Novel Phenylalanine Dehydrogenases from Sporosarcina ureae and Bacillus sphaericus

PURIFICATION AND CHARACTERIZATION*

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NAD⁺-dependent phenylalanine dehydrogenase was first found to occur in Brevisibacterium sp. by Hummel et al. in 1984 (1). The enzyme catalyzes reversible oxidative deamination acting on L-phenylalanine. Much attention is being paid to the enzyme not only because its occurrence was completely unknown until the discovery by Hummel et al., but because it appears to be useful as an industrial catalytic in the asymmetric synthesis of L-phenylalanine and related L-amino acids from their keto analogs (2). Enzymes that catalyze this type of reaction include glutamate dehydrogenase (EC 1.4.1.2-4), alanine dehydrogenase (EC 1.4.1.1), and leucine dehydrogenase (EC 1.4.1.9, etc. Glutamate dehydrogenase is widely distributed and few organisms lack this enzyme. Glutamate dehydrogenase of animal origin functions with both NAD⁺ and NADP⁺, whereas most of the other organisms possess the enzymes specific either for NAD⁺ or NADP⁺ (3). The distribution of alanine dehydrogenase (4–9) and leucine dehydrogenase (9, 10) is limited to microorganisms. Both of them are NAD⁺-dependent, and high activities are observed in Bacillus species, namely in B. sphaericus IFO 3525 (4, 10).

In a study of the stereoelectivity in hydride transfer from the C-4 of nicotinamide ring, Alizade et al. (11) and Ohshima and Soda (4) showed that Bacillus alanine dehydrogenase is A-stereospecific in the hydride transfer. However, other amino acid dehydrogenases such as glutamate dehydrogenase (12), leucine dehydrogenase (10), and meso-a,-disaminopimelate dehydrogenase (13) are all B-stereospecific. Benner and co-workers (14, 15) have recently demonstrated that the stereochemistry in dehydrogenases acting on alcohols follows a rule; the stability of the ketone in the oxidation-reduction reactions determines the stereochemical preference in the hydride transfer.

These situations have prompted us to study phenylalanine dehydrogenase. We first screened for the enzyme activity among a number of microorganisms from soil samples and culture collections and soon found that the enzyme activity was very narrowly distributed among aerobic spore-forming Gram-positive bacteria. As an initial step in understanding the primary structure of the enzyme and its catalytic properties, we decided to purify the enzyme to homogeneity. In a preliminary paper, we purified the enzyme in crystalline form from Sporosarcina ureae (16).

This paper describes the purification to homogeneity and physical, catalytic, and immunological properties of the enzyme from S. ureae and B. sphaericus, in comparison with other amino acid dehydrogenases.

EXPERIMENTAL PROCEDURES

Materials—DEAE-Toyopearl, Butyl-Toyopearl and TSK-Gel G 3000 SW were purchased from Toyo Soda (Japan), Sephadex G-200 superfine from Pharmacia (Sweden), carboxypeptidase Y [(EC 3.4.12.-,) Saccharomyces cerevisiae], alcohol dehydrogenase [(EC 1.1.1.1), S. cerevisiae] and marker proteins for molecular weight determination from Oriental Yeast (Japan), and leucine dehydrogenase (EC 1.4.1.9), B. Sphaericus) and horseradish peroxidase (EC 1.11.1.7) from Toyobo (Japan). Candida boidinii No. 2201 was kindly provided by Professor Y. Yogi of Tokyo University. Formate dehydrogenase (EC 1.2.1.2) was purified from C. boidinii according to the method of Kato et al. (17). L-Amino acid oxidase (EC 1.4.3.2), Botrops atra, aldehyde dehydrogenase (EC 1.2.1.5), S. cerevisiae, sodium phenylpyruvate, imidazolopropionic acid·HCl, indole-3-propionic acid, t-allo-isoleucine, L-phenylalaninamide·HCl, L-tyrosinamide·HCl, L-phenylalanine hydroxamate, L-tyrosine hydroxamate, L-tyrosine methylster, and dansylated amino acids were purchased from Sigma, L-phenylalanine methylster, L-phenylalaninol, and l-hydroxypyruvylpyruvic acid were from Aidrich, and L-tyrosine was from Daiichi Kagaku Yukuhin (Japan). Hydroxypapitate and INT were from Wako Pure Chemicals (Japan). Coomassie Brilliant Blue R-250 was purchased from Fluka (Switzerland). [3H]Ethanol (>99%) was purchased from Nakarai Chemicals (Japan). Amphotale used for isoelectric focusing were the products of LKB-Produkter AB. The membrane filter (Diaflo Ultrafilter, PM-30) was obtained from Amicon. All other chemicals were from commercial sources and used without further purification.

Screening of Phenylalanine Dehydrogenase Producing Strains

The abbreviations used are: INT, 2(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.

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Phenylalanine dehydrogenase activities were measured among yeast and bacterial strains from type culture collections and microorganisms isolated from soil samples. Strains from type culture collections included 57 strains of yeasts and 81 strains of 29 genera of bacteria. They were obtained from stock cultures of the Laboratory of Fermentation Physiology, Faculty of Agriculture, Kyushu University, Japan (KU), the Institute for Fermentation, Osaka, Japan (IFO), the National Collections of Marine Bacteria, United Kingdom (NCMB), and our laboratory (SCRC). They included the following genera of yeasts: Pichia, Hansenula, Saccharomyces, Lipomyces, Saccharomycodes, Hanseniaspora, Cryptococcus, Torulaspora, Candida, Kloecheria, Trigonopsis, Rhodotorula. The bacterial genera tested included Escherichia, Klebsiella, Enterobacter, Citrobacter, Serratia, Proteus, Salmonella, Hafnia, Acetobacter, Acinetobacter, Chromobacterium, Pseudomonas, Xanthomonas, Protaminobacter, Moraxella, Alcaligenes, Achromobacter, Flavobacterium, Agrobacterium, Azotobacter, Bacillus, Sporosarcina, Micrococcus, Corynebacterium, Arthrobacter, Brevibacterium, Cellulomonas, Microbacterium and Mycobacterium. A number of L-phenylalanine-assimilating microorganisms were isolated from soil samples by an enrichment culture technique. The bacterial strains were cultured aerobically at 30°C for 24 h on 200 ml of a medium composed of 1.0% L-phenylalanine, 1.0% peptone (Kykoto, Tokyo), 0.5% yeast extract (Oriental Yeast, Tokyo), 0.2% K₂HPO₄, 0.1% NaCl, and 0.025% MgSO₄·7H₂O in tap water, adjusted to pH 6.0. The medium composition was killed at pH 6.0. The cells were centrifuged, washed once with physiological saline, and suspended in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. The cells were disrupted by ultrasonication for 20 min with a Koubota-Shoji 9-kHz ultrasonic oscillator. The disrupted cells were centrifuged at 14,000 x g for 20 min. The supernatant was dialyzed against 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol.

Enzyme Assay and Definition of Units—Activity of NAD⁺-dependent phenylalanine dehydrogenase was assayed at 25°C by measuring the rate of NADH oxidation at 340 nm in a cuvette placed in the beam of 1-cm light path. The reaction mixture contained 100 μmol of glycine-KCl-KOH buffer, pH 10.4, 2.5 μmol of NAD⁺, 10 μmol of L-phenylalanine and enzyme solution in a total volume of 1.0 ml. The enzyme activity for the reductive amination was measured at 25°C by the disappearance of NADH at 340 nm in the reaction mixture containing 100 μmol of Tris-HCl buffer, pH 9.5, 0.1 μmol of NADH, 200 μmol of NH₄Cl, 10 μmol of sodium phenylnitroprusside and enzyme solution in a total volume of 1.0 ml. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 pmol of NAD⁺ per minute. The COOH-terminal amino acid was determined with carboxypeptidase Y (27). Carboxypeptidase Y (2.5 nmol) was added to 68-109 μmol of SDS-denatured enzyme in 1 ml of 0.05 M potassium-acetate, pH 5.5, and inactivated at 25°C. At intervals 100-μl aliquots were withdrawn and put into Eppendorf tubes containing 20 μl of 30% trichloroacetic acid. The amino acids in the deproteinized mixture were analyzed by an amino acid analyzer (Kyowa Seimitsu, K-101, Japan).

Purification of Phenylalanine Dehydrogenase from S. urea SCRC-R04—S. urea SCRC-R04 was cultivated aerobically at 30°C for 20 h. All the enzyme purification procedures were performed at temperatures lower than 5°C. Potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol was used throughout the purification.

Cells (about 700 g wet weight) from 70 liters of culture were suspended in 0.1 M buffer. The cells were disrupted for 20 min (30 h total) by a Koubota-Shoji 9-kHz sonic oscillator. The disrupted cells were centrifuged at 14,000 x g for 20 min. The supernatant was dialyzed against 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 M EDTA and 5 mM 2-mercaptoethanol. The dialyzed enzyme solution was applied to a DEAE-Toyopearl 650M column (6.5 x 50 cm) and eluted with 0.1 M buffer. The active fractions were combined, dialyzed, and applied to the second hydroxyapatite column (4.5 x 7 cm) and eluted likewise. The active fractions were concentrated by ultrafiltration.

To the enzyme-free extract was added 5% potassium sulfate, to a concentration of 1 g of potassium sulfate to 1 g of protein. After stirring for 30 min, the precipitate formed was removed by centrifugation at 14,000 x g for 20 min. To the supernatant was added ammonium sulfate to 30% saturation to remove inactive residues and then brought to 60% saturation. The active precipitate collected by centrifugation was dissolved in 1 liter of 0.01 M buffer and dialyzed against seven changes of 25-liter buffer (0.01 M). The dialyzed enzyme solution was applied to a DEAE-Toyopearl 650M column (6.5 x 50 cm) and eluted with 0.1 M buffer. The active fractions were combined, dialyzed, and applied to the second hydroxyapatite column (4.5 x 7 cm) and eluted likewise. The active fractions were concentrated by ultrafiltration.

The enzyme solution was added ammonium sulfate to 30% saturation to remove inactive residues and then brought to 60% saturation. The active precipitate collected by centrifugation was dissolved in 1 liter of 0.01 M buffer containing ammonium sulfate to 30% saturation, and eluted with a linear gradient of ammonium sulfate (30-0% saturation) in 0.1 M buffer. The active fractions were dialyzed, concentrated by ultrafiltration, and applied to the second hydroxyapatite column (4.5 x 7 cm) and eluted likewise. The active fractions were concentrated by ultrafiltration.

Purification of Phenylalanine Dehydrogenase from B. sphaericus SCRC-R79a—B. sphaericus SCRC-R79a was cultivated as described above. The enzyme was extracted by centrifugation of the supernatant of the Sporosarcina enzyme. Potassium phosphate buffer, pH 7.9, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol was used throughout the procedures. All the purification procedures were performed at a temperature lower than 5°C unless indicated otherwise.
Phenylalanine Dehydrogenase

Cells (about 1 kg wet weight) from 100 liters of culture were suspended in 0.1 M buffer and disrupted for 20 min (41 h total) by the sonic oscillator. The disrupted cells were removed by centrifugation at 14,000 x g for 20 min. The cell-free extract was heated for 10 min in a water bath at 50 ºC. The extract was cooled in ice and centrifuged at 14,000 x g for 20 min to remove denatured protein. To the supernatant was added ammonium sulfate to 30% saturation, and the precipitate formed was removed by centrifugation at 14,000 x g. The precipitate formed on addition of ammonium sulfate to 60% saturation was dissolved in a 0.01 M buffer and dialyzed in the same buffer. The enzyme solution was applied on a DEAE-Toyopearl 650M column (6.5 x 36 cm) which had been equilibrated with 0.01 M buffer. After washing the column with a 0.1 M buffer, the enzyme was eluted with the same buffer containing 0.1 M NaCl. The active fractions were combined, dialyzed, and applied to the second DEAE-Toyopearl (4.5 x 9.5 cm) and eluted likewise. The enzyme was dialyzed and placed on a hydroxypatite column (4.5 x 8 cm) which had been equilibrated with 0.01 M buffer. The enzyme was eluted with a linear concentration gradient of from 0.01 to 0.4 M buffer. The active fractions were pooled and concentrated by ultrafiltration and placed on a column of Sephadex G-200 (2.2 x 123 cm) equilibrated with 0.05 M buffer containing 0.1 M NaCl. The active fractions were combined and concentrated by ultrafiltration.

Isoelectric Focusing—Isoelectric focusing was performed according to the method of Vesterberg (28). Electrophoresis was carried out at 5 ºC for 70 h using Ampholite at a pH range of 3.5 to 10.

Preparation of Antibody—The purified B. sphaericus enzyme (0.34 mg) in 1.0 ml of a 4 M potassium phosphate buffer, pH 7.9, containing 57 mM NaCl was emulsified with an equal volume of the complete Freund’s adjuvant (Miles Laboratories). The emulsion was injected into multiple subcutaneous sites on the back of a male Japanese white rabbit weighing 2.0 kg. After 4 weeks, a booster of 0.51 mg of the purified enzyme in 10 mM potassium phosphate buffer, pH 7.9, was injected into the ear vein. Anti-phenylalanine dehydrogenase activity of the sera was tested by an enzyme-linked immunoabsorbent assay kit (Kirkegaard and Perry Laboratories (U. S. A.)). On the 6th day after the booster injection, blood was obtained from the ear vein and allowed to clot. The serum was centrifuged at 1700 x g for 10 min and stored at -20 ºC.

Immunochromatography—Ouchterlony plates were made using a 1% Agar Purified (Difeo) in 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 0.01% sodium azide (29). Immunotitrations were carried out using as an antigen the purified enzyme or crude preparations obtained by the ammonium sulfate fractionation (0.3-0.6 saturation). The reaction mixture contained various amounts of an antibody and a fixed unit of the enzyme in 0.01 M potassium phosphate buffer, pH 7.0, in a final volume of 0.6 ml. The mixture was incubated for 30 min at 37 ºC and then stood for 12 h at 5 ºC. After centrifugation, the residual activity of each supernatant was measured.

Preparations of Deuterated NADH Derivatives—[3-2H]Leucine was prepared by reductive amination of a-ketoisocaproate with a deuterated NADH to regenerate dehydrogenase activity and to aldehyde dehydrogenase with [U-3H]ethanol as a substrate. The reaction mixture contained 1.52 g (10 mmol) of a-ketoisocaproate, 1.43 g (2 mmol) of NAD+, 200 mg (4 mmol) of hydrazine-H2O, 400 units of leucine dehydrogenase (B-stereospecific) (10, 12) and 5 mmol of glycine-KCl-KOH, pH 10.6. [4A-2H]NADH was prepared by incubating 800 mg (1.17 mmol) of [U-3H]ethanol, 1.43 g (2 mmol) of NAD+, 200 mg (4 mmol) of hydrazine-H2O, 2000 units of alcohol dehydrogenase (A-stereospecific) (12) and 5 mmol of Tris-HCl, pH 8.5. [4A-2H]NADH and [4B-2H]NADH were recovered as barium salts by precipitation adding 99% ethanol (30).

Distribution of Phenylalanine Dehydrogenase—As shown in Table I, the activity of the enzyme was found in four strains of the genus Bacillus and two strains of the genus Sporosarcina. They belong to aerobic Gram-positive endospore-formers.

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**Table I**

**Formation of phenylalanine dehydrogenase by microorganisms**

The cultivation of microorganisms and assay of the enzyme activity were carried out as described under “Experimental Procedures.” The *Bacillus* and *Sporosarcina* strains which had no or less than 4 units/liter of culture of phenylalanine dehydrogenase activity included *B. subtilis* IAM-3018, AKU-216, AKU-222, AKU-224, and *SCC-207*; *B. natto* AKU-205 and AKU-206; *B. cereus* IFO-3001 and IAM-1226; *B. sphaericus* IFO-3341, IFO-3525, IFO-3526, IFO-3527, IFO-3528, IFO-12622, and IAM-1228; *B. brevis* IAM-3331; *B. roseus* AKU-208; *B. aneurinolyticus* IAM-1077, AKU-201 and AHU-1354; *B. circulans* IFO-3328; *B. megaterium* IFO-3970 and IAM-1032; *B. pumilus* AKU-207; *B. licheniformis* IAM-11034; *B. altei* IFO-3343; *B. thuringiensis* IFO-3951; *B. thiaminolyticus* IAM-1054; *B. coagulans* IFO-3886 and IFO-12714; *B. stearothermophilus* IFO-12550, IFO-12983, and IFO-13737; *B. lubeiskii* NCMB-1524; and *S. ureae* IFO-12698.

**Table II**

**Summary of purification of phenylalanine dehydrogenase from *S. ureae* SCRC-R04**

The concentration of the purified enzyme after the DEAE-Toyopearl step was determined from the absorbance at 280 nm using the extinction coefficient (ε280 = 16.1) which was obtained by dry weight determination.

**Table III**

**Summary of purification of phenylalanine dehydrogenase from *B. sphaericus* SCRC-R79a**

The concentration of the purified enzyme after the DEAE-Toyopearl step was determined from the absorbance at 280 nm using the extinction coefficient (ε280 = 11.7) which was obtained by dry weight determination.
Purification of Phenylalanine Dehydrogenase—S. ureae SCRC-R04 and B. sphaericus SCRC-R79a which were isolated from soil samples were chosen as likely sources of the enzyme. Summaries of the purification procedures for the two enzymes from S. ureae and B. sphaericus are shown in Tables I and II, respectively. The Sporosarcina enzyme was purified about 1500-fold with a 70% yield and the Bacillus enzyme 1600-fold with a 55% yield from the cell-free extracts.

Crystallization of Phenylalanine Dehydrogenase—The purified enzyme samples were concentrated to be about 30 mg/ml. Solid ammonium sulfate was added to the enzyme solutions to make a 30% saturated solution. Ammonium sulfate was carefully added to the solutions little by little over a period of 3 to 7 days until the solution became silky. The solutions were kept at 5 °C for a few days to obtain crystalline enzymes. They were isolated in plate and needle form from S. ureae SCRC-R04 and B. sphaericus SCRC-R79a, respectively. Figs. 1 and 2 show the photomicrographs of the crystalline enzymes.

Purity of the Enzyme—Both the enzymes were judged to be homogeneous by the criteria of native and SDS-polyacrylamide gel electrophoreses, HPLC on Toyo Soda G-3000 SW column, and isoelectric focusing, as all of the results gave a single band or a single peak. The mobility of the native enzyme stained with Coomassie Brilliant Blue on the polyacrylamide gel coincided well with a band formed when the gel was stained for the enzyme activity. An antibody against the purified Bacillus enzyme gave a single precipitation line on an Ouchterlony double diffusion plate with a crude extract of B. sphaericus SCRC-R79a, proving that the purified enzyme was immunologically pure.

Identification of Reaction Product and Stoichiometry—The stoichiometry of the Sporosarcina enzyme reaction was examined with a reaction mixture containing 100 μmol glycine-KCl-KOH buffer, pH 10.4, 5 μmol of NADH, 5 μmol of L-phenylalanine and Sporosarcina enzyme in a total volume of 1.0 ml. The reaction was carried out at 30 °C for 10 min. Because simultaneous determination of L-phenylalanine, NADH, phenylpyruvate, and ammonia was not possible, they were analyzed two at a time in several different runs. Consumption of L-phenylalanine and formation of phenylpyruvate were both 1.46 μmol, when the reaction was terminated by the addition of 1 ml of 1 N HCl. Formation of phenylpyruvate and NADH were 1.38 and 1.23 μmol, respectively, as terminated by the addition of 1 ml of 99% ethanol. When the reaction was terminated by the addition of 1 ml of 10% (w/v) sodium carbonate, the amount of NADH and ammonia formed was 1.36 and 1.25 μmol, respectively. These results indicated that phenylpyruvate, NADH, and ammonia were formed stoichiometrically with the consumption of L-phenylalanine, NADH, and H2O.

The identity of L-phenylalanine in the reductive amination reaction was confirmed by its isolation. The reaction mixture contained 22 mmol of sodium phenylpyruvate, 49 mmol of ammonium formate, 0.29 mmol of NADH, 18 mmol of Tris-HCl, pH 8.5, 43 units of purified Sporosarcina enzyme, and 49 units of partially purified formate dehydrogenase in a total volume of 300 ml. After the mixture was incubated at 30 °C for 24 h, L-phenylalanine formed in the mixture was isolated and crystallized by a procedure involving deproteinization by trichloroacetic acid, column chromatographies on Amberlite IR-120 (H+), and Amberlite IRA-400 (OH−). The results of the elementary analysis of isolated L-phenylalanine was:

<table>
<thead>
<tr>
<th>C</th>
<th>H</th>
<th>N</th>
<th>O</th>
<th>Calculated</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>66</td>
<td>6.61</td>
<td>8.48</td>
<td></td>
<td>65.43</td>
<td>65.33</td>
</tr>
</tbody>
</table>

Molecular Weight, NH2-, and COOH-terminal Residues and Isoelectric Point—The molecular weight of the native Sporosarcina enzyme was calculated to be approximately 310,000 by gel filtration on HPLC. A molecular weight of 305,000 was obtained by the sedimentation equilibrium method, assuming a partial specific volume of 0.74. The molecular weight of the subunits was calculated to be 39,000 by comparing the mobility on SDS-polyacrylamide disc gel electrophoresis to that of standard proteins. Only a valine derivative was detected in the NH2-terminal amino acid analysis of the Sporosarcina enzyme. The amino acids released by carboxypeptidase Y digestion of the Sporosarcina enzyme appeared in the order of glutamic acid, lysine, and isoleucine. Thus, the enzyme probably consists of eight subunits identical in molecular weight. The enzyme had an isoelectric point of pH 5.3.

The molecular weight of the native Bacillus enzyme was calculated to be approximately 340,000 by HPLC and sedimentation equilibrium analyses. NH2-terminal amino acid analysis of the Bacillus enzyme was shown to be alanine. Amino acids released in the COOH-terminal amino acid digestion of the Bacillus enzyme were in the order of phenylalanine, histidine, and glutamine. The molecular weight of the subunit of the enzyme was estimated to be 39,090 by SDS-polyacrylamide gel electrophoresis. Therefore, the enzyme is composed of eight (or nine) identical subunits. The isoelectric point of the Bacillus enzyme was estimated to be 4.3.

Absorption Spectrum—The absorption spectra of both the purified Sporosarcina and Bacillus enzymes in 0.01 M potassium phosphate buffer, pH 7.0, showed the maximum absorbance at 273 nm with a small shoulder at 283 nm.

Effect of Temperature and pH on the Enzyme Activity—
The effect of temperature on the activity of the enzyme was examined. The Sporosarcina enzyme exhibited the maximal activity at 40°C and pH 10.5. Seventy-five percent of the activity was retained after incubation at 40°C and pH 9.0 for 10 min, whereas the enzyme was completely inactivated at 50°C for 10 min. The enzyme exhibited the maximal activity for oxidative deamination of L-phenylalanine at pH 10.5 and for reductive amination of phenylpyruvate at pH 9.0. No loss of activity was found at pH 9.0 after incubation at 30°C for 1 h.

On the other hand, the Bacillus enzyme exhibited the maximal activity at 50°C and pH 10.5. No loss of activity was detected after incubation at 55°C and pH 9.0 for 10 min, whereas at 60°C, most of the activity was lost. The enzyme exhibited the maximal activity for oxidative deamination of L-phenylalanine and reductive amination of phenylpyruvate at pH 11.3 and 10.3, respectively. No loss of activity was found between pH 9.0 and 11.3 after incubation at 30°C for 1 h. More than 80% activity remained at pH 4.0 in the same condition.

Substrate Specificity and Kinetic Properties—The enzyme activity toward various amino acids was examined at a concentration of 10 mM as shown in Table IV. L-Phenylalanine, L-phenylalanine methyl ester, and L-norleucine were active as a substrate for the Sporosarcina enzyme. Amino acids which were inactive as substrates for the Sporosarcina enzyme included glycine, L-alanine, L-histidine, L-arginine, L-lysine, L-ornithine, L-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, L-proline, L-serine, L-threonine, L-cysteine, L-tyrosinamide, L-tyrosine hydroxamate, D-phenylalanine, D-tyrosine, DL-phenylglycine, tert-L-leucine, and DL-threo-phenylserine. A marked difference between the Sporosarcina and the Bacillus enzymes is that the latter is considerably active toward L-tyrosine. Aromatic L-amino acids other than L-phenylalanine and L-tyrosine and aliphatic amino acids were only slightly active for the Bacillus enzyme. Amino acids which were inactive as substrates for the Bacillus enzyme included glycine, L-alanine, L-histidine, L-arginine, L-lysine, L-ornithine, L-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, L-proline, L-serine, L-threonine, L-cysteine, tert-L-leucine, D-phenylalanine, D-tyrosine, DL-phenylglycine, and DL-threo-phenylserine. NAD+ was active as a cofactor for both the enzymes, whereas NADH was not. The enzymes showed lower substrate specificities in the reductive amination reaction than that observed in the oxidative deamination reaction. Table V shows the substrate specificities of the Sporosarcina and the Bacillus enzymes in the reductive amination reactions. In addition to phenylpyruvate, α-ketovalerate, α-keto-γ-methylthiobutyrate, p-hydroxyphenylpyruvate, α-ketoisocaproate were active as substrates for the Sporosarcina enzyme. For the Bacillus enzyme, p-hydroxyphenylpyruvate, phenylpyruvate and α-keto-γ-methylthiobutyrate were active as substrates. Pyruvate, α-ketoglutarate, and α-keto-γ-methylthiobutyrate were active in both the enzymes. L-Glutamine, L-asparagine, methylamine, dimethylamine, trimethylamine, and ethylamine (each at 200 mM) did not replace ammonium ion in the reductive amination reaction catalyzed by the Bacillus enzyme.

Kinetic studies were performed to determine the Michaelis constant (Km) and maximum reaction velocities (Vmax). Using the Bacillus enzyme, the velocity was measured at various concentrations of L-phenylalanine and NAD+. As shown in

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**TABLE IV**

Substrate specificity of phenylalanine dehydrogenase

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>S. ureae SCRC-R04</th>
<th>B. sphaericus SCRC-R79a</th>
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<tr>
<td></td>
<td>Relative activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Km (mM)</td>
<td>Vmax (units/mg)</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>100</td>
<td>0.096% 114%</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>5.4%</td>
<td>72% 112%</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>5.5%</td>
<td>1.2% 112%</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.1%</td>
<td>0.1% 112%</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>4.8%</td>
<td>3.0% 112%</td>
</tr>
<tr>
<td>L-Phenylalanine methyl ester</td>
<td>8.3% 72% 112%</td>
<td>1.4% 112%</td>
</tr>
<tr>
<td>L-Valine</td>
<td>3.1%</td>
<td>1.4% 112%</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>2.3%</td>
<td>1.3% 112%</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0.7%</td>
<td>0.45% 112%</td>
</tr>
<tr>
<td>L-allo-isoleucine</td>
<td>4.3%</td>
<td>0.3% 112%</td>
</tr>
<tr>
<td>L-α-amino-n-butyric acid</td>
<td>1.6% 112%</td>
<td>1.6% 112%</td>
</tr>
<tr>
<td>L-Norvaline</td>
<td>6.0%</td>
<td>1.3% 112%</td>
</tr>
<tr>
<td>L-Norleucine</td>
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<td>20.0% 112%</td>
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<tr>
<td>L-Phenylalaninamide</td>
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<td>2.5% 112%</td>
</tr>
<tr>
<td>L-Tyrosinamide</td>
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<td>1.4% 112%</td>
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<td>L-Phenylalanine methyl ester</td>
<td>35% 72% 112%</td>
<td>20.3% 112%</td>
</tr>
<tr>
<td>L-Tyrosine methyl ester</td>
<td>1.4% 72% 112%</td>
<td>1.4% 112%</td>
</tr>
<tr>
<td>L-Phenylalanine hydroxamate</td>
<td>0.4% 72% 112%</td>
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</tr>
<tr>
<td>L-Tyrosine hydroxamate</td>
<td>4% 72% 112%</td>
<td>3.2% 112%</td>
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<tr>
<td>L-Phenylalaninol</td>
<td>9.4%</td>
<td>0.6% 112%</td>
</tr>
</tbody>
</table>

* Value determined from the secondary plots of intercepts against reciprocal concentration of the substrate.
* Measured at 1.4 mM.
* Measured at 0.3 mM.
* Apparent value determined from double reciprocal plot at a fixed concentration of NAD+ (2.5 mM).
Fig. 3, the intersecting patterns are consistent with a sequential mechanism. From the secondary plots, the $K_m$ value for NAD$^+$ and L-phenylalanine were calculated as 0.17 and 0.22 mM, respectively. The $K_m$ value for L-tyrosine was calculated as 0.50 mM. In a similar manner, the $K_m$ values of the Sporosarcina enzyme for NAD$^+$ and L-phenylalanine were calculated as 0.14 and 0.096 mM, respectively. $K_m$ values of the Sporosarcina enzyme for NADH and ammonia in the reductive amination were calculated from secondary plots of intercepts against a substrate at a fixed concentration of NH$_4$Cl (400 mM) or NADH (0.4 mM), to be 0.072 and 85 mM, respectively. The $K_m$ values of the Bacillus enzyme for NADH and ammonia were calculated in a similar manner to be 0.025 and 78 mM, respectively. Tables IV and V show the $K_m$ and $V_{max}$ values thus calculated.

The mean value of the apparent equilibrium constant ($K_{eq}^{0.100}$) of [phenylpyruvate][NADH][H$^+$][L-phenylalanine][NADH][H$_2$O]) was determined using the Bacillus enzyme to be 1.4 x $10^{-15}$ at the pH range of 7.0-11.5 and 25°C.

Effect of Metal Ions and Inhibitors—The effect of metal ions, inhibitors, and amino acids on the enzyme activity was studied. The enzyme activity was measured after the enzyme was preincubated at 25°C for 10 min with various compounds. The activities of both the Sporosarcina and Bacillus enzymes were completely inhibited by sulfhydryl reagents such as AgNO$_3$ (at 1 mM) and p-chloromercuribenzoate (at 0.02 mM). The Sporosarcina enzyme was more sensitive to sulfhydryl reagents than the Bacillus enzyme, when reacted with HgCl$_2$ (inhibition at 0.01 mM: Sporosarcina enzyme 100%, Bacillus enzyme 62%), 5,5'-dithio-bis(2-nitrobenzoic acid) (inhibition at 0.17 mM: Sporosarcina enzyme 100%, Bacillus enzyme 26%), and N-ethylmaleimide (inhibition at 1 mM: Sporosarcina enzyme 79%, Bacillus enzyme 0%). The activities of both the enzymes were not lost upon preincubation with the following chelating or carbonyl reagents (at 1 mM unless otherwise noted), such as EDTA, 8-oxyquinoline, o,o'-dipyridyl, o-phenanthroline (at 0.5 mM), sodium azide, hydroxylamine (at 10 mM), or KCN (at 0.5 mM). The Sporosarcina enzyme was inhibited by amino acids such as L-leucine, D-phenylalanine, D-tryptophan (at 5 mM), D-methionine, D-norleucine, and D-leucine (inhibition at 10 mM except for D-tryptophan: 39, 77, 65, 25, 34, and 31%, respectively). The Bacillus enzyme was also inhibited by amino acids such as L-isoleucine, L-leucine, L-valine, L-cysteine, D-phenylalanine, D-leucine, D-tryptophan (5 mM), D-methionine, D-norleucine, and D-tyrosine (at 1 mM) (inhibition at 10 mM except for D-tryptophan and D-tyrosine: 75, 58, 23, 41, 89, 17, 11, 83, 63, 52, and 29%, respectively).

Stability—Both the enzymes are stable at 4°C over a period of at least 1 month in 0.01 M potassium phosphate buffer, pH 7.0 containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol, and at least 2 years in crystalline forms.

Immunochemical Properties—As shown in Fig. 4, the antibody raised against the purified Bacillus enzyme formed a single precipitation line with the partially purified enzymes from B. sphaericus SCRC-R79a, Bacillus sp. SCRC-R53b, and Bacillus sp. SCRC-101A. The precipitation lines completely fused with each other. These bacteria appear to form immunologically identical enzymes. The antibody did not cross-react with the partially purified enzymes from S. ureae SCRC-R04, Bacillus sp. SCRC-114D, purified leucine dehydrogenase from B. sphaericus, and the crude extract of B. sphaericus IFO-3525, which contains both alanine and leucine dehydrogenase activities (4, 10), and B. stearothermophilus IFO-12550 (32). As shown in Fig. 5, immunotitration experiments of partially purified phenylalanine dehydrogenases from various strains were made with the antibody. The titration curves for B. sphaericus SCRC-R79a and Bacillus sp. SCRC-R53b enzymes were similar. However, the inactivation of the enzyme from Bacillus sp. SCRC-114D required more than seven times the antibody than that for B. sphaericus SCRC-R79a. The antibody did not inactivate any significant activity of the purified Sporosarcina enzyme. Thus, B. sphaericus SCRC-R79a, Bacillus sp. SCRC-R53b, and Bacillus sp. SCRC-101A enzymes are immunologically identical, whereas Bacillus SCRC-114D has only partial similarity to them. The Sporosarcina enzyme and other dehydrogenases are immunologically different from the Bacillus enzyme.

Stereochemistry of Hydride Transfer from NADH—To investigate the stereochemistry of the hydride transfer reaction between NADH and phenylpyruvate in the phenylalanine dehydrogenase reaction, two enantiomers of the deuterated NADH derivative [4A-'H]NADH and [4B-'H]NADH were enzymatically prepared, and the incorporation of deuterium into L-phenylalanine was examined. When [4A-'H]NADH was used as a substrate in the reductive amination reaction by the Bacillus enzyme, no incorporation of deuterium into position 2 of L-phenylalanine was detected by mass spectrometry. On the other hand, 0.7 atoms of deuterium was incorporated into one molecule of L-phenylalanine when reacted with [4B-'H]NADH. Thus, the stereochemistry of the
purification and characterization of the enzyme. NAD+-dependent phenylalanine dehydrogenases isolated from Sporosarcina and Bacillus in crystalline form exhibit the stoichiometry of typical L-amino acid dehydrogenase catalyzing the reversible oxidation and reduction reactions acting on L-phenylalanine, placing them in the oxidoreductase category with the systematic name L-phenylalanine:NAD+ oxidoreductase, deaminating, (EC 1.4.1.1). In the present investigation, NAD+-dependent phenylalanine dehydrogenase was found to be very narrowly distributed among microorganisms. The enzyme activity was not found in the yeast or bacterial genera tested but in some specific aerobic spore-forming Gram-positive bacterial strains such as S. ureae SCRC-R04 and B. sphaericus SCRC-R79a, which had been found among L-phenylalanine-assimilating bacteria isolated from soil. The enzyme activity was not found in the deposited Bacillus strains we tested.

The properties of phenylalanine dehydrogenase from S. ureae SCRC-R04 and B. sphaericus SCRC-R79a are summarized in Table VI. The molecular weight of the Sporosarcina enzyme was calculated to be 305,000 and 310,000 by ultracentrifugal analysis and gel filtration on HPLC, respectively, and the Bacillus enzyme to be 340,000 in either method. The molecular weight of the subunit of the enzymes, determined by SDS-polyacrylamide gel electrophoresis, was about 39,000 for both the enzymes. The results of NH2- and COOH-terminal amino acid analyses demonstrate that the enzymes consist of possibly eight (or nine) polypeptide chains. They are similar in specific activity, molecular weight, and subunit structure but different immunologically from each other and in catalytic properties such as substrate specificity and behavior toward heat and pH. The Bacillus enzyme was more stable than the Sporosarcina enzyme with respect to heat and pH. The pH optima of the former enzyme was more on the alkaline side than the latter.

The phenylalanine dehydrogenases from S. ureae and B. sphaericus are thus characterized by their octameric (or non-meric) structure, whereas those of previously reported Bacillus alanine dehydrogenase (molecular weight of subunit, 38,000) and leucine dehydrogenase (molecular weight of subunit, 41,000) are mostly hexameric (4, 10, 32, 34), with the single exception in leucine dehydrogenase from B. cereus (molecular weight of subunit, 39,000) whose subunit structure was found to be octameric (35, 36). The stereochemistry of hydride transfer from NAD(P)H is B-stereospecific, i.e. the pro-S hydrogen of NAD(P)H is transferred to α-keto acids, in most amino acid dehydrogenases reported so far, such as glutamate dehydrogenase (12), leucine dehydrogenase (10), and meso-α,ε-diaminopimelate D-dehydrogenase (13). However, alanine dehydrogenase is an A-stereospecific enzyme. This is the only exception among the amino acid dehydrogenases (4, 11). In this report, the stereochemistry of hydride transfer from NADH in Bacillus phenylalanine dehydrogenase was proved to be B-stereospecific. Therefore, phenylalanine dehydrogenase from B. sphaericus SCRC-R79a shares a common stereospecificity in the hydride transfer from NADH with leucine dehydrogenase from B. sphaericus IPO-3525. Thus, phenylalanine dehydrogenase resembles leucine dehydrogenase from B. cereus (35, 36), with respect to the molecular weight and the number of subunits and possibly to the stereochemistry of hydride transfer from NADH.

The equilibrium constants for the glutamate dehydrogenase, alanine dehydrogenase, leucine dehydrogenase, and phenylalanine dehydrogenase are 8.56 x 10^-14 (37); 1.36 x 10^-14

2 Value at 0.1 ionic strength with NAD+ as a cofactor.

**DISCUSSION**

We have described in detail the distribution, purification, physicochemical, immunological, and catalytic properties of phenylalanine dehydrogenases from S. ureae SCRC-R04 and B. sphaericus SCRC-R79a. The enzyme was first found in *Brevibacterium* sp. by Hummel et al. in 1984 (1). Apart from some details using a crude extract there are no reports on

**TABLE VI**

Comparison of properties of phenylalanine dehydrogenase purified from S. ureae SCRC-R04 and B. sphaericus SCRC-R79a

<table>
<thead>
<tr>
<th>Property</th>
<th>Value with enzyme from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. ureae SCRC-R04</td>
</tr>
<tr>
<td>Specific activity of initial preparation (μmol/mg/min)</td>
<td>84.1</td>
</tr>
<tr>
<td>Molecular weight</td>
<td></td>
</tr>
<tr>
<td>Sedimentation equilibrium</td>
<td>305,000</td>
</tr>
<tr>
<td>Gel filtration on HPLC</td>
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</tr>
<tr>
<td>Molecular weight of subunit</td>
<td>39,000</td>
</tr>
<tr>
<td>Number of subunits</td>
<td>8</td>
</tr>
<tr>
<td>pH</td>
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</tr>
<tr>
<td>Oxidative deamination</td>
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</tr>
<tr>
<td>Reductive amination</td>
<td>9.0</td>
</tr>
<tr>
<td>Km (mM) value for</td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0.096</td>
</tr>
<tr>
<td>L-Tyrosine</td>
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</tr>
<tr>
<td>Phenylpyruvate</td>
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</tr>
<tr>
<td>p-Hydroxyphenylpyruvate</td>
<td>0.14</td>
</tr>
<tr>
<td>NAD+</td>
<td>0.072</td>
</tr>
<tr>
<td>NADH</td>
<td>85</td>
</tr>
<tr>
<td>Ammonia</td>
<td>85</td>
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</tbody>
</table>

Hydride transfer from NADH to phenylpyruvate catalyzed by the *Bacillus* phenylalanine dehydrogenase was proved to be B-stereospecific.
seems to be no correlation between the stereochemistry of the charged polar groups. The substrate specificity of the dehydrogenases.

addition to L-phenylalanine. The enzymes showed high affinity for L-phenylalanine. No activity is seen with D-amino acids, or respectively, whereas the other Bacillus amino acid dehydrogenase have one or two order lower affinities than phenylalanine dehydrogenase for their best substrates. The $K_m$ values for alanine dehydrogenase for L-phenylalanine is 18.9 mM for L-phenylalanine (4), and the $K_m$ value of leucine dehydrogenase for L-leucine is 1 mM for L-leucine (10). In the reductive amination, both the enzymes showed relatively generous substrate specificity, as has been reported in other amino acid dehydrogenases (4, 10).

The enzymes were not inactivated by metal chelating or carbonyl reagents but by sulfhydryl reagents. This observation is a common character among amino acid dehydrogenases. The catabolism of L-alanine to pyruvate during the vegetative growth on L-alanine in Bacillus subtilis was detected in phenylalanine dehydrogenase-producing Bacillus sp. DSM 2448, Hummel et al. (1) suggested that the enzyme is responsible for the degradation of L-phenylalanine and not for the synthesis of the amino acid. In S. ureae SCRC-R04, however, L-phenylalanine aminotransferase activity with oxaloacetate as an amino acceptor, was detected during the growth on a medium containing L-phenylalanine at a range between 0.001 and 0.006 units/mg, while phenylalanine dehydrogenase was highly induced (0.033 units/mg). The formation of phenylalanine dehydrogenase was growth-associated, indicating that the enzyme is responsible for the catabolism of L-phenylalanine. On the other hand, when the strain was grown in a M9 medium supplemented with 0.1% yeast extract, the phenylalanine dehydrogenase activity was detected only in the exponential phase at a level as high as 0.059 units/mg, much higher than L-phenylalanine aminotransferase activity (less than 0.001 units/mg). Considering that the equilibrium of the phenylalanine dehydrogenase is favored toward the synthesis of amino acid, it is probable that the enzyme also functions in the anabolism of L-phenylalanine, depending on the environment.

The immunochemical studies revealed that three immunologically different phenylalanine dehydrogenases occur in five Sporosarcina and Bacillus strains isolated from soil. The present study indicates that there still may be more new enzymes on earth to be exploited, which may even act on a very common metabolite.

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REFERENCES

2 All the values were calculated assuming the conventional concentration of water ($[\text{H}_2\text{O}] = 1 \text{ M}$).
3 Y. Asano and A. Nakazawa, unpublished data.