroxymyristic acid formed by the reaction of $P450_{BS\beta}$ is nearly enantiomerically pure *R*-form (Fig. 6). *S*-Form of β-hydroxymyristic acid could not be detected, but *S*-form of α-hydroxymyristic acid was about 22% of the total amount of α-hydroxyl product. In yeast, Venter *et al*. (16) demonstrated that 3-hydroxy-polyenoic fatty acid was *R*-form and suggested that the *R*-form of β-hydroxy fatty acid is formed by other metabolic reactions such as direct oxygenation by P450, because the normal β-hydroxyl intermediate in β-oxidation is the *S*-form. Thus, at least β-hydroxy fatty acid produced by the reaction of $P450_{BS\beta}$ may be utilized to synthesize acylpeptides, antibiotic compounds produced by *Bacillus* species, although the physiological roles of α-hydroxyl fatty acid in this bacterium remain to be elucidated.

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