

Acyloin Formation by Benzoylformate Decarboxylase from *Pseudomonas putida*

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Whole cells and cell extracts of *Pseudomonas putida* grown in a medium containing ammonium mandelate have the capacity to produce the acyloin compound 2-hydroxypropiophenone when incubated with benzoylformate and acetaldehyde. Benzaldehyde and benzyl alcohol were formed as reaction by-products. The enantiomeric excess of the 2-hydroxypropiophenone product was found to be 91 to 92%. The absolute configuration of the enzymatically prepared product at the carbinol carbon was found to be *S*. The thiamine PP₁-linked enzyme benzoylformate decarboxylase, purified to give a single protein band on polyacrylamide gel electrophoresis, was shown to be responsible for the catalysis of this novel condensation reaction.

The advantages of using enzymes in the synthesis of organic compounds relate to their versatility, high reaction rates, and regio- and stereospecificity and the relatively mild reaction conditions involved (15). Stereospecificity is especially important in the synthesis of bioactive molecules, as only one of the enantiomeric molecular forms usually manifests bioactivity. Although enzymes which catalyze asymmetric carbon-carbon bond formation are of great importance in bioorganic synthesis, only a few examples of such biotransformations are currently known (23).

The conversion of benzaldehyde to optically active L-phenylacetyl carbinol by yeast fermentation involves enzyme-catalyzed carbon-carbon condensation, which is a key step in the industrial manufacture of (-)-ephedrine (14). L-Phenylacetyl carbinol formation is mediated by the pyruvate decarboxylase complex (27). Pyruvate is decarboxylated to active acetaldehyde, which forms the carbinol product in the presence of benzaldehyde as a cosubstrate (11, 24). The normal reaction of this enzyme and the acyloin-forming reaction are illustrated in Fig. 1. While yeast pyruvate decarboxylase is known to be relatively specific for pyruvate as a substrate, a range of substituted aromatic aldehydes may be used in place of benzaldehyde as cosubstrates to produce the corresponding optically active L-acetyl aromatic carbinols (17, 21).

Yeast pyruvate decarboxylase (EC 4.1.1.1) is by far the best-characterized enzyme among thiamine PP₁-linked α -ketoacid decarboxylases. Limited investigations have been carried out with two other enzymes which decarboxylate benzoylformate and phenylpyruvate. Benzoylformate decarboxylase, phenylpyruvate decarboxylase, and pyruvate decarboxylase are all tetramers having similar subunit molecular weights (1, 2, 9, 26). However, no common epitopes could be detected by antibodies raised against benzoylformate decarboxylase or pyruvate decarboxylase (3).

The ability of nonoxidative α -ketoacid decarboxylases requiring thiamine PP₁, other than pyruvate decarboxylase, to produce acyloin compounds in the presence of an aldehyde cosubstrate has not, to the best of our knowledge, been

reported. Benzoylformate decarboxylase (EC 4.1.1.7), which is involved in the catabolism of aromatic compounds by metabolically versatile bacteria such as *Pseudomonas putida* and *Acinetobacter calcoaceticus* (2, 25), normally converts benzoylformate to benzaldehyde. Some of the properties of this enzyme are described by Hegeman (13).

In order to extend the range of acyloin compounds available via enzyme biotransformation, we have investigated the capacity of *P. putida* cells containing benzoylformate decarboxylase, and also the purified enzyme, to produce acyloin compounds in the presence of benzoylformate and acetaldehyde. The substrates and possible products of this reaction are illustrated in Fig. 1. The expected acyloin reaction product, 2-hydroxypropiophenone, is a tautomer of phenylacetyl carbinol.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *P. putida* ATCC 12633 was maintained on nutrient agar plates. The mandelate medium for induction of benzoylformate decarboxylase was a modification of the medium of Hegeman (12, 13) and consisted of the following (in grams per liter): ammonium mandelate, 3; nitrioloacetic acid, 0.2; MgSO₄ · 7H₂O, 0.58; CaCl₂ · 2H₂O, 0.067; (NH₄)₆Mo₇O₂₄ · 4H₂O, 0.0002; FeSO₄ · 7H₂O, 0.002; KH₂PO₄, 3.4; Na₂HPO₄ · 7H₂O, 6.7; and yeast extract, 1.0. An agar colony was loop inoculated into a tube containing 5 ml of mandelate medium. Following a 24-h incubation at 30°C, the tube's contents were inoculated into a 250-ml Erlenmeyer flask containing 50 ml of mandelate medium. Flasks were incubated at 30°C on an orbital shaker set at 200 rpm. Erlenmeyer flask cultures were transferred a minimum of four times at 24-h intervals into fresh flask media at an inoculum rate of 5% (vol/vol), and the cells were then harvested from flasks by centrifugation at 4,000 × g after a 16- to 20-h incubation. Cells were washed with 50 mM sodium phosphate buffer, pH 6.0, and recentrifuged, and the pellets were stored frozen until required.

Cell extraction. Thawed cells (14.76 g [wet weight]) were resuspended in 50 mM sodium phosphate, pH 6.0, containing 1 mM EDTA and 0.1 mM dithiothreitol (buffer 1) to a final volume of 74 ml and sonicated in an ice-water bath for three 2-min intervals with a Braun Sonic 2000 set at 170 W. The crude enzyme extract was recovered after cell debris

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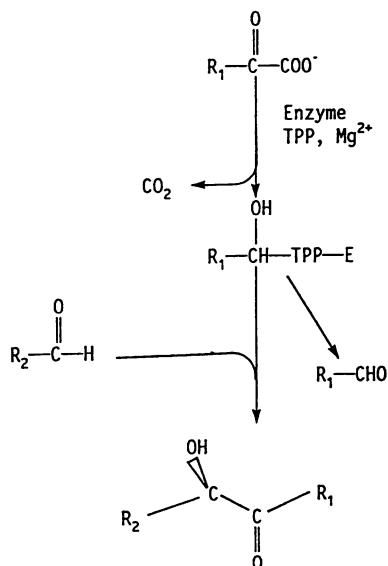


FIG. 1. Production of acyloins by biotransformation. (Upper diagram) The enzyme is yeast pyruvate decarboxylase. R₁, CH₃; R₂, Ph. The product is (*R*)-phenylacetyl carbinol. (Lower diagram) The enzyme is benzoylformate decarboxylase. R₁, Ph; R₂, CH₃. The expected product is 2-hydroxypropiophenone. (Assuming the stereochemistry of the reaction was the same as for yeast pyruvate decarboxylase, the *R* enantiomer would be expected.)

was removed by centrifugation at $9,000 \times g$ for 15 min at 5°C.

Enzyme purification. The crude enzyme extract was heated from 5 to 50°C over a 2-min period, held at this temperature for 5 min, cooled in ice to 5°C, and centrifuged at $9,000 \times g$ for 15 min at 5°C to recover the supernatant. A 45 to 75% ammonium sulfate cut was prepared, with (NH₄)₂SO₄ additions carried out at 4°C followed by holding for 15 min prior to centrifugation at $9,000 \times g$ as described before. The pellet was resuspended in a minimal volume of buffer 1 and recentrifuged, and the clear supernatant was applied to a previously equilibrated Toyo Pearl HW-55 column (2.6 by 20 cm). Eluted fractions containing benzoylformate decarboxylase activity were pooled and applied to a DEAE Toyo Pearl column (1 by 10 cm), which bound the enzyme. The column was eluted stepwise with 100-ml volumes of buffer 1 containing 50, 100, and 150 mM NaCl, with the enzyme being eluted in a sharp peak at 150 mM NaCl.

Preparative polyacrylamide gel electrophoresis (PAGE) was carried out by using a 7% gel concentration according to the method of Ornstein (22) and Davis (5). The thickness of the gel was 1.5 mm, and electrophoresis was carried out with Tris-glycine buffer, pH 8.3, with a current of 15 mA at 5°C for 2 h. The gel was stained with 0.1% Coomassie brilliant blue G250 in methanol-acetic acid-water (10:10:40, vol/vol/vol) for 1 h at 40°C and destained with methanol-acetic acid-water (10:10:20, vol/vol/vol) at 40°C to identify protein bands. The PAGE-purified enzyme was recovered from an unstained gel by slicing and maceration in buffer 1.

Enzyme biotransformations and enzyme assay conditions. Unless otherwise stated, the concentrations of constituents in the enzyme biotransformation reaction mixture (in millimoles per liter) were as follows: sodium phosphate (pH 6.0), 200; benzoylformate, 100; acetaldehyde, 200; thiamine PP₁, 1.5; and magnesium chloride, 2.5. Also included were *P.*

putida cells (0.015 g [dry weight] of cells per ml) or the equivalent as cell extract or purified enzyme. Biotransformation reactions were carried out at 30°C, and the reaction was stopped by boiling for 5 min. Product formation was determined by gas chromatography (GC).

In order to monitor acyloin-forming activity during enzyme purification, the above biotransformation procedure was also used with a 30-min incubation (in an enzyme dilution range in which acyloin formation was proportional to the enzyme concentration). Acyloin formation was measured colorimetrically by using the method of Groger and Erge (10), with racemic 2-hydroxypropiophenone as the standard. One unit of acyloin-forming activity is defined as the amount of enzyme producing 1 μmol of product per min at 30°C under these conditions.

Benzoylformate decarboxylase activity was monitored by using the coupled assay of Barrowman and Fewson (2). Activities were measured by monitoring the reduction of NAD at 340 nm in the presence of an excess of aldehyde dehydrogenase. Reaction mixtures prepared in 3-ml volumes consisted of (final concentrations) 200 mM potassium phosphate buffer, pH 6.0, 1 mM NAD, 0.1 mM thiamine PP₁ (TPP), 1.0 U of yeast aldehyde dehydrogenase (Sigma, St. Louis, Mo.), benzoylformate decarboxylase, and 5 mM benzoylformate (adjusted to pH 6.0) added last to initiate the reaction. The temperature of incubation was 30°C. The increase in absorbance due to the reduction of NAD was measured at 340 nm in a 1-cm light path in a Shimadzu UV-120-02 spectrophotometer (Kyoto, Japan) equipped with a chart recorder. One unit of acyloin-forming activity is the amount of enzyme activity catalyzing the formation of 1 μmol of aldehyde per min at 30°C.

Protein determination. The protein concentration was determined by using the method of Bradford (4). A standard protein calibration curve was prepared with bovine serum albumin.

GC analysis. For GC determination, 2 ml of the biotransformation reaction mixture was extracted three times with 2-ml volumes of glass-distilled ether. The ether extracts were pooled and concentrated under nitrogen to 1 ml. An internal standard of 0.5 ml of cyclohexanone was added to 2 ml of the reaction mixture. This extract was analyzed in a Shimadzu GC (model 14A) equipped with flame ionization detection and a chromatopac C-R6A integrator. The GC contained a fused silica megabore column (30 m by 0.52 mm; inside diameter) coated with a 1-μm thickness of 25% cyanopropyl-25% phenyl-50% methyl polysiloxane (Durabon 225; Chromatographic Specialties, Brockville, Ontario, Canada). Operating conditions comprised column and injector temperatures of 150°C and a detector temperature of 200°C, with helium as the carrier gas. Chemically synthesized racemic 2-hydroxypropiophenone was used as the standard.

Extraction of (-)-2-hydroxypropiophenone from the biotransformation. The biotransformation broth (40 ml) from *P. putida* was extracted with ether (3 times, 150 ml each time). The ether extracts were washed with saturated NaHCO₃ (2 times, 100 ml each time), brine (100 ml), and then dried over Na₂SO₄. Analysis of the ether extracts by thin-layer chromatography (silica gel-hexane ethyl acetate, 2:1) revealed the presence of both benzaldehyde and 2-hydroxypropiophenone by comparison with authentic samples. The ether extracts were concentrated to dryness in vacuo (at 0.05 mm Hg) to provide 30.0 mg of 2-hydroxypropiophenone contaminated by a small amount of benzaldehyde (CHO signal at δ 9.5 in the ¹H nuclear magnetic resonance (NMR)

spectrum, 5 mol%). $[\alpha]_D = -76.6^\circ$ (c 0 to 02 g/ml, CHCl_3). For spectral data, see below.

Preparation of (\pm)-2-hydroxypropiophenone. An authentic sample of racemic 2-hydroxypropiophenone was prepared from propiophenone according to the method of McCormick et al. (20). The synthetic sample was spectroscopically identical to that produced enzymatically: $^1\text{H NMR}$ (200 MHz, CDCl_3) δ 1.4 (d, $J = 7.5$ Hz, 3H), 3.75 (d, 7.5 Hz, 1H, exchanges with D_2O), 5.15 (quintet, $J = 7.5$; Hz, 1H), 7.4 to 7.7 (complex m, 3H), 7.92 (dd, $J = 8.0, 2.0$, 2H). Infrared (thin film) 3,460s, 3,061w, 2,978w, 2,934w, 1,685s, 1,596m, 1,575m, 1,449m, 1,272m, 1,224m, 1,130s, 1,074m, 1,027m, 971s, 897w, 792w, 760w, 700s cm^{-1} .

Determination of optical purity of ($-$)-2-hydroxypropiophenone (from *P. putida*). Both racemic 2-hydroxypropiophenone (see above) and optically active material produced enzymatically were separately acetylated by using the following procedure.

A solution of triethylamine (124 mg, 4.3 mmol) and acetyl chloride (0.22 ml, 3.05 mmol) in CH_2Cl_2 (2.0 ml) was added via syringe to a stirred solution of 2-hydroxypropiophenone (139 mg, 0.95 mmol) in CH_2Cl_2 (5.0 ml) at 0°C . After 30 min, the mixture was diluted with ether (20 ml) and washed with 0.5 M HCl (three times, 30 ml each time), saturated NaHCO_3 (three times, 30 ml each time), and brine (30 ml). The ether phase was dried over MgSO_4 and filtered, and the filtrate was concentrated in vacuo to provide pure material. $^1\text{H NMR}$ (200 MHz, CDCl_3) δ 1.5 (d, $J = 8.6$ Hz, 3H), 2.05 (s, 3H), 5.90 (quartet, $J = 6.8$ Hz, 1H), 7.2 to 7.6 (complex m, 3H), 7.94 (dd, $J = 7.8, 1.8$ Hz, 2H).

For the determination of optical purity, $^1\text{H NMR}$ spectra of the above products, separately dissolved in benzene- d_6 , were recorded in the presence of 4 mol% of the chiral shift reagent tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato]europium(III) [$\text{Eu}(\text{hfc})_3$] (8). Separate signals in a ratio of 1:1 due to the acetate methyl group were observed at δ 1.98 and 1.93 ppm at 200 MHz and 25°C for the racemic material. For the acetate derivative of the enzymatically produced material, these same two signals were present in a ratio of 24:1. This corresponds to an enantiomeric excess of 92%. We have verified this result in two independent trials and have additionally demonstrated that the isolation and derivatization procedure does not lead to racemization with optically pure material.

Preparation of (*S*)-($-$)-2-hydroxypropiophenone. An authentic sample of (*S*)-($-$)-2-hydroxypropiophenone was prepared by using the method of Davis and Haque (6) and converted to the acetate derivative as described above. A $^1\text{H NMR}$ spectrum of this material in the presence of the chiral shift reagent $\text{Eu}(\text{hfc})_3$ (see above) exhibited two resonances at δ 1.98 and 1.93 ppm in a ratio of 2.4:1 (41% ee; lit.^{3a} 43% ee).

RESULTS

Production of the acyloin compound 2-hydroxypropiophenone from benzoylformate with acetaldehyde as the cosubstrate was initially observed with induced whole cells of *P. putida*. GC was employed to monitor the time course of the fermentation process, and the distribution of volatile products as a function of incubation time is depicted in Fig. 2.

A high initial rate of benzaldehyde and 2-hydroxypropiophenone formation was observed in the first 15 min of the biotransformation. A peak concentration of benzaldehyde, 42.7 $\mu\text{mol/ml}$, was observed at 90 min, after which a decline in benzaldehyde concentration was noted. A maximum

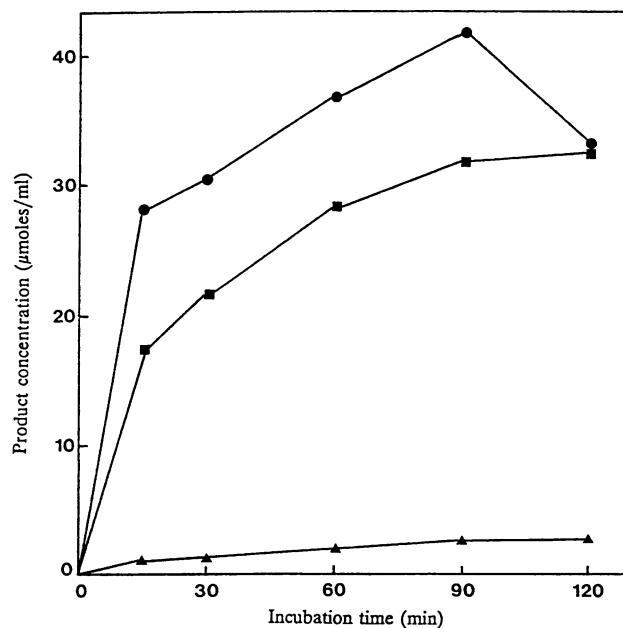


FIG. 2. Pattern of production of 2-hydroxypropiophenone (■), benzaldehyde (●), and benzyl alcohol (▲) from benzoylformate by whole cells of *P. putida* ATCC 12633.

2-hydroxypropiophenone concentration of 32.7 $\mu\text{mol/ml}$ occurred at 120 min. The biotransformation reaction was also monitored over time when whole cells were replaced with a crude cell extract prepared from the same quantity of cells

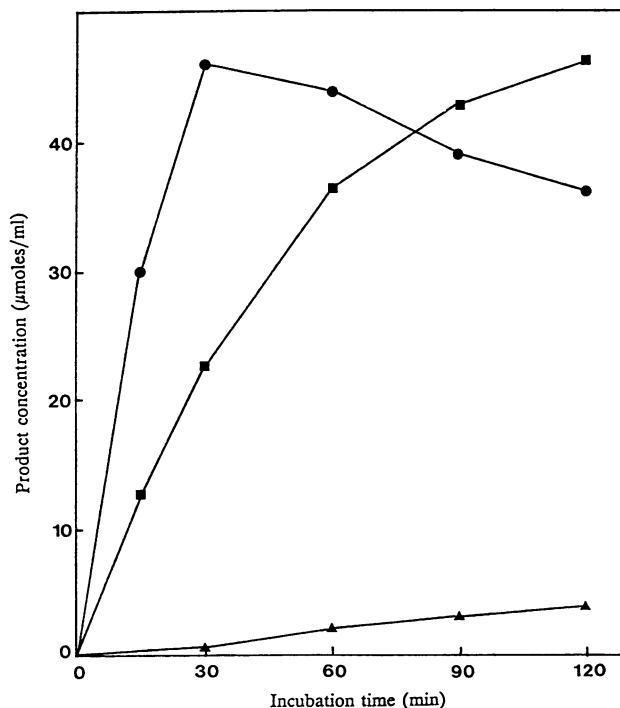


FIG. 3. Pattern of production of 2-hydroxypropiophenone (■), benzaldehyde (●), and benzyl alcohol (▲) from benzoylformate by crude cell extracts of *P. putida* ATCC 12633.

(Fig. 3). Benzaldehyde formation was at its maximum level after 30 min, after which a gradual decrease was observed. The 2-hydroxypropiophenone concentration increased over the course of the biotransformation. The maximum concentrations of benzaldehyde and 2-hydroxypropiophenone achieved during this biotransformation were 45.8 and 46.3 $\mu\text{mol/ml}$, respectively. Thus, production of 2-hydroxypropiophenone was more efficient with crude cell extracts rather than whole cells. In both the whole-cell and crude-cell-extract biotransformations, some benzyl alcohol was also formed, reaching concentrations of 2.7 and 3.9 $\mu\text{mol/ml}$, respectively, at 120 min.

2-Hydroxypropiophenone was isolated from the fermentation broth as described in Materials and Methods and was identified from ^1H NMR and infrared spectra and by comparison to an authentic sample that was independently prepared by using a published method (20).

The by-products, benzaldehyde and benzyl alcohol, were identified by comparing their GC retention times with those of authentic samples and also by GC-mass spectrometry analysis of the final product mixture after extraction with ether.

The enantiomeric excess of 2-hydroxypropiophenone produced under these conditions was found to be 91 to 92% by ^1H NMR spectroscopy of its *O*-acetyl derivative in the presence of a chiral shift reagent (8).

It was possible to determine the absolute configuration of the enzymatically derived product. An authentic sample of (*S*)-2-hydroxypropiophenone (41% ee) was prepared by using a published method (6, 7), and a ^1H NMR spectrum of its acetate derivative was recorded in the presence of the chiral shift reagent. By comparing the two spectra, it was concluded that the absolute configuration at the carbinol carbon of the derived material was also *S*. In addition, the specific optical rotation of this material was also determined, and it was in agreement with that reported for the *S* enantiomer of this compound (6).

The main rationale for carrying out purification studies was to illustrate the coincidence of benzoylformate decarboxylase and acyloin-forming activity. Benzoylformate decarboxylase was purified from cell homogenates by heat treatment, ammonium sulfate precipitation, Toyo Pearl HW55 gel filtration, DEAE Sephadex, and preparative PAGE electrophoresis. The active enzyme fraction eluted from DEAE Sephadex was separated on PAGE to give a band stained intensely with Coomassie blue and many more faint bands (Fig. 4, lane 1). A single benzoylformate decarboxylase activity band corresponding to the protein band stained intensely with Coomassie blue was observed and recovered. This material was rerun on PAGE (Fig. 4, lane 2). During the purification process, benzoylformate decarboxylase activity was measured by using the linked assay of Barrowman and Fewson (2), and, in addition, 2-hydroxypropiophenone-forming activity was monitored by using the colorimetric assay of Groger and Erge (10) (Table 1). The ratio of acyloin-forming activity and benzoylformate decarboxylase activity remained relatively constant throughout the purification protocol, and the presence of both activities in the PAGE-purified preparation indicated the capacity of benzoylformate decarboxylase also to form the acyloin compound. On average, the rate of acyloin formation was found to be 45% of the rate of benzaldehyde formation in the assay procedures used.

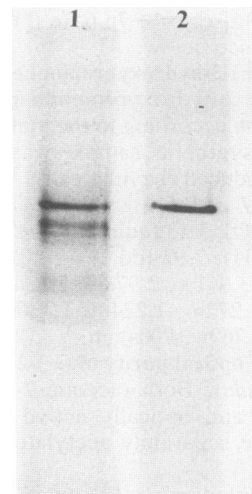


FIG. 4. Coomassie blue-stained protein bands on PAGE gels of the benzoylformate decarboxylase fraction. Shown are the fractions eluted from DEAE-Sephadex (lane 1) and preparative PAGE (lane 2).

DISCUSSION

In this study, the ability of benzoylformate decarboxylase to form the acyloin compound 2-hydroxypropiophenone when incubated with benzoylformate and acetaldehyde has been demonstrated for the first time. The normal reaction product of the enzyme, benzaldehyde, and the acyloin product were both detected after biotransformation. Under the reaction conditions tested, the initial rate of benzaldehyde production was higher than the rate of acyloin production, but similar concentrations of both products were observed as the reaction progressed. It was also noted that benzaldehyde concentration declined after reaching a peak and that low levels of benzyl alcohol were also produced. During pyruvate decarboxylase-mediated production of *L*-phenylacetyl carbinol, some of the benzaldehyde substrate was reduced to benzyl alcohol by yeast oxidoreductases (18). Thus, part of the later decline in benzaldehyde concentration observed in our studies may be due to its reduction to benzyl alcohol by *P. putida* oxidoreductases. In addition, if benzaldehyde can also form a benzoylformate decarboxylase-TPP-active aldehyde substrate intermediate similar to the pyruvate decarboxylase active acetaldehyde complex (16), some of the decline in benzaldehyde may also be due to acyloin formation.

It was interesting that the absolute configuration at the carbinol carbon of (*S*)-2-hydroxypropiophenone produced by *P. putida* is opposite to that of (*R*)-phenylacetyl carbinol prepared by using baker's yeast. The lack of complete stereoselectivity (91 to 92% ee of *S*) is considered to be an intrinsic property of the enzyme reaction and is not due to slow nonenzymatic racemization of the 2-hydroxypropiophenone product. The result was highly reproducible. In addition, in more recent biotransformation studies with the enzyme system from *A. calcoaceticus*, enantioselectivity was essentially complete (>98% ee of *S*) (unpublished work). The mechanism for the key C-C bond-forming step in both cases is the reaction of the TPP-bound active aldehyde, derived from either pyruvate (with phenylacetyl carbinol) or benzoylformate (with 2-hydroxypropiophenone), with the cosubstrate, benzaldehyde or acetaldehyde (Fig. 1). The

TABLE 1. Summary of the protein recovery stages involved in purifying benzoylformate decarboxylase from *P. putida* to a single PAGE band

Fraction	Total protein (mg)	Results of:					
		Benzoylformate decarboxylase assay			Acylain-forming assay		
		Total activity (U)	Sp act (U/mg)	Purification factor	Total activity (U)	Sp act (U/mg)	Purification factor
Crude	1,200.00	361.80	0.30	1.0	156.00	0.13	1.0
Heat treated	470.00	342.70	0.73	2.4	136.30	0.29	2.2
45–75% (NH ₄) ₂ SO ₄	279.00	ND ^a	ND	ND	125.50	0.45	3.5
HW55 peak	158.00	183.30	1.16	3.9	85.30	0.54	4.2
DEAE peak	21.80	120.70	5.54	18.5	70.00	3.21	21.0
PAGE band ^b	0.06	0.94	16.80	55.9	0.36	6.43	49.5

^a ND, not determined.

^b Less than 1 mg of protein was applied to the PAGE gel, <5% of the enzyme units recovered from the DEAE step.

present results demonstrate that the cosubstrates that bind at the active sites of these TPP-linked decarboxylase enzymes must be different in the two cases.

The acylain condensation of aromatic and α,β -unsaturated aldehydes with pyruvic acid [or CH₃(CH₂)_nCOCO₂H], $n = 1$ and 2] mediated by baker's yeast has been extensively studied (23). In all cases, the absolute configuration at the carbinol carbon in the product is the same, *R*. Thus, the present work is significant in that products that are enantiomeric at this carbon can be simply prepared by using a different enzyme system.

Enzymes which can accept a variety of related substrates increase the attractiveness of using biotransformation systems in organic synthesis. Pyruvate decarboxylase manifests relatively high levels of specificity toward its α -ketoacid substrate (16) but can accommodate a variety of acylain-forming cosubstrates (19, 21). Thus, pyruvate decarboxylase can produce acylain compounds with a fixed aliphatic group and a variable aromatic group. Aromatic α -ketoacid decarboxylases such as benzoylformate decarboxylase and phenylpyruvate decarboxylase may offer the potential to produce acylain compounds with fixed aromatic groups and variable aliphatic groups. The delineation of substrate and cosubstrate specificities of these enzyme systems is an important area for further research.

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