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Synthesis of Chiral Arogenic Acid via Immobilized Microbial Proteins

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Although L-(8*S*)-arogenate has been recognized as a potential precursor of L-phenylalanine or L-tyrosine biosynthesis for only a few years, it is widely distributed in nature. The biochemical formation of arogenate has involved its isolation from the culture supernatant of a mutant strain of *Neurospora crassa*, a lengthy procedure of 20-day duration. We now report an improved approach using immobilized crude enzyme extracts from a cyanobacterium. The starting materials, chorismic acid or prephenic acid, are readily available, and overall yields ranging from 40 to 60% are obtained. The whole procedure takes only 1 day. Crude, unfractionated enzyme extracts from *Synechocystis* sp. ATCC 29108 are immobilized on a phenoxyacetyl cellulose solid support. The hydrophobic binding of the extract proteins did not denature chorismate mutase or prephenate aminotransferase, the enzymes catalyzing the conversion of chorismate to prephenate and prephenate to arogenate, respectively. This microbial system was ideally suited for preparation of arogenate, since other enzyme activities which might compete for prephenate or chorismate as substrates, or which might further metabolize arogenate, were absent or inactive under the conditions used. In addition to the substrates prephenate or chorismate, pyridoxal-5′-phosphate (the coenzyme required for transamination), as well as leucine (amino donor for transamination of prephenate), was added. The reaction product, arogenate, was separated from the starting materials by preparative thin-layer chromatography.

INTRODUCTION

For a long time the biosynthetic steps leading to the essential amino acids, phenylalanine and tyrosine, were thought to follow a universal pattern. Rearrangement of chorismic acid **1** yielded compound **2** (Fig. 1), believed to be the last nonaromatic precursor and therefore appropriately named prephenic acid (*1*). Enzymatic dehydration of prephenate coupled with decarboxylation would form the intermediate phenylpyruvate, **3**, thence converted by transamination to form L-phenylalanine, **4**. Tyrosine biosynthesis from prephenate requires two steps: (i) oxidative decarboxylation of prephenate to produce 4-hydroxyphenylpyruvate **5**, and (ii) transamination of 4-hydroxyphenylpyruvate. Indeed, biosynthesis of phenylalanine and tyrosine in such familiar microbes as *Escherichia coli* and *Bacillus subtilis* does involve phenylpyruvate and 4-hydroxyphenylpyruvate as obligatory intermediates which proceed from prephenic acid.

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In 1974, Jensen and co-workers (*2*) found that at least many species of cyanobacteria did not utilize 4-hydroxyphenylpyruvate as the precursor of tyrosine. A new intermediate termed pretyrosine was isolated and tentatively assigned a structure consistent with a nonaromatic amino acid derived from transamination of prephenate. Due to the instability of pretyrosine, its difficult accessibility from isolation, and the large number of functional groups, the structure was rigorously proven only in 1980 by Zamir *et al.* (*3*). In addition, pretyrosine was eventually found in some organisms [e.g., *Pseudomonas diminuta* (*4*), *Euglena gracilis* (*5*), and *Pseudomona aeruginosa* (*6, 7*)], to act as a precursor for phenylalanine synthesis as well. It was therefore renamed arogenic acid (*3*), to connote its potential to serve as precursor for biosynthesis of either amino acid. At one extreme (Fig. 2), only the arogenate pathway is present for the biosynthesis of phenylalanine and tyrosine (*E. gracilis*). Decarboxylation and dehydration of arogenate produces phenylalanine, whereas tyrosine is derived by an oxidative decarboxylation of arogenate. Another extreme case (*6, 7*) is presented by *P. aeruginosa,* where dual pathways exist for the production of phenylalanine (the phenylpyruvate and the arogenate pathways), as well as two pathways for the synthesis of tyrosine (the 4-hydroxyphenylpyruvate and the arogenate pathways). *P. diminuta* (*4*) uses the arogenate pathway for L-phenylalanine biosynthesis, but uses the 4-hydroxyphenylpyruvate pathway for L-tyrosine biosynthesis. Cyanobacteria and coryneform bacteria show the reverse picture, tyrosine originating from the arogenate pathway and L-phenylalanine from the phenylpyruvate pathway.

The growth and modified purification procedures (*3, 8*) for accumulation and

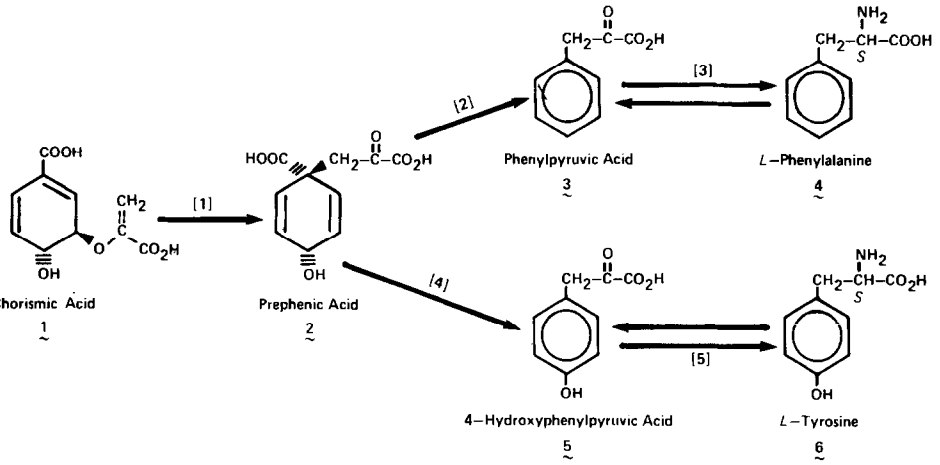

FIG. 1. Biosynthesis of L-phenylalanine and L-tyrosine in procaryotes such as *E. coli*. The rearrangement of chorismic acid **1** to prephenate **2** is catalyzed by chorismate mutase [1]. The conversion of prephenic acid **2** to phenylpyruvate **3** is catalyzed by the enzyme prephenate dehydratase [2]. Phenylpyruvate **3** is transaminated to L-phenylalanine **4** via phenylpyruvate aminotransferase [3]. Prephenate dehydrogenase [4] catalyzes the oxidative decarboxylation of prephenic acid **2** to give 4-hydroxyphenylpyruvate **5**. This latter compound is transaminated to L-tyrosine in the presence of 4-hydroxyphenylpyruvate aminotransferase [5].

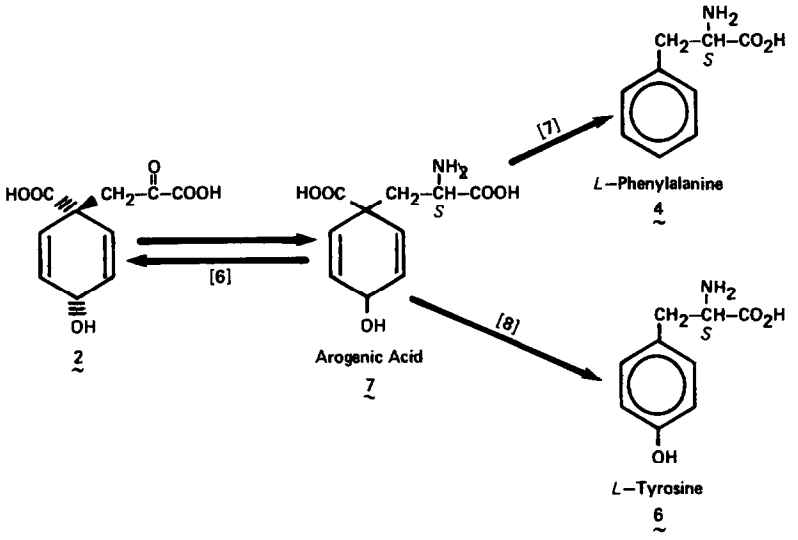

FIG. 2. The arogenate sequences for biosynthesis of L-phenylalanine and L-tyrosine. Transamination of prephenate **2** with prephenate aminotransferase [6] yields arogenic acid **7**. The conversion of arogenic acid to L-phenylalanine **4** is catalyzed by the enzyme arogenate dehydratase [7]. The formation of L-tyrosine **6** is catalyzed by arogenate dehydrogenase [8]. The pathway diagrammed is exemplified in nature by *Euglena gracilis* (*5*).

isolation of L-arogenate from the culture supernatant of a mutant strain of *Neurospora crassa* (75001/5212/C-167) require a 3-week process. Also, arogenate being acid labile, pH wandering must be avoided at all times during this period or the isolation yield can be negligible. Total synthesis of the arogenate molecule has been accomplished (*9*), as has been the total synthesis of prephenate (*14, 15*). However, chemical synthesis probably would not be the most practical procedure for obtaining arogenate in quantities adequate for biochemical and physiological studies. In this study we report a new and convenient preparative synthesis of chiral arogenic acid as well as of [^{14}C]arogenic acid via immobilization of unfractionated cyanobacterial enzymes, starting either from chorismate or prephenate. Fortunately, chorismate (*10*) or prephenate (*11, 12*) is easily obtained from cultures of microbes bearing appropriate genetic blocks. Prephenate has also been obtained through immobilization of a partially purified chorismate mutase on polyamine agarose using chorismic acid as a substrate (*13*).

EXPERIMENTAL PROCEDURES

Materials

Phenoxyacetyl cellulose was obtained from Regis Chemical Company, Morton Grove, Illinois. Chorismate was isolated as the free acid from *Klebsiella pneumoniae 62-1* (*10*), and disodium prephenate was isolated from a tyrosine auxotroph of

Salmonella typhimurium (*12*). All other biochemicals and all chemical reagents were of the best quality available from commercial sources.

Methods

Preparation of Crude Enzyme Extract from Synechocystis sp

Stock cultures of *Synechocystis* sp. ATCC 29108 were maintained on 2% agar (Difco) slants of Cg10 medium (*16*) containing (in grams per liter): EDTA, 0.003; $FeCl_2$, 0.004; $MgSO_4 \cdot 7\ H_2O$, 0.25; KNO_3, 1.0; $CaNO_3 \cdot 4H_2O$, 0.025; K_2HPO_4, 0.050; glycylglycine, 1.0; and 1 ml of trace elements (TE-5). Mixture TE-5 contained (in grams per liter): HBO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4$, 0.222; MoO_3, 0.018; $CuSO_4 \cdot 5\ H_2O$, 0.079; $CoCl_2$, 0.10. Cultures were grown at 30°C in a flask with continuous gassing (air enriched with 2% CO_2) for about 5 days. The cells were harvested by centrifugation at 10,000 rpm for 10 min and washed with 50 m*M* potassium phosphate buffer, pH 7.5 (*17*). The cell pellets can then be frozen at −20°C and kept indefinitely. To rupture the cells the pellets were thawed, 50 m*M* potassium phosphate buffer at pH 7.5 and 1 m*M* dithiothreitol (DTT) were added, and sonication (five 30-sec pulses with intermittent cooling) were carried out. Centrifugation at 45,000 rpm for an hour was carried out to pellet cell debris. The supernatant was passed through a 30 × 2.5-cm Sephadex G-25 column in order to remove low-molecular-weight organic compounds, e.g., cofactors. The resultant supernatant of soluble enzymes is denoted as crude extract. At that stage, the extract can be frozen and kept indefinitely.

Immobilization of Crude Extract Enzymes on Phenoxyacetyl Cellulose

Approximately 7 g of phenoxyacetyl cellulose in an ethanol slurry were packed into a column of 1.5 cm diameter (45-ml packed column). The column was rinsed with 200 ml of distilled water to remove the ethanol and equilibrated with sodium phosphate buffer (50 m*M*, pH 7.8). The *Synechocystis* sp. crude extract (4.5 ml) was loaded onto the column. The protein content of this crude extract determined by the Bio-Rad protein assay (*18*) was 24.1 mg per ml, the specific activity of chorismate mutase was 2.1 nmol/min/mg, and the specific activity of prephenate aminotransferase was 8.7 nmol/min/mg. Unbound proteins were removed by rinsing the column with 50 ml of buffer. The eluate lacked detectable activity for prephenate aminotransferase and chorismate mutase.

Protein Determination

Protein was determined by the method of Bradford (*18*) using Bio-Rad protein dye reagent (Coomassie brilliant blue G-250) and Bio-Rad protein standard. Five milliliters of diluted Bio-Rad protein dye concentrate (4 parts distilled water:1 part concentrate) was added to 0.1 ml of extract, Bio-Rad protein standard (0.2–1.4 mg/ml), and blanks (50 m*M* sodium phosphate buffer, pH 7.5). After 5 min, absorbtion at 595 nm was read on a Gilford 250 spectrophotometer. Protein in the extract was determined by comparison to the standard curve obtained.

Aminotransferase Assay

The activity of the aminotransferase was determined using phenylalanine and α-ketoglutarate in the presence of the coenzyme pyridoxal phosphate. The resulting phenylpyruvate was determined from its uv absorbance at 320 nm (ϵ = 17,500).

To 75 μl of enzyme extract (about 5 mg protein/ml), 50 μl, of 8 m*M* phenylalanine, 50 μl of 4 m*M* α-ketoglutarate, and 25 μl of 0.2 m*M* pyridoxal-5′-phosphate were added and the mixture incubated for 20 min at 37°C. The reaction was quenched with 0.8 ml of 2.5 *M* NaOH, and the resulting phenylpyruvate was determined from the absorbance at 320 nm.

Prephenate Aminotransferase Assay

To a mixture of 50 μl 8 m*M* leucine, 50 μl 4 m*M* disodium prephenate, 25 μl 0.2 m*M* pyridoxal-5′-phosphate was added 75 μl enzyme extract, and reaction at 37°C was allowed to proceed for 20 min. The arogenate produced was estimated by dansylation as follows.

To 20-μl samples were added 10 μl of tritiated dansylchloride [prepared as a 9 : 1 mixture of [^{3}H]dansyl chloride in acetone (1 mCi/1 ml; 26.08 mCi/mmol) and unlabeled 5% dansyl chloride in acetone] and 2 μl of 3.4 *M* potassium carbonate buffer (pH 10.2). After incubation for 30 min at 37°C, 3 μl of 5.1 *M* $HClO_4$ was added to convert dansyl arogenate to dansyl phenylalanine. In order to ensure this conversion, the mixture was further incubated for 15 min at 37°C. One microliter of 3.4 *M* potassium carbonate buffer was added as well as 1 μl of dansyl phenylalanine. The sample was lyophilized. The residue was dissolved in 50 μl methanol, and 5 μl of the methanolic solution were spotted on a 5 × 5-cm polyamide plate (Chen-Chin polyamide, Pierce Chemicals). The thin-layer chromatographic(TLC) plate was eluted with H_2O : NH_4OH (4 : 1) followed by elution in the perpendicular direction with benzene : acetic acid : pyridine (50 : 5 : 1). The dansyl phenylalanine spot was excised into 100 μl formic acid, 500 μl H_2O, and 7 ml aquasol and counted in a scintillation counter. The amount of arogenate formed can be determined by comparing the disintegrations per minute of the sample to a standard curve defined by the disintegrations per minute of dansyl phenylalanine standards plotted vs phenylalanine concentration.

Chorismate Mutase Assay

A reaction mixture (200 μl) containing 175 μl of 1.1 m*M* chorismate (43.3 μg) and 25 μl enzyme, both in 50 m*M* sodium phosphate buffer (pH 7.5) was incubated at 37°C for 20 min. Any prephenate formed was then converted to phenylpyruvate by incubating the reaction mixture at 37°C for 15 min after addition of 0.1 ml of 1 *N* HCl. The absorbance of phenylpyruvate at 320 nm was measured after addition of 0.7 ml of 2.5 *N* NaOH. An extinction coefficient of 17,500 (*11*) was used for calculation.

Enzymatic Quantitation of Chorismic Acid

The radioactive chorismic acid was assayed by conversion to anthranilic acid

using an adaptation of the initial rate method of Egan and Gibson (*20*). An extract containing anthranilate synthase was prepared from a culture of *Anabaena* ATCC 29151, originally obtained from the American Type Culture Collection (Rockville, Md.). For each example and standard, the following reagents were added to an Aminco-Bowman spectrophotofluorometer cell: 25 μl enzyme extract, 25 μl of 160 m*M* L-glutamine, 25 μl of 80 m*M* $MgSO_4$, and 100 μl of 50 m*M* sodium phosphate buffer (pH 7.5). After placing the cell in the fluorometer at 37°C, 25 μl of chorismate was added and the initial rate of anthranilate formation was followed (excitation at 313 nm, emission at 390 nm). The concentration of [^{14}C]chorismate was determined by comparison to a standard curve obtained in the linear range for the assay with this enzyme.

Preparative Synthesis of Arogenic Acid from Prephenate

A reaction mixture consisting of 5.5 ml of 5.2 m*M* sodium prephenate (7.1 mg), 73 m*M* L-leucine (52.7 mg), and 0.03 m*M* pyridoxal phosphate (0.04 mg) was prepared from buffered (50 m*M* sodium phosphate, pH 7.5) stock solutions. A 5.0-ml aliquot of this mixture (26.2 m*M* sodium prephenate, 6.4 mg) was applied to the column containing the immobilized crude extract. Since the distribution of prephenate aminotransferase activity on the column bed was not known, a peristaltic pump was used to recycle the mixture continuously through the column at a rate of 0.5 ml/min. The shortest possible length of 1/16 in.-i.d. tubing was used in the pump circuit to keep dilution of the reaction mixture to a minimum. After 17 hr (overnight), all the fluid in the pump circuit (about 20 ml) was collected in 5-ml fractions while additional buffer was added to the top of the column. When 40 ml of effluent had been collected the fractions were assayed for prephenate and arogenate. Fractions containing these compounds were pooled and loaded onto an ion-exchange column to separate arogenic acid from prephenic acid.

Preparative Synthesis of Arogenic Acid from Chorismate

Stock solutions containing 5.5 ml of 5.0 m*M* chorismic acid (6.2 mg), 75 m*M* L-leucine (54.1 mg), and 0.03 m*M* pyridoxal phosphate (0.04 mg) from buffered 50 m*M* sodium phosphate (pH 7.7) were prepared. A 5.0-ml aliquot of this mixture (5.6 mg chorismic acid) was applied to the column containing the immobilized proteins. A peristaltic pump was used to recycle the mixture continuously through the column at 0.5 ml/min. After 16 hr (overnight) all the fluid in the pump circuit (about 20 ml) was collected while additional buffer was added to the top of the column. When 35 ml had been collected, the effluent was assayed for arogenate and prephenate.

Prephenate was measured by uv after acidic conversion to phenylpyruvate: an aliquot of the eluate (0.1 ml) was acidified with 0.1 ml of 1 *N* HCl and from the uv spectrum (λ 320 nm, ϵ = 17,500) phenylpyruvate concentration was estimated. A sensitive method for detection and quantitation of arogenate was used. The aliquot is dansylated at basic pH with an excess of dansyl chloride. The fluorescent dansyl arogenate obtained was easily detected by radial thin-layer chromatography (*19*). Quantitation was then effected by eluting the dansyl arogenate

crescent, measuring fluorescence, and comparing it with a calibration curve constructed by use of known amounts of dansyl arogenate. Alternatively, either a radioactive dansyl assay or high-performance liquid chromatography (HPLC) was used for quantitation of arogenate. The radioactive dansyl assay employs [^{3}H]dansyl chloride of high specific activity (1 mCi/ml, 26.08 mCi/mmol) performing the reaction as outlined above, excising the dansyl arogenate crescent from the TLC plate, and measuring radioactivity in 15 ml of scintillation fluid (Aquasol, New England Nuclear) using a Delta 300-Tracor scintillation counter. For the HPLC assay, the dansylated mixture was loaded onto a LiChrosorb RP18 10 μm column (25 cm × 1.4 mm) (Altex Scientific Company, Berkeley, Calif.). A linear gradient was formed by pumping through 20 m*M* Na_2HPO_4, NaH_2PO_4 buffer (pH 6.6) with increasing concentrations of acetonitrile (10–32%). The flow rate was 1.5 ml/min. Dansyl arogenate was detected at about 15% acetonitrile concentration.

Purification of Arogenate

Arogenate in the eluate from the immobilized enzyme was purified either by preparative (TLC) or by ion-exchange chromatography depending on the amounts used. For microscale (up to 50–100 mg) preparative TLC can be used. Larger scale can be accommodated by ion-exchange separation.

(*a*) *Preparative TLC.* Arogenate in the eluate from the immobilized enzyme column was separated from chorismate, prephenate, L-leucine, and L-phenylalanine by TLC. The eluate was first lyophilized, and a few milliliters of methanol (made slightly basic with 1 *M* NaOH) were added to the residue to redissolve the arogenate. After centrifugation to remove buffer salts, the solutes in the supernatant were separated on 20 × 20-cm Kieselguhr 60-F254 silica gel plates using an ethanol : $CHCl_3$: NH_4OH (20 : 4 : 6) solution as solvent. The arogenate band had the lowest R_f (0.40) and was well separated from prephenate (R_f, 0.48), the next closest band.

(*b*) *Ion-exchange column.* Arogenate in the eluate from the immobilized enzyme column was separated from residual chorismate, prephenate, L-leucine, and L-phenylalanine by ion-exchange chromatography. Bio-Rad AG1-X8 (Cl^-) was used in a 0.8 × 15-cm column, equilibrated to pH 8.6 with 0.1% *N*-ethylmorpholine. After adjusting the eluate to pH 8.6 (with 1 *N* NaOH) the column was loaded and washed with 0.1% *N*-ethylmorpholine solution. Arogenate was separated by eluting the column with a linear gradient consisting of 0.1% *N*-ethylmorpholine and 1 *N* NaCl in 0.1% *N*-ethylmorpholine solution at a flow rate of 0.4 ml/min. Arogenate eluted between L-leucine and prephenic acid.

(*c*) *Yield of arogenate.* The yield of purified arogenate obtained starting from prephenate was 60%. In 1 day, we could make ~20–30 mg of arogenate. The yield of purified arogenate starting from the earlier precursor, chorismic acid, was 42%. We have made up to 15 mg of arogenate in a day from chorismate.

Preparation of [^{14}C]Chorismate

[^{14}C]Chorismate was prepared from an adaptation of Gibson's isolation method

(*10*). The growth from a nutrient agar plate (Difco) of *K. pneumoniae* 62-1 was added to 300 ml of growth medium A under aseptic conditions, and incubated with shaking at 30°C until an optical density of about 1.3 at 620 nm was obtained. The culture was then centrifuged and the cells were resuspended in 300 ml of accumulation medium B to which 1 mCi of [^{14}C]glucose (230 mCi/mmol) (Schwartz–Mann) had been added. The suspension was incubated with shaking for 16 hr at 30°C. After centrifuging at 8000 rpm, the pH of the supernatant was adjusted to 7.5 by addition of 10 *N* NaOH. The supernatant was then pumped through a 0.8 × 10-cm column of approximately 3 g of Bio-Rad AG1-X8 (Cl^-), 200–400 mesh, at a rate of 1.5 ml/min. The column was washed with 50 ml of distilled water. Chorismate was eluted with 1 *M* NH_4Cl (pH adjusted to 8.5) at a flow rate of 0.5 ml/min, and collected in 2-ml fractions. Chorismate was detected in the eluate fractions by measurement of the absorbance of 1 : 100 dilutions at 275 nm (ϵ = 2630). Fractions containing ammonium chorismate were pooled, acidified with 1 *N* HCl to pH 1.5, and saturated with NaCl. An amount of ether equal to twice the volume of the pooled fractions was divided into several portions and used to extract the chorismate. The ether extracts were then combined and the ether removed by rotary evaporation at 20°C. Water was then removed by lyophilization. After dissolving the lyophilized residue in 10 ml of anhydrous ether, the chorismate was partially purified by crystallizing out some impurities with a dry ice–acetone bath. The supernatant containing most of the chorismate was drawn off. The remaining ether was then evaporated. The amount of chorismate produced was measured by enzymatic conversion to anthranilic acid and was found to be 25 mg. This agreed reasonably well with the value obatined from the uv spectrum of chorismate. After numerous crystallizations, the constant specific activity was 399,454 dpm/mg.

Preparation of [^{14}C]*Arogenate*

[^{14}C]Arogenate was obtained from [^{14}C]chorismate using the same immobilized crude extract, 75 m*M* L-leucine and 0.03 m*M* pyridoxal phosphate. After 16 hr, 35 ml of effluent was collected and assayed for arogenate and prephenate. After multiple purifications by preparative TLC the constant specific activity of [^{14}C]arogenate was 100,000 dpm/mg.

RESULTS AND DISCUSSION

Arogenate, a Major Intermediate in Aromatic Amino Acid Biosynthesis

Comparative enzymological data have revealed that the arogenate pathways of tyrosine and/or phenylalanine synthesis are widely employed in nature by both microorganisms and plants (*2, 4–8, 26*). The structure of arogenate (*3*) is that of an intricate small molecule with various functional groups. The lability of the molecule (it is converted to phenylalanine at slightly acidic pH) mandates preparation of the compound in a short time. In order to carry out a series of enzymological and physiological experiments, an improved procedure for preparation of

arogenate was needed, especially one which would also be appropriate for isolation of [^{14}C]arogenate. This goal has been accomplished using immobilized enzyme proteins.

Selection of an Arogenate-Producing Microorganism

The following steps led to a decision between microbes which potentially might serve as an ideal source of extract proteins for immobilization: (i) selection of a microbial organism which can convert prephenate to arogenate, i.e., has an active prephenate aminotransferase, (ii) elimination of those microorganisms in which prephenate is a substrate for other reactions that compete in crude extracts with prephenate aminotransferase, (iii) elimination of any candidate microorganisms where arogenate is enzymatically metabolized after it is produced, and (iv) the choice of a solid support that binds prephenate aminotransferase without interference with catalysis. The organism which best fit these criteria was found to be *Synechocystis* sp. (ATCC 29109), a cyanobacterium. The biosynthetic pathways that govern the production of aromatic amino acids in this organism are shown in Fig. 3.

The first reaction shown, the rearrangement of chorismate to prephenate, is catalyzed by chorismate mutase. It is an enzymatic Claisen rearrangement, in essence an irreversible step. The next reaction, catalyzed by prephenate aminotransferase [6], exists as an obligatory step in tyrosine synthesis in this organism. Although enzymes [2] and [8] are also immobilized, their catalytic activities were avoided as follows. The further metabolism of arogenate by enzyme [8] was negated in the absence of $NADP^+$, an obligatory cofactor requirement. Enzyme [2] of *Synechocystis* sp. exhibits an absolute requirement for L-tyrosine, an allosteric activator molecule; catalytic activity is nil in the absence of tyrosine.

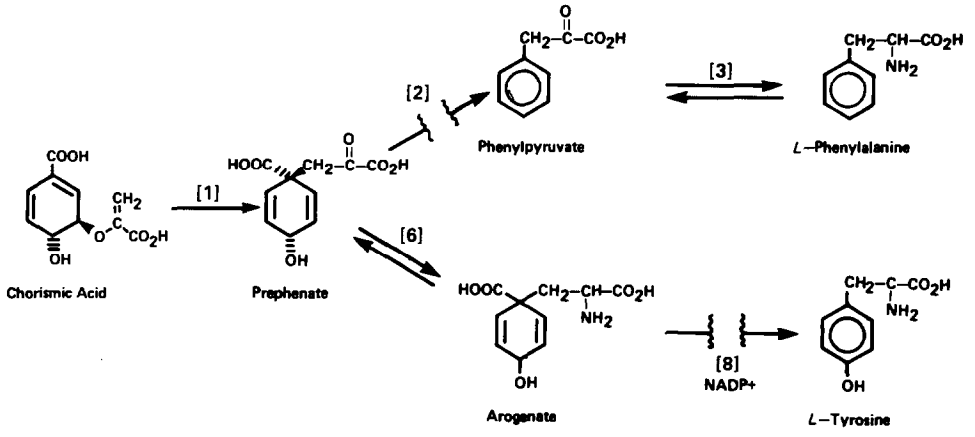

FIG. 3. Biochemical pathways utilized by *Synechocystis* sp. for L-tyrosine and L-phenylalanine biosynthesis. Prevention of the function symbolized by the interrupted arrows (see text) of enzymes [2] and [8] channels the flow of prephenate exclusively to the dead-end formation of arogenate. Enzymes: [1] chorismate mutase; [2] prephenate dehydratase; [3] phenylpyruvate aminotransferase; [6] prephenate aminotransferase; and [8] arogenate dehydrogenase.

Hence, if a crude, unfractionated extract of *Synechocystis* sp. is passed through a coarse Sephadex column (G-25) to fractionate away small organic molecules such as $NADP^+$ or tyrosine, it can be used successfully as a source of immobilized prephenate aminotransferase without interference from other enzyme activities.

Immobilization of Enzyme Proteins

Phenoxyacetyl cellulose, a support expected to bind proteins via hydrophobic interactions, was selected in order to minimize the possibility of denaturation. Previous investigators have succeeded in immobilizing purified enzymes such as phosphatases, lipase, and chymotrypsin on phenoxyacetyl cellulose with near 100% retention of activity (*22–27*). Since isolation and purification of chorismate mutase and prephenate aminotransferase requires painstaking biochemical expertise as well as time-consuming and tedious experimentation, we chose to immobilize enzyme proteins in crude, unfractionated extracts. A whole-cell extract of *Synechocystis* sp. was successfully immobilized on a phenoxyacetyl cellulose column with no detectable losses in the activities of chorismate mutase and prephenate aminotransferase. The crude extract could be kept frozen with no loss of enzyme activity. Chorismate mutase was stable up to 3 months, whereas prephenate aminotransferase was stable for at least 6 months.

Prephenate aminotransferase, like many aminotransferases, exhibited considerable substrate ambiguity. L-Leucine was among the best amino-donor substrates in combination with prephenate. L-Leucine was preferable to L-glutamate, another good amino-donor substrate, because of the difficulty in separating residual glutamate from arogenate. Passage through the column of a substrate mixture of prephenate, L-leucine, and pyridoxal phosphate led to formation of arogenic acid in a yield of 60%. This preparative synthesis takes at most 1 day and is very reproducible. It is feasible to start with chorismic acid, since the immobilized chorismate mutase was quite active. Indeed, it is possible to make 10 mg of arogenate in 1 day from chorismate. The yield of arogenate from chorismate was about 42%. In either case, the arogenate obtained is purified either by preparative TLC or by passage through an ion-exchange resin. [^{14}C]Arogenate was also prepared from [^{14}C]chorismate using the same immobilized enzyme column.

The major advantages of this method for preparation of arogenic acid are: (a) high yield, (b) no need for enzyme purification, (c) time involved in the preparation is very short, and (d) the reproducibility is very high. Another attractive advantage of immobilized proteins is the potential to produce novel derailment metabolites (*28*) by supplying enzymes with substrate analogs. The lack of specificity of some of the enzymes involved can result in the production of an unusual metabolite which otherwise is not a natural product. For example, epiprephenate (courtesy of Dr. S. Danishefsky) is transformed by immobilized prephenate aminotransferase to epiarogenate (Zamir, unpublished data).

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