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# CHORISMATE MUTASE/PREPHENATE DEHYDRATASE FROM *ESCHERICHIA COLI*: SUBCLONING, OVERPRODUCTION AND PURIFICATION

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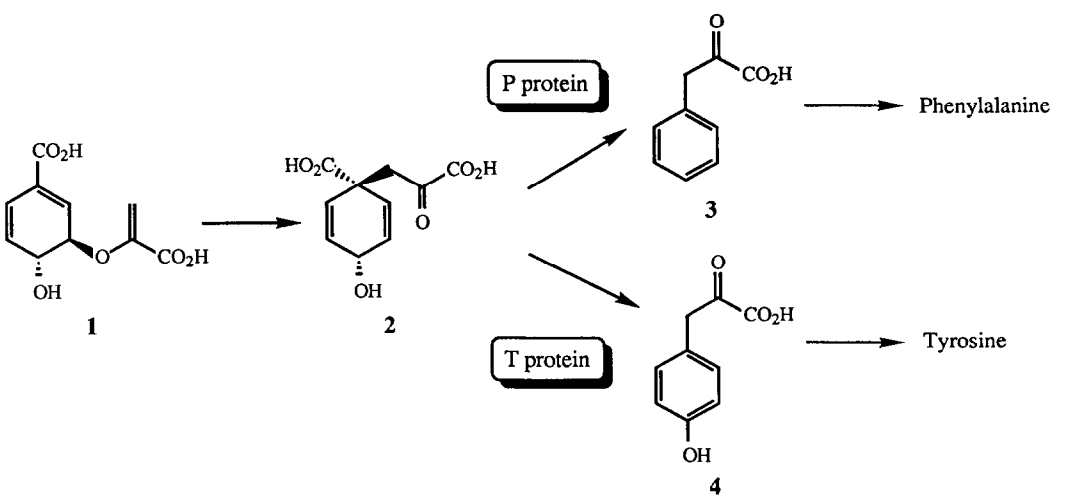
**Abstract:** Efficient overproduction of chorismate mutase/prephenate dehydratase (20% of soluble protein) has been achieved using recombinant DNA techniques. A new purification procedure utilizing hydrophobic and ion-exchange chromatography yielded homogeneous enzyme in two steps.

## INTRODUCTION

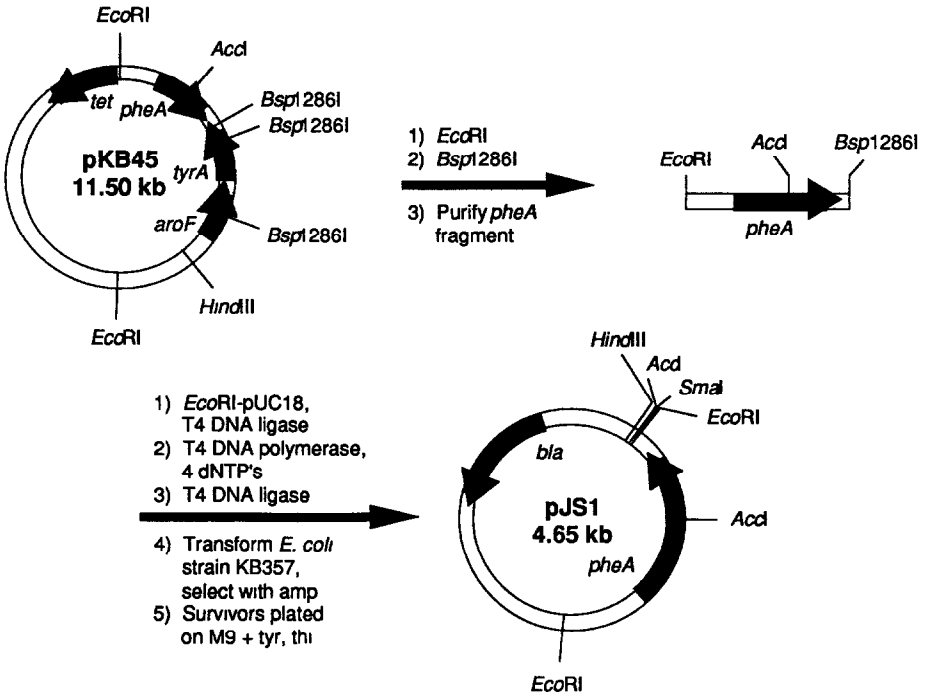
The Claisen rearrangement of chorismic acid **1** to prephenic acid **2** (Scheme 1) represents the first committed step in the biosynthesis of phenylalanine and tyrosine in bacteria and plants.[1,2] This conversion has attracted much attention from chemists, both because it is an unusually facile Claisen rearrangement and because it is the sole example of an enzyme-catalyzed pericyclic reaction: chorismate mutases accelerate this reaction by more than a million-fold.[3] While much effort has gone into studying isotope effects[4,5] and devising inhibitors,[6] the catalytic mechanism of these enzymes remains obscure. Mechanistic and physical studies in this area would be facilitated by an efficient source of pure mutase.

**SCHEME 1**




Bifunctional chorismate mutases produced by the bacterium *Escherichia coli* have been studied most thoroughly.[7] In addition to the abovementioned Claisen rearrangement, these enzymes also catalyze either the decarboxylation and dehydration of **2** to form phenylpyruvate **3**, or the oxidative decarboxylation of **2** to form

**SCHEME 2**




*p*-hydroxyphenylpyruvate **4**. Genes encoding both enzymes have been cloned[8] and sequenced.[9] While Bhosale *et al.* reported overproduction of the T protein (approximately 15% of soluble protein)[10], there have been no reports of P protein overproduction. We were especially interested in the P protein since both biochemical[11-13] and genetic[14] studies indicated that the two active sites of this enzyme were essentially independent. Here we describe the overproduction and purification of *E. coli* P protein.

## CONSTRUCTION OF A PLASMID FOR P PROTEIN OVEREXPRESSION

We planned to overproduce the P protein by cloning its gene (*pheA*) into a high-copy number plasmid containing a strong *E. coli* promoter (Scheme 2). The source of the *pheA* gene was plasmid pKB45, a derivative of pMB9 which contained a 6 kilobase (kb) segment of *E. coli* chromosomal DNA inserted at its unique *Eco*RI site.[8] Plasmid pKB45 was digested sequentially with restriction enzymes *Eco*RI and *Bsp*1286I[15] and the 1.9 kb segment containing *pheA* was purified by agarose gel electrophoresis. The vector, plasmid pUC18,[16] was prepared by digestion with *Eco*RI. The two DNA's were treated with T4 DNA ligase; a linear molecule in which the *pheA* gene had been joined with vector was the desired product. Since the *Eco*RI and *Bsp*1286I ends were not complementary, both ends were blunted by treating the ligation mixture with T4 DNA polymerase[17] in the presence of all four dNTP's. In this manner, the *Eco*RI 5' overhang was filled in by the polymerase and the *Bsp*1286I 3' overhang was removed by the 3'-5' exonuclease activity of this bifunctional enzyme. Finally, the mixture was treated with T4 DNA ligase to circularize the DNA. The final ligation mixture was used to

transform *E. coli* strain KB357[18] (relevant genotype: Δ[*pheA-tyrA*], *thi*). Transformants were initially selected by plating on rich medium containing ampicillin; survivors were then plated on glucose minimal plates containing only tyrosine and thiamine. Plasmid DNA from colonies which grew under the latter conditions (where a plasmid-borne *pheA* gene complemented the chromosomal deletion) was isolated by alkaline mini-prep[19] and analyzed by restriction digestions. A plasmid, designated pJS1, was identified with the desired structure. This plasmid was used for all overexpression experiments.

## OVERPRODUCTION AND PURIFICATION OF THE P PROTEIN

It proved difficult to grow KB357(pJS1) reproducibly, therefore the plasmid was moved into *E. coli* strain KS474 (relevant genotype: *degP*41 [Δ*Pst*I-Kan])[20] by a standard calcium chloride-mediated transformation. The resulting strain, KS474(pJS1), was used for all overexpression experiments. After considerable experimentation, optimal growth conditions were determined consisting of M9 minimal medium[21] supplemented with isopropyl–β-D-galactoside (IPTG) and ampicillin, with glucose as carbon source. Under these conditions, P protein represented approximately 20% of soluble protein in a crude extract of KS474(pJS1).

Since the previously-reported procedures[22,23] were either lengthy or irreproducible, a new procedure for purifying the P protein was devised (Table 1) which required two chromatographic steps and afforded a 26% recovery of mutase activity. Using the procedure of Gething *et al.*,[23] a crude extract of KS474(pJS1) was prepared and nucleic acids were precipitated with streptomycin sulfate. The supernatant was chromatographed on Phenyl-Sepharose, a hydrophobic support to which P protein adsorbed very strongly (1:1 $H_2O$:ethylene glycol was required to elute the enzyme). In contrast, the P protein bound very poorly to phenylalanyl-sepharose. Fractions containing appreciable mutase activity were pooled and applied to a column of Q-Sepharose (an anion exchange resin). After washing with starting buffer, the column was developed with a linear salt gradient. Essentially homogeneous P protein was recovered from this column (Figure 1). The amino acid composition[9] and final specific activities[23] (mutase, 45 U/mg; dehydratase, 21 U/mg) of overexpressed P protein agreed well with literature values.

**TABLE 1**

*PURIFICATION OF CHORISMATE MUTASE/PREPHENATE DEHYDRATASE*

| Sample | Volume (mL) | Protein conc. (mg/mL) | Total Protein (mg) | Mutase Activity (U/mL) | Total Activity (U) | Specific Activity (U/mg) | Yield |
|---|---|---|---|---|---|---|---|
| Crude extract | 16 | 11 | 180 | 110 | 1760 | 10 | 100% |
| After streptomycin sulfate | 17 | 6.8 | 116 | 88 | 1500 | 13 | 85% |
| After Phenyl-Sepharose | 50 | 0.34 | 17 | 13 | 650 | 38 | 37% |
| After Q-Sepharose | 2.5 | 4.0 | 10 | 180 | 450 | 45 | 26% |

In summary, *E. coli* chorismate mutase/prephenate dehydratase has been efficiently overproduced by recombinant DNA techniques. Further, a simplified purification procedure has been developed so that 10 mg of homogeneous enzyme can be easily obtained from a one-liter bacterial culture. Efforts to grow crystals of this intriguing enzyme suitable for X-ray analysis are in progress and results will be reported in due course.

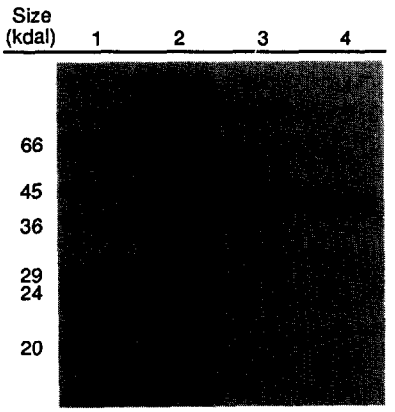



Figure 1: SDS-polyacrylamide gel (12%) summarizing P protein purification. After running, the gel was stained with Coomassie Blue as described in the Experimental section. (1) molecular weight markers; (2) crude extract of KS474(pJS1); (3) after chromatography on phenyl-Sepharose; (4) after chromatography on Q-Sepharose.

## EXPERIMENTAL SECTION

General. Rich medium (LB)[21] was used for routine growth of bacteria. For plates, agar was added at a concentration of 15 g per liter. When required for plasmid maintenance, ampicillin or tetracycline was added at concentrations of 200 and 15 μg/mL, respectively. Restriction endonucleases were purchased from Amersham Corp. (Arlington Heights, IL), New England Biolabs (Beverly, MA) or United States Biochemical Corp. (Cleveland, OH). T4 DNA polymerase was obtained from IBI (New Haven, CT). Recombinant DNA procedures were carried out essentially as described by Maniatis *et al.*[21] Both Phenyl- and Q-Sepharose were products of Pharmacia (Piscataway, NJ). SDS gel electrophoresis was carried out as described by Laemmli;[24] gels were stained after running with Coomassie Brilliant Blue R for 30 min at room temperature, then de-stained overnight. Protein concentrations were estimated by the method of Bradford[25] using bovine serum albumin as a standard. Amino acid analysis was performed on a PICO-TAG analyzer (Waters Associates, Milford, MA).
Enzyme assays. Mutase and dehydratase assays were performed as described by Gething *et al.*[23] Chorismic acid was isolated from *Klebsiella pneumoniae* strain 62-1.[26]
Growth of KS474(pJS1). An overnight culture of KS474(pJS1) grown in LB + ampicillin was diluted 1:100 into 1 L of M9 medium supplemented with IPTG (1 m*M*) and ampicillin (100 μg/mL) in a baffled 2.8 L Fernbach flask. Sterile glucose was added to a final concentration of 0.4%. The culture was shaken at 250 rpm in a rotary shaker at 30°C. After 17 h, this culture reached an optical density at 600 nm of 2.0. At this time, the cells were harvested by centrifugation (7,000 x g for 20 min at 4 °C), then re-suspended in 20 mL of 100 m*M* Tris-Cl (pH 8.2), 5 m*M* EDTA. After centrifugation as above, 2.7 g of wet cells were obtained.
Purification of P protein from KS474(pJS1). **All purification steps were performed at 4°C.** The cells were re-suspended in 15 mL of 100 m*M* Tris-Cl (pH 8.2), 5 m*M* EDTA, then disrupted in a French pressure cell (Aminco, Inc.) at 15,000-20,000 psi. Debris was removed by centrifugation at 31,000 x g for 30 min at 4°C. Nucleic acids were precipitated from the supernatant (16 mL) by adding 0.15 volume (2.4 mL) of freshly-prepared 30% (w/v) streptomycin sulfate solution in four equal portions. After incubating for 2 h, the precipitate was removed by centrifugation at 20,000 x g for 30 min at 4°C. The salt concentration of the supernatant (17 mL) was adjusted to approximately 400 m*M* by adding 375 mg of solid NaCl and its pH was adjusted to 8.2 with 1 *M* $NH_4OH$. The mixture was applied to a column of Phenyl-Sepharose (1.5 x 22 cm) which had been equilibrated with 10 m*M* Tris-Cl (pH 8.2), 5 m*M* 2-mercaptoethanol, 0.5 m*M* EDTA, 400 m*M* NaCl. After the sample had been applied, the column was washed with 75 mL each of starting buffer, starting buffer minus NaCl, distilled water, and 10 m*M* Tris-Cl (pH 8.2), 5 m*M* 2-mercaptoethanol, 0.5 m*M* EDTA, 10% ethylene glycol, then with 100 mL of 50% aqueous ethylene glycol. The flow was 45 mL/h and 6-mL fractions were collected. A small amount of mutase activity eluted in the distilled water wash, but the bulk was removed by 50% ethylene glycol. Fractions from the final wash exhibiting substantial mutase activity were pooled (50 mL) and applied to a column of Q-Sepharose (1.5 x 16 cm) which had been equilibrated with 10 m*M* Tris-Cl (pH 7.4), 5 m*M* 2-mercaptoethanol, 0.5 m*M* EDTA. After washing with 70 mL of starting buffer, the column was developed with a linear NaCl gradient formed by 400 mL each of starting buffer and starting buffer plus 800 m*M* NaCl. The flow was 30 mL/h and 6-mL fractions were collected. The P protein eluted at approximately 260 m*M* NaCl.

Mutase-active fractions were pooled and concentrated with a CX-30 ultrafiltration membrane (Millipore, Bedford, MA). Purified P protein was stored at 4°C since freezing and thawing was found to cause precipitation, even at high enzyme concentrations.

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