Short Communication

Bacillus Phenylalanine Dehydrogenase Produced in *Escherichia coli* —Its Purification and Application to L-Phenylalanine Synthesis—

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We previously reported the first purification to homogeneity of a phenylalanine dehydrogenase from a soil isolate, *Sporosarcina ureae* SCRC-R04.[1)] Subsequently, we isolated and identified *Bacillus sphaericus* SCRC-R79a which produced another phenylalanine dehydrogenase.[2)] The equilibrium of the enzyme reaction strongly favors amino acid synthesis,[2,3)] thus the enzyme is useful for the preparation of L-amino acids from their keto analogs.[3,4)]

In this communication, we describe the purification and identification of the *B. sphaericus* phenylalanine dehydrogenase produced in an *Escherichia coli* transformant, and the synthesis of L-phenylalanine from phenylpyruvate using the *E. coli* and *Candida boidinii* cells.

Plasmid pBPDH3 comprises a 4.0 kb fragment of the phenylalanine dehydrogenase gene in pBR322.[5)] The 1.3 kb fragment from the pBPDH3 insert was subcloned into pUC9[5)] to give pBPDH1-DBL. Both plasmids contain endogeneous promoter and ribosomal recognition sequences originating from *B. sphaericus* SCRC-R79a, and can direct the synthesis of phenylalanine dehydrogenase in *E. coli*. The details of the cloning of the phenylalanine dehydrogenase gene and analysis of the plasmids will be reported elsewhere. *E. coli* RR1[5)] and JM 109[6)] were used as hosts, and cultivated with shaking at 37°C for 12 hr in LB medium,[5)] pH 7.5, supplemented with 50 μg/ml ampicillin. The transformation of *E. coli* was carried out as described by Hanahan.[7)]

E. coli transformant JM 109/pBPDH1-DBL expressed about 120-fold higher total phenylalanine dehydrogenase activity per liter of culture (7,200 units*/l) than *B. sphaericus* SCRC-R79a.[2)] The enzyme was purified about 9-fold up to the specific activity of 179 units/mg* with a 56% yield from the cell-free extract by a procedure involving heat treatment, ammonium sulfate fractionation, and column chromatographies on DEAE-Toyopearl and Sephadex G-200, as described previously.[2)] The enzyme was found to be homogeneous on disc gel electrophoresis (7.5%, pH 8.9). The molecular weight of the enzyme was estimated to be 310,000 ~ 340,000 by gel filtration on a G 3000 SW HPLC column (0.75 × 60 cm, Toyo Soda, Japan), eluted with 0.1 M potassium phosphate buffer, pH 7.0, containing 0.2 M NaCl. The molecular weight of the subunit of the enzyme was determined to be approximately 39,000 on SDS-disc gel electrophoresis (10%, pH 7.2). The enzyme appeared to have an octameric structure. The enzymes purified from *B. sphaericus*[2)] and the *E. coli* transformant electrophoresed to exactly the same positions on polyacrylamide and SDS-polyacrylamide gels. Both of them were eluted at the same position as to molecular weight on a G 3000 SW column, and at the same ionic strength on a DEAE-5PW column (0.75 × 7.5 cm, Toyo Soda, Japan) in 0.02 M Tris–HCl, pH 8.0, with a linear gradient of 0.15 M to 0.5 M NaCl. Figure 1 shows that the antibody against the purified enzyme from *B. sphaericus* formed a single precipitation line with the purified *E. coli* enzyme, and the line fused with that with the purified *B. sphaericus* enzyme. Therefore,

* The enzyme activity and protein were determined, and the units defined as described previously.[2)]

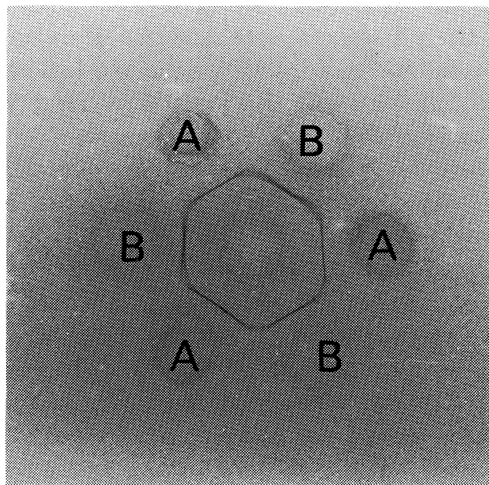

FIG. 1. Ouchterlony Double Diffusion of the Phenylalanine Dehydrogenases.

Ouchterlony plates were made using 1% Agar Purified (Difco) in 10 mM potassium phosphate buffer, pH 7.0, containing 130 mM NaCl and 0.01% sodium azide. The center well contained the antibody against the phenylalanine dehydrogenase from *B. sphaericus* SCRC-R79a[2] (1 μl of antiserum). The outer wells contained the purified enzymes as follows: A, enzyme from *B. sphaericus* SCRC-R79a[2] (3.0 μg); B, enzyme from *E. coli* JM 109/pBPDH1-DBL (3.3 μg). The agar plate was stained with Amido Black 10B.

they share the same antigenic determinants. The enzyme sample was passed through a Phenyl-5PW column (0.75 × 7.5 cm, Toyo Soda, Japan), and fractionated with a linear gradient of 20 ~ 80% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid. The enzyme sample (about 300 μg) was analyzed with an automatic protein sequencer 470A (Applied Biosystem, U.S.A.). Identification of phenylthiohydantoin derivatives was carried out with a HPLC system SP 8100 XR (Spectra Physics, U.S.A.) on a reversed phase column, Senshu Pack Aquasil SEQ-4 (0.46 × 30 cm, Senshu Kagaku, Japan). The N-terminal amino acid sequence of the enzyme purified from *B. sphaericus* was determined to be Ala^1-Lys-Gln-Leu-Glu^5-Lys-Ser-Ser-Lys-Ile^{10}-Gly-Asn-Glu-Asp-Val^{15}-Phe-Gln-Lys-Ile-Ala^{20}-Asn-X-X-Gln-Ile^{25}-Val-Phe-X-Asn (X denotes an undetermined amino acid residue). The N-terminal amino acid sequence of the enzyme produced by *E. coli* was the same at amino acid residues No. 1 to 12, 17 and 18, that were determinable on the sequencing. From these results, we can conclude that plasmid pBPDH1-DBL encodes the *B. sphaericus* phenylalanine dehydrogenase gene, and the gene is expressed in *E. coli* JM 109/pBPDH1-DBL. It is noteworthy that the specific activity of the final enzyme preparation from the *E. coli* transformant (179 units/mg) was higher that of the *Bacillus* enzyme (111 units/mg),[2] although the reason for this has not been elucidated.

We previously demonstrated that phenylalanine dehydrogenase acts as an excellent catalyst for the asymmetric synthesis of L-phenylalanine and related L-amino acids, in combination with formate dehydrogenase from *C. boidinii.*[3] However, the amount of the enzyme formed by the wild strain was insufficient for practical use.[1~3] We here developed a simple and convenient synthetic method for L-phenylalanine, utilizing cells of *C. boidinii*[8] and an *E. coli* transformant which expresses a high amount of phenylalanine dehydrogenase. The *E. coli* transformant, RR1/pBPDH3, employed in the following experiment showed about 40-fold higher total enzyme activity per liter of culture than that of *B. sphaericus* SCRC-R79a. A reaction mixture containing 250 μmol Tris–HCl, pH 8.5, 183 μmol sodium phenylpyruvate, 2.5 μmol NAD^+, 2 mmol ammonium formate, and 3 mg and 30 mg acetone–dried cells of *E. coli* RR1/pBPDH3 and *C. boidinii*, respectively, in a total volume of 3.0 ml, was incubated at 30°C for 53 hr. Solid sodium phenylpyruvate (183 μmol each), at 7, 20 and 32 hr, and solid ammonium formate (1.2 mmol each), at 24 and 32 hr, were added after the reaction was started. Microbiological assaying of the reaction mixture using *Pediococcus acidilactici* ATCC 8042 revealed that the phenylpyruvate was stoichiometrically converted into L-phenylalanine (40 mg/ml).

Since phenylalanine dehydrogenase has become abundantly available due to the recombinant DNA technique, the synthetic method described above may be applicable to the

large-scale preparation of L-phenylalanine and related L-amino acids.

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