Eur. J. Biochem. *168*, 153–159 (1987)

Phenylalanine dehydrogenase of *Bacillus badius*

Purification, characterization and gene cloning

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(Received March 23/June 2, 1987 – EJB 87 0344)

Phenylalanine dehydrogenase produced by *Bacillus badius* IAM 11059 was purified from the crude extract of *B. badius* to homogeneity, as judged by disc gel electrophoresis. The enzyme has an isoelectric point of 3.5 and a relative molecular mass, M_r, of 310000–360000. The enzyme is composed of identical subunits with an M_r 41000–42000. The substrate specificity of the enzyme in the oxidative deamination reaction was high for L-phenylalanine, but rather low in the reductive amination reaction, with phenylpyruvate, *p*-hydroxyphenylpyruvate, and 2-oxohexanoate. The gene for the enzyme was cloned into *Escherichia coli* with plasmid pBR322 as a vector. The enzyme was expressed in high level in *E. coli*. The enzyme produced by *E. coli* transformant was purified to homogeneity and shown to be identical to that of *B. badius* IAM 11059 with respect to the specific activity, M_r, subunit structure and amino acid composition.

NAD^+-dependent phenylalanine dehydrogenase (EC 1.4.1.–) catalyzes the reversible oxidation-reduction reactions for L-phenylalanine [1, 2]. The enzyme is speculated to play a role in the degradation of L-phenylalanine, since it was found to be inducible, and a constitutive L-phenylalanine aminotransferase activity was also detected in the cell extract of *Sporosarcina ureae* (Y. Asano, unpublished work). Among the amino acid dehydrogenases, $NADP^+$-dependent glutamate dehydrogenase appears to have diverse physiological action. The enzyme catalyzes the biosynthesis of L-glutamate [3].

During the screening program of the phenylalanine dehydrogenases among a number of yeast and bacterial strains, we found that the enzyme was very narrowly distributed among gram-positive aerobic spore-forming bacteria (Y. Asano, unpublished work). We described the first purification and crystallization of the novel phenylalanine dehydrogenase from *S. ureae* SCRC-R04, which was isolated from soil [2]. During the further screening of the enzyme activity among the microorganisms in the stock cultures, we found that *Bacillus badius* IAM 11059 exhibits higher specific activity and total phenylalanine dehydrogenase activity per culture than the previously reported strain [2]. The occurrence of the enzyme was rare even in the genus *Bacillus*. We characterized the phenylalanine dehydrogenase and cloned the gene encoding the enzyme not only to study the evolutionary relationship among the $NAD(P)^+$-dependent amino acid dehydrogenases and other dehydrogenases, but also to produce the enzyme catalyst for the asymmetric synthesis of L-phenylalanine and related L-amino acids [4].

We here report the purification and characterization of phenylalanine dehydrogenase from *B. badius* IAM 11059, and the cloning in *E. coli* of the gene for the enzyme.

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Enzymes. Phenylalanine dehydrogenase (EC 1.4.1.–); lysozyme (EC 3.2.1.17); adenylate kinase (EC 2.7.4.3); L-lactate dehydrogenase (EC 1.1.1.27); enolase (EC 4.2.1.11); glutamate dehydrogenase (EC 1.4.1.2); site-specific endodeoxyribonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I and *Sma*I (EC 3.1.21.4); alkaline phosphatase (EC 3.1.3.1); T_4 ligase or poly(deoxyribonucleotide):poly(deoxyribonucleotide) ligase (AMP-forming) (EC 6.5.1.1); DNA polymerase I or deoxynucleoside-triphosphate:DNA deoxynucleotidyl transferase (DNA-directed) (EC 2.7.7.7); DNase I or deoxyribonuclease I (EC 3.1.21.1); ribonuclease A or pancreatic ribonuclease (EC 3.1.27.5); *Streptomyces griseus* proteinase or microbial metalloproteinase (EC 3.4.24.4).

MATERIALS AND METHODS

Materials

Restriction endonucleases, T_4 DNA ligase, DNA polymerase (*E. coli*), DNase I (bovine pancreas) and dNTPs were purchased from Takara Shuzo (Japan) and Toyobo (Japan), calf intestine alkaline phosphatase from Boehringer Mannheim (FRG). [α-^{32}P]dCTP was from Amersham (USA). DEAE-Toyopearl and HPLC columns G-3000 SW, DEAE-5PW and Phenyl-5PW were purchased from Toyo Soda (Japan), Sephadex G-200 from Pharmacia (Sweden), marker proteins for molecular mass determination from Oriental Yeast (Japan), marker DNA from Nippon Gene (Japan), and ampholites from LKB Produkter AB (Sweden). Nitrocellulose filter was purchased from Advantech (Japan) and Schleicher & Schüll (FRG). The membrane filter (Diaflo, PM 30) was purchased from Amicon (USA). 5-Bromo-4-chloro-3-indoyl-β-D-galactoside, ribonuclease A (bovine pancreas), proteinase (*Streptomyces griseus*), *E. coli* DNA and oxo analogs of amino acids were purchased from Sigma (USA), and calf thymus DNA from Boehringer Mannheim. 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazoliumchloride was purchased from Dojin Chemicals (Japan), and fluorinated phenylalanine derivatives from Tokyo Kasei (Japan). All other chemicals were of analytical grade.

Bacterial strains, plasmids and cultivation

Bacillus badius IAM 11059 was obtained from the Institute for Applied Microbiology (University of Tokyo, Japan). It

was cultivated with rotary shaking in a medium containing 2 g L-phenylalanine, 5 g yeast extract (Oriental Yeast, Tokyo), 10 g peptone (Kyokuto, Tokyo), 2 g K_2HPO_4, 1 g NaCl and 0.2 g $MgSO_4 \cdot 7\,H_2O$ per 1 l tap water, pH 7.0, for 20 h at 30 °C. *Escherichia coli* RR1 [5] and JM 103 [6] were used as hosts and cultivated with shaking in LB medium [7], supplemented with 50 μg/ml ampicillin, for 12 h at 37 °C. pBR322 [5] and pUC19 [8] were used as vectors.

DNA techniques

For the isolation of chromosomal DNA, cells of *B. badius* IAM 11059 were harvested after 6 h cultivation at an absorbance at 610 nm of 0.8. Total DNA of *B. badius* was isolated according to the procedure as described by Doi [9], including spheroplast formation by lysozyme, cell lysis by SDS treatment, phenol extraction, ribonuclease A and proteinase treatments, and ethanol precipitation. Plasmid was isolated by the alkaline extraction procedure [10]. Restriction endonucleases and other enzymes were used as recommended by the suppliers. DNA was nick-translated [11] with DNase I and DNA polymerase I in the presence of [α-^{32}P]dCTP and other dNTPs, and used as a probe for DNA fragments encoding phenylalanine dehydrogenase gene which were prepared by digestion of the chromosomal DNA with *Eco*RI. Other techniques used were essentially as described by Maniatis et al. [7].

Cloning and screening of recombinants

The chromosomal DNA isolated from *B. badius* IAM 11059 was digested with *Eco*RI and the digested DNA was fractionated by size on 5% polyacrylamide gel. The fragments with a size about 2 – 9 kb were recovered by electroelution and then ligated with *Eco*RI-digested and dephosphorylated pBR322. Competent *E. coli* RR1 cells were prepared and transformed with the ligation mixture according to the method of Hanahan [12], and transformants were selected for expression of ampicillin resistance. The visualization of the phenylalanine dehydrogenase activity expressed in the transformants was carried out essentially as described by Inagaki et al. [13], with modifications. The production of insoluble formazan was initiated by the catalysis of phenylalanine dehydrogenase to yield NADH. The hydride of NADH was transferred non-enzymatically to 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride to form water-insoluble formazan in the presence of phenazine methosulfate as an electron carrier [14]. A sheet of nitrocellulose filter (Advantech, 82 mm in diameter) with about 2000 colonies of recombinants was floated on the surface of 1 ml of 10 mg/ml lysozyme solution containing 10 mM EDTA at pH 6.0 [15] until it wetted from beneath, and incubated at 30 °C for 30 min. The filter was transferred onto a filter paper to remove the residual lysozyme solution and then put on a dry plastic tray. The filter on the tray was frozen at – 20 °C and thawed at room temperature. This procedure was repeated three or four times to destroy the spheroplasts. The filter was incubated at 50 °C for 10 min, kept moistened from beneath with 0.1 M Tris/HCl, pH 8.0, to diminish the formation of formazan presumably caused by the respiratory chain enzymes. The filter was then floated on the surface of 1 ml of a mixture containing 10 mM L-phenylalanine, 0.40 mM NAD^+, 0.54 mM 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride and 0.33 mM phenazine methosulfate in Tris/HCl, pH 8.5, and stood at room temperature. Colonies that developed a crimson color of formazan in a few minutes were picked up. The phenylalanine dehydrogenase activity in the cell-free extract of the positive transformants was measured under the standard assay conditions.

Enzyme assay

The enzyme activity in the oxidative deamination reaction was assayed by the reduction of NAD^+, with L-phenylalanine as a substrate. The standard enzyme activity in the oxidative deamination reaction was measured in a reaction mixture (1.0 ml) containing 10 mM amino acid, 100 mM glycine/KCl/KOH, pH 10.4, 2.5 mM NAD^+, and the enzyme. The reaction mixture used for the kinetic study of the oxidative deamination reaction contained 100 mM glycine/KCl/KOH, pH 10.4, various concentrations of L-phenylalanine and NAD^+, and the enzyme. The enzyme activity in the reductive amination reaction was assayed by the oxidation of NADH in a reaction mixture (1.0 ml) containing 10 mM 2-oxo acid, 200 mM NH_4Cl/NH_4OH, pH 9.0, 0.1 mM NADH and the enzyme. The reaction mixture (1.0 ml) used for the kinetic study of the reductive amination reaction contained 100 mM glycine/KCl/KOH, pH 9.2, various concentrations of phenylpyruvate, NADH and NH_4Cl, and the enzyme. The reaction was monitored by the change in absorbance at 340 nm with a Hitachi 228A spectrophotometer. A reaction mixture to give a linear change in absorbance for at least 30 s was employed in the kinetic study, and the absorbance change for the initial 5 s was used for the calculation of the enzyme activity. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol NADH/min in the oxidative deamination. Protein was determined by the method of Lowry [16] or from the absorbance at 280 nm.

Purification of phenylalanine dehydrogenase from B. badius *IAM 11059*

B. badius IAM 11059 was cultivated in a 2-l flask containing 600 ml medium. Washed cells (68 g wet weight) from 13 lots of 600-ml cultures were suspended in 0.6 l 0.1 M buffer. The buffer used throughout the purification procedure was potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol unless otherwise stated. All the procedures were carried out at 0 – 5 °C except for heat treatment and HPLC column chromatography. The cells were suspended in 0.1 M buffer and disrupted for 20 min (totally 160 min) by a Kubota Syoji-9 KHz sonic oscillator. The cell debris was removed by centrifugation at 14000 × *g* for 20 min. The temperature of the cell-free extract was brought to 50 °C and incubated at the same temperature for 10 min. The cell-free extract was rapidly chilled with ice, and insoluble material formed was removed by centrifugation. The supernatant was dialyzed against 0.01 M buffer and fractionated with ammonium sulfate (30 – 60% saturation). The enzyme solution was dialyzed and applied on a DEAE-Toyopearl column (4.5 × 17 cm) equilibrated with 0.01 M buffer. The active enzyme fractions eluted with 0.1 M buffer containing 0.1 M NaCl were dialyzed and concentrated by ultrafiltration (Amicon, PM 30), and then applied to a Sephadex G-200 column (2.2 × 120 cm), which had been equilibrated with 0.05 M buffer containing 0.1 M NaCl. The active fractions were combined and concentrated by ultrafiltration. About 3 mg each of the partially purified enzyme was applied to a Toyo Soda DEAE-5PW column (0.75 × 7.5 cm) in an HPLC system (Toyo Soda, SP-8700), eluted at room temperature by

a linear gradient of 0.15–0.5 M NaCl in 0.02 M Tris/HCl buffer, pH 8.0, at a flow rate of 0.5 ml/min, and the active fractions were collected.

Purification of phenylalanine dehydrogenase from E. coli *RR1/pBB19*

E. coli RR1/pBB19 was cultivated in LB medium supplemented with 50 μg/ml ampicillin at 37°C for 12 h. Wet cells from three 600-ml cultures were suspended in 130 ml 0.1 M buffer. The cells were sonicated for 15 min (totally 1 h) and cell debris was removed by centrifugation. The cell-free extract was heated at 50°C for 10 min and fractionated with ammonium sulfate (30–60% saturation). The active pellet was dialyzed, applied to a DEAE-Toyopearl column (2.8 × 18 cm) and eluted with 0.1 M buffer containing 0.1 M NaCl. The active fractions concentrated by ultrafiltration were applied to a Sephadex G-200 column (2.2 × 123 cm), equilibrated with 0.05 M buffer containing 0.1 M NaCl. The active fractions were pooled, dialyzed and concentrated by ultrafiltration.

Other methods

The molecular mass of the enzyme was estimated by the HPLC system with a G 3000 SW column (0.75 × 60 cm) at a flow rate of 0.5 ml/min with an elution buffer consisting of a 0.1 M potassium phosphate buffer, pH 7.0 containing 0.2 M NaCl. The molecular mass of the enzyme was calibrated [17] with reference to the following proteins: cytochrome *c* (M_r = 12400), adenylate kinase (M_r = 32000), enolase (M_r = 67000), lactate dehydrogenase (M_r = 142000) and glutamate dehydrogenase (M_r = 290000). Polyacrylamide gel electrophoresis was carried out according to the method of Davis [18]. The molecular mass of the subunit was determined by sodium dodecyl sulfate (SDS) disc gel electrophoresis [19] with oligomers of cytochrome *c* ($M_r = 12400 \times n$, $n = 1-4$, 6) as marker proteins. Isoelectric focusing was carried out as described by Vesterberg [20]. For amino acid analysis, the purified enzymes were further subjected to a Phenyl-5PW column (0.75 × 7.5 cm, Toyo Soda, Japan) at a flow rate of 1.0 ml/min with a linear gradient of 20–80% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid as a solvent. The enzyme was hydrolyzed in 6 M HCl containing 4% thioglycolic acid at 135°C for 3 h in an evacuated sealed tube. The hydrolyzate was analyzed by an amino acid analyzer (Hitachi 835, Japan).

RESULTS AND DISCUSSION

Purification of phenylalanine dehydrogenase

Table 1 summarizes the purification of phenylalanine dehydrogenase. The enzyme was purified about 150-fold with a 27% yield from the cell-free extract. The total activity per liter culture and the specific activity in the cell-free extract were about five and seven times, respectively, higher than that from *S. ureae* SCRC-R04 [2] under the same culture conditions.

Purity, isoelectric point and absorption spectrum

The enzyme was found to be homogeneous by polyacrylamide and SDS/polyacrylamide disc gel electrophoreses (Fig. 1). It was eluted as a single peak in the HPLC with G 3000 SW, DEAE-5PW and Phenyl-5PW columns. Ampholite electrofocusing gave only one absorption peak of protein (pI 3.5) and it was identical to phenylalanine dehydrogenase. The purified enzyme showed an absorption maximum at 278 nm.

Table 1. *Purification of phenylalanine dehydrogenase from* B. badius *IAM 11059*
The concentration of the purified enzyme after the DEAE-Toyopearl step was determined from the absorbance at 280 nm using the absorption coefficient $A^{1\%}_{1cm} = 6.3$

Step	Total activity	Total protein	Specific activity	Yield
	units	mg	units/mg	%
Cell-free extract	2390	5200	0.460	100
Heat treatment	2200	3810	0.577	92
Ammonium sulfate (30–60%)	1830	1350	1.36	77
DEAE-Toyopearl	1460	123	11.9	61
Sephadex G-200	1390	34.3	40.5	58
DEAE-5PW	653	9.38	67.8	27

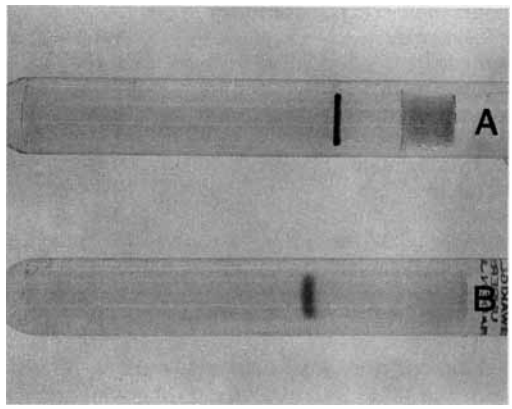

Fig. 1. *Polyacrylamide disc gel electrophoresis of the purified enzyme from* B. badius *IAM 11059*. (A) Purified enzyme (10 μg) was electrophoresed in the absence of SDS at a current of 2 mA. (B) Purified enzyme was incubated in the presence of 1% SDS and 3% 2-mercaptoethanol at 95°C for 3 min. The enzyme (3 μg) was electrophoresed in the presence of 0.1% SDS at a current of 8 mA. The gels were stained with Coomassie brilliant blue R-250

Molecular mass and subunit structure

The M_r of the enzyme was estimated to be 310000–360000 by gel filtration on HPLC by G 3000 SW column. The M_r of the subunit of the enzyme was estimated to be 41000–42000 by SDS/polyacrylamide disc gel electrophoresis. The native enzyme appears to have an octameric structure. The molecular mass and the subunit structure of the enzyme are similar to those of *S. ureae* SCRC-R04 [2], although the octameric structure is not common in amino acid dehydrogenases. Only leucine dehydrogenase of *B. cereus* [21] is reported to be octameric, while most of the other amino acid dehydrogenases are hexameric [3, 22, 23], tetrameric [3], dimeric [24], or monomeric [25].

Substrate specificity and kinetic properties

The substrate specificity of the enzyme in the oxidative deamination was high for L-phenylalanine as shown in

Table 2. *Substrate specificity of phenylalanine dehydrogenase in the oxidative deamination*
The enzyme activity was measured as described in Materials and Methods at 10 mM substrate concentration unless otherwise indicated

Amino acid	Relative activity
	%
L-Phenylalanine	100
L-Tyrosine (1.2 mM)	9.0
L-Tryptophan	4.0
L-Histidine	0.40
L-Methionine	8.0
L-Ethionine	7.0
L-Valine	4.0
L-Leucine	3.0
L-Isoleucine	0.20
L-Alloisoleucine	4.3
L-2-Amino-*n*-butyric acid	1.0
L-2-Aminopentanoic acid (L-norvaline)	5.0
L-2-Aminohexanoic acid (L-norleucine)	19
L-Phenylalaninamide	9.0
L-Tyrosinamide	0.10
L-Phenylalanine methyl ester	38
L-Tyrosine methyl ester	0.40
L-Phenylalanine hydroxyamate	1.0
L-Tyrosine hydroxamate	0.10
L-Phenylalaninol	9.4
DL-*o*-Fluorophenylalanine	2.0
DL-*m*-Fluorophenylalanine	11
DL-*p*-Fluorophenylalanine	34

Table 2. The enzyme was active toward L-phenylalanine methyl ester (38% relative to L-phenylalanine), DL-*p*-fluorophenylalanine (34%), L-2-aminohexanoic acid (L-norleucine) (19%) and DL-*m*-fluorophenylalanine (11%). The following amino acids were inert as substrates: D-phenylalanine, phenylglycine, glycine, L-alanine, L-serine, L-threonine, L-proline, L-glutamic acid, L-aspartic acid, L-glutamine, L-asparagine, DL-lysine, L-arginine and L-ornithine. Kinetic studies were carried out to determine the Michaelis constant (K_m) and maximum reaction velocity (V). The initial velocity of L-phenylalanine oxidation was studied with various concentrations of NAD^+ at fixed concentrations of L-phenylalanine. As shown in Fig. 2A, intersecting lines in the upper left quadrant in the double reciprocal plots were consistent with a sequential mechanism. The K_m value for L-phenylalanine was calculated to be 0.088 mM from a secondary of the intercepts at the ordinate against reciprocal concentration of L-phenylalanine. K_m value for NAD^+ was calculated to be 0.15 mM by a similar procedure from the replots of Fig. 2A. The V for the enzyme activity in the oxidative deamination, L-phenylalanine as a substrate, was calculated to be 71.6 μmol min^{-1} mg^{-1}. $NADP^+$ exhibited only 0.74% activity relative to NAD^+.

In the reductive amination, the enzyme acted rather broadly on 2-oxo acids as shown in Table 3. The enzyme was active toward *p*-hydroxyphenylpyruvate (53% relative to phenylpyruvate), 2-oxohexanoate (31%), 2-oxo-4-methylthiobutyrate (16%), 2-oxo-4-methylpentanoate (13%) and 2-oxovalerate (12%). The following substrates were inert: benzoylformate, pyruvate, 3-hydroxypyruvate, 2-oxoglutarate, and imidazolepyruvate. Double-reciprocal plots of velocity against NH_4Cl concentration at various fixed concentrations of phenylpyruvate and a constant concentration of NADH (at 0.4 mM) gave straight lines that intersected in the upper-left quadrant (Fig. 2B). The apparent K_m value for phenylpyruvate was calculated to be 0.106 mM from the secondary plot of the intercepts at the ordinate against reciprocal concentration of phenylpyruvate. The apparent V value in the reductive amination, phenylpyruvate as a substrate, was calculated to be 737 μmol min^{-1} mg^{-1}. The apparent K_m value for NH_4Cl was calculated to be 127 mM by a similar procedure from the replots of Fig. 2B. Double-reciprocal plots of velocity against phenylpyruvate concentrations at various fixed concentrations of NADH and a constant concentration of NH_4Cl (at 400 mM) gave parallel straight lines (Fig. 2C). The apparent K_m value for NADH was calculated to be 0.21 mM from the secondary plot of the intercepts at the ordinate against reciprocal concentrations of NADH. L-Glutamine, L-asparagine and methylamine (at 200 mM) could not replace ammonium ion.

Other properties

The pH optimum for the oxidative deamination of L-phenylalanine and for the reductive amination of phenylpyruvate were pH 10.4 and pH 9.4, respectively. The optimum temperature for the oxidative deamination of L-phenylalanine was 65°C at pH 10.4; 75% of the activity was retained after incubation at 30°C and pH 8.0 for 1 h. About half of the enzyme activity was lost after incubation at 55°C and pH 8.0 for 10 min. The effect of metal ions and inhibitors on the enzyme activity was investigated. The enzyme activity was measured after the enzyme was preincubated at 25°C for 10 min. The enzyme was not affected by chelating and carbonyl reagents (at 10 mM unless otherwise noted), such as EDTA, 2,2′-dipyridyl, *o*-phenanthroline (at 0.5 mM), 8-oxyquinoline (at 0.2 mM), potassium cyanide (at 0.1 mM), sodium azide and hydroxylamine. It was markedly inhibited by sulfhydryl reagents such as $AgNO_3$ (at 1 mM), $HgCl_2$ (at 0.01 mM) and *p*-chloromercuribenzoate (at 0.037 mM) and the remaining activities were 34%, 0% and 0%, respectively.

Cloning of phenylalanine dehydrogenase gene

The gene for phenylalanine dehydrogenase from *B. badius* was isolated from a plasmid pool containing *Eco*RI fragments of *B. badius* chromosomal DNA ligated into the *Eco*RI site of pBR322 and introduced in *E. coli* RR1 by transformation. About 50 of the approximately 23000 transformants exhibited phenylalanine dehydrogenase activity as shown by the development of the crimson color on the nitrocellulose filter screening assay. Rapid isolation of plasmids from 12 randomly chosen transformants revealed that one contained a 7-kb insert, while the others had a 3.8-kb insert. Plasmid pBB1 was obtained from one of the transformants that contained the 3.8-kb *Eco*RI insert in the *Eco*RI site of pBR322. The pBB1 was digested with various restriction enzymes and the resulting fragments were analyzed by agarose and polyacrylamide gel electrophoresis. The physical map of pBB1 is shown in Fig. 3. The insert had single sites for *Sal*I and *Pst*I, and two sites for *Hind*III. No site was detected for *Bam*HI or *Sma*I. The insert was subcloned in the *Eco*RI site of pUC19 to give pBB19. The phenylalanine dehydrogenase gene was subcloned to the *Eco*RI-*Pst*I site of pUC19 to give pBB191 with a 3.2-kb insert, eliminating a 0.6-kb *Eco*RI-*Pst*I fragment. pBB191 can transform *E. coli* into being phenylalanine-dehydrogenase-positive. To ensure that the phenylalanine dehydrogenase gene in pBB191 originated from *B. badius*, the chromosomal DNA was digested with *Eco*RI and hybridized

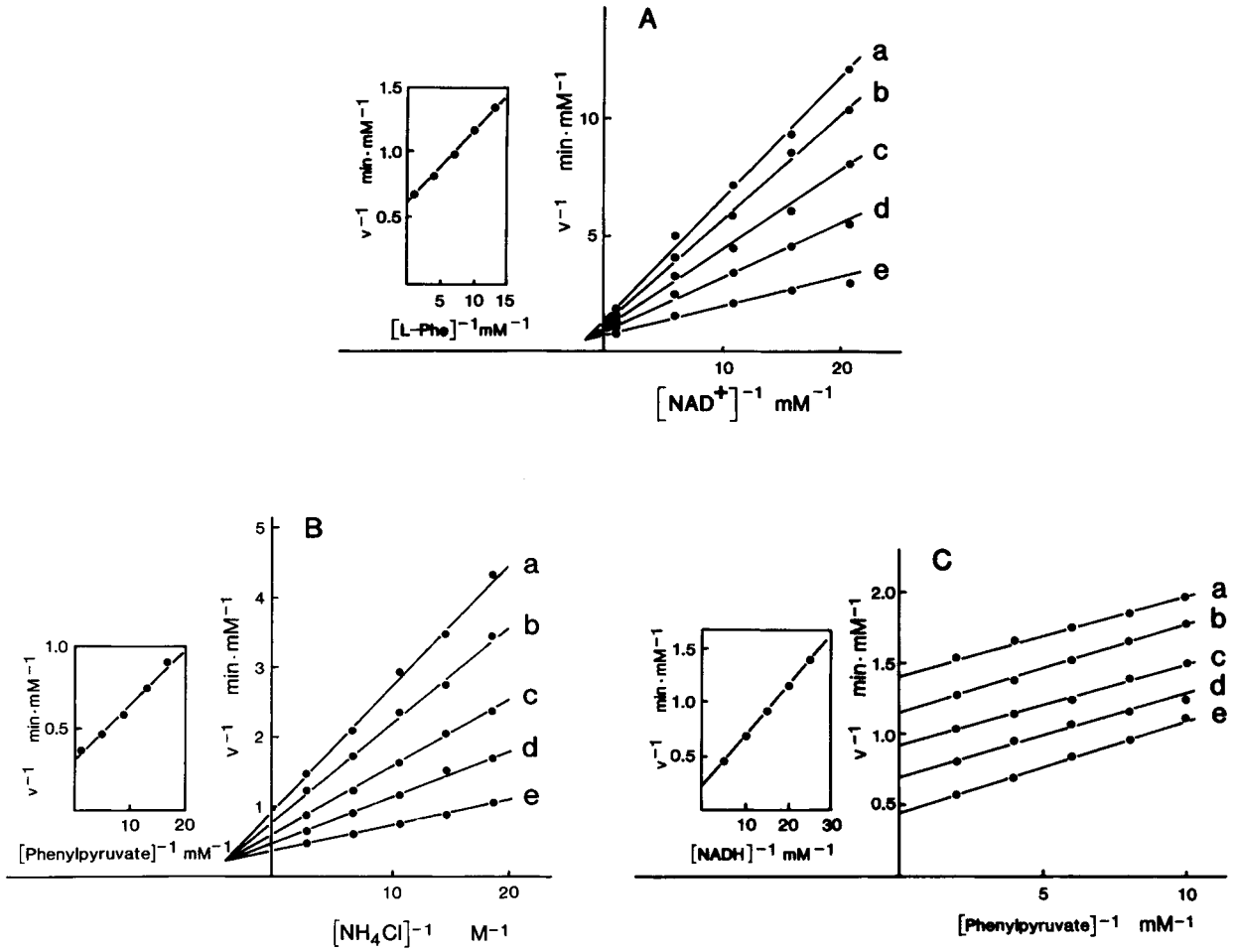

Fig. 2. *Effect of substrate concentration on the phenylalanine dehydrogenase activity.* (A) Double-reciprocal plots of initial velocity against NAD^+-concentration at a series of fixed concentrations of L-phenylalanine. The oxidative deamination reactions were carried out under the standard reaction conditions. The concentrations of L-phenylalanine used were (a) 0.077 mM, (b) 0.10 mM, (c) 0.14 mM, (d) 0.25 mM and (e) 1.0 mM. The concentrations of NAD^+ used were 0.048, 0.063, 0.091, 0.17 and 1.0 mM. The amount of enzyme used was 0.06 unit. Velocity (v) is expressed as μmol NADH formed/min. The insert is a secondary plot of intercept against L-phenylalanine concentration. (B) Double-reciprocal plots of initial velocity against NH_4Cl concentration at a series of fixed concentrations of phenylpyruvate. The reductive amination reactions were carried out under the standard reaction conditions except that various amounts of phenylpyruvate and NH_4Cl were used. The concentrations of phenylpyruvate used were (a) 0.059 mM, (b) 0.077 mM, (c) 0.11 mM, (d) 0.20 mM and (e) 1.0 mM. The concentrations of NH_4Cl used were 0.053, 0.067, 0.091, 0.14 and 0.33 M. In B and C, the amount of enzyme used was 0.006 unit and velocity (v) was expressed as μmol NADH consumed/min. The insert is a secondary plot of intercept against phenylpyruvate concentration. (C) Double-reciprocal plots of initial velocity against phenylpyruvate concentration at a series of fixed concentrations NADH. The concentrations of NADH used were (a) 0.040 mM, (b) 0.050 mM, (c) 0.067 mM, (d) 0.10 mM and (e) 0.20 mM. The concentrations of phenylpyruvate used were 0.10, 0.13, 0.17, 0.25 and 0.50 mM. The insert is a secondary plot of intercept against NADH concentration

Table 3. *Substrate specificity of phenylalanine dehydrogenase in the reductive amination*
The enzyme activity was measured as described in Materials and Methods with 10 mM 2-oxo acid unless otherwise indicated

2-Oxo acid	Relative activity
	%
Phenylpyruvate	100
p-Hyroxyphenylpyruvate (2.5 mM)	53
2-Oxo-4-methylthiobutyrate	16
2-Oxo-4-methylpentanoate	13
DL-2-Oxo-3-methylvalerate	4.0
2-Oxobutyrate	3.0
2-Oxovalerate	12
2-Oxohexanoate	31

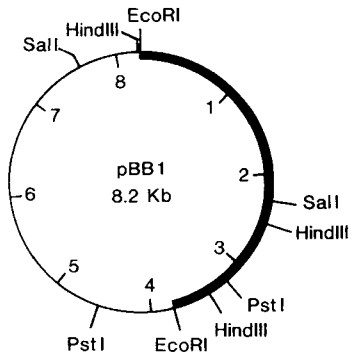

Fig. 3. *Physical map of pBB1.* The heavy line represents *B. badius* chromosomal DNA, and the light line represents DNA from the pBR322. Map units are in kb

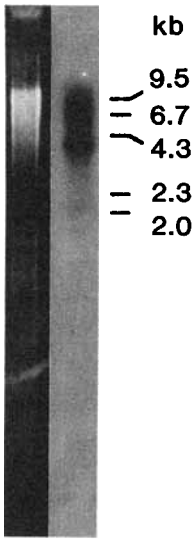

Fig. 4. *Southern blot analysis of the* B. badius *chromosomal DNA. B. badius* chromosomal DNA was completely digested with *Eco*RI and applied to 1.0% agarose gel electrophoresis. DNA fragments were probed with ^{32}P-labeled 3.2-kb *Eco*RI – *Pst*I fragment. Southern hybridization was carried out as described by Maniatis et al. [7]. (A) *B. badius* DNA stained with ethidium bromide. (B) Autoradiogram of DNA hybridized with ^{32}P-labeled *Eco*RI – *Pst*I fragment

Table 4. *Amino acid composition of phenylalanine dehydrogenase from* B. badius *and* E. coli *RR1/pBB19*
About 60 μg enzyme was subjected to amino acids analysis. Tryptophan was not determined

Amino acid residue	Phenylalanine dehydrogenase from	
	B. badius IAM 11059	*E. coli* RR1/pBB19
	mol/100 mol	
Asx	11.3	11.3
Thr	5.0	4.7
Ser	4.3	4.1
Glx	11.0	11.6
Gly	12.6	12.5
Ala	11.4	11.2
Val	5.8	6.0
Cys	1.2	1.3
Met	3.7	3.9
Ile	4.3	4.4
Leu	7.1	7.1
Tyr	3.5	3.5
Phe	4.3	4.2
Lys	6.6	6.6
His	1.6	1.5
Arg	3.5	3.4
Pro	2.8	2.7

with the 3.2-kb *Eco*RI – *Pst*I fragment. As shown in Fig. 4, the *B. badius* chromosomal DNA digested with *Eco*RI gave a single band top hybridize with the *Eco*RI – *Pst*I fragment. It is evident that the DNA insert in pBB191 is derived from *B. badius* chromosomal DNA.

Purification and properties of phenylalanine dehydrogenase produced by E. coli *RR1/pBB19*

The total activity and the specific activity of the enzyme detected in the cell-free extract of *E. coli* RR1/pBB19 were 6890 units/l culture and 7.05 units/mg, respectively. The enzyme was purified about 10-fold up to the specific activity of 67.9 units/mg with a 54% yield from the cell-free extract. The amount of the enzyme in the cell-free extract of *E. coli* RR1/pBB19 was elevated 24-fold per liter culture over that of the *B. badius*, and comprised 9.6% of the total extractable cellular protein.

The apparent molecular mass of the enzyme produced in *E. coli* was indistinguishable from that of the enzyme from *B. badius*, as they were eluted at exactly the same position in the gel filtration in G 3000 SW column. Only a single band was stained in SDS/polyacrylamide gel and the M_r of the subunit of the enzyme produced by *E. coli* was about 41000. Both of the enzymes from *B. badius* and *E. coli* were eluted at exactly the same ionic strength from a DEAE-5PW column. The N-terminal amino acid sequences of both the enzymes were determined to be identical by automated Edman degradation (data not shown). Furthermore, the amino acid composition of *B. badius* was similar to that of the enzyme produced by *E. coli* as shown in Table 4. The identity of the *B. badius* and the *E. coli* enzymes was thus confirmed with respect to the specific activity of the purified enzyme, the molecular mass, the subunit structure, and the amino acid composition. These results, together with the result of the Southern blot analysis, provide the evidence that the gene for the *B. badius* phenylalanine dehydrogenase was cloned and expressed in *E. coli*.

To examine whether the expression of the phenylalanine dehydrogenase gene is dependent on its endogenous promoter, the enzyme activities in the cell extract of *E. coli* JM 103/pBB19 grown at 37°C for 10 h with or without 0.5 mM 5-bromo-4-chloro-3-indoyl-β-D-galactoside were measured. There was no notable difference in the enzyme activity between the two cultures (17.0 units/mg with the glycoside, 16.5 units/mg without), indicating that the phenylalanine dehydrogenase gene was transcribed from its own promoter, since *E. coli* JM 103 expresses the β-galactosidase promoter in the presence of 5-bromo-4-chloro-3-indoyl-β-D-galactoside [26].

Sequencing of the gene and a comparison of the primary structure between various nicotinamide-cofactor-dependent dehydrogenases including amino acid dehydrogenase should provide the basis for increasing our understanding of the catalytic properties and the structure of phenylalanine dehydrogenase.

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