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# Purification and Characterization of the Sesquiterpene Cyclase Aristolochene Synthase from *Penicillium roqueforti*

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The sesquiterpene cyclase, aristolochene synthase, has been purified from *Penicillium roqueforti* by gel filtration and anion-exchange chromatography. Isolation was facilitated by a change in the elution behavior of the enzyme during gel filtration at different steps in the purification. The purified enzyme had a specific activity of 70 nmol/min/mg protein. The molecular weight as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was $M_r$ 37,000. The native molecular weight as determined by gel filtration chromatography was $M_r$ 48,000. The requirement for $Mg^{2+}$ could be partially substituted with 0.01 mM $Mn^{2+}$, but higher concentrations were inhibitory. Pyrophosphate, a competitive inhibitor of most terpene cyclases, had no effect on enzyme activity up to a concentration of 5.0 mM. The maximum activity was observed between pH 6.25 and pH 7.50, and the $K_m$ for farnesyl pyrophosphate was 0.55 ± 0.06 μM. © 1989 Academic Press, Inc.

The enzyme aristolochene synthase catalyzes the cyclization of *trans,trans*-farnesyl pyrophosphate (1) to aristolochene (2). Aristolochene is a bicyclic sesquiterpene reported to occur in *Aristolochia indica* (1), *Syntermes* soldiers (2), and the fungus *Aspergillus terreus* (3). It is the apparent precursor of a family of sesquiterpenes produced by *Penicillium roqueforti* (3). The most toxic of these, PR-toxin,[3] is lethal to rats and mice by either intraperitoneal or oral administration and is produced by isolates of *P. roqueforti* from blue cheese and other sources (4, 5). The biosynthesis of PR-toxin (6, 7) and the structurally similar capsidiol (8) and petasin (9) has been studied. These investigations support a scheme in which a germacrene A (3) intermediate is formed by the initial cyclization of FPP. Subsequent cyclization then proceeds through a eudesmane carbocation (4) and involves the migration of a methyl group from C10 to C5 (Scheme I). Since isolation of the relevant enzymes had not previously been reported, some question existed as to whether the two proposed cyclization steps were catalyzed by the same enzyme. The isolation of aristolochene synthase demonstrates that, in the case of aristolochene, a single enzyme performs both cyclizations.

There are probably 200 different sesquiterpene cyclases from microbial and plant

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[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

[3] Abbreviations used: PR-toxin, *Penicillium roqueforti*-toxin; FPP, pyrophosphate ester of 3,7,11-trimethyl-2*E*,6*E*,10-dodecatrienol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mopso, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazene-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; GC/MS gas chromatography/mass spectrometry; DTT, dithiothreitol; EI, electron ionization; CI, chemical ionization.



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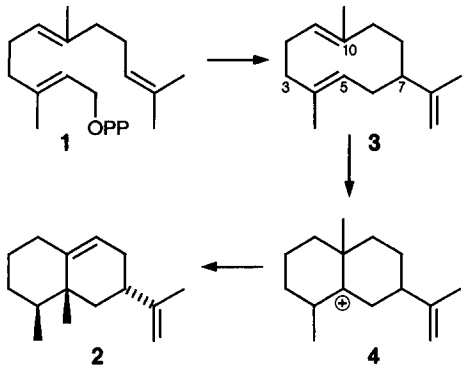


SCHEME I. Conversion of *trans,trans*-farnesyl pyrophosphate (1) to aristolochene (2).

sources based on the estimated number of structurally distinct sesquiterpenes (10). Investigations of these enzymes (11–13), with one exception (14), have employed cell extracts and partially purified enzyme preparations. As a result, considerable information exists on the mechanisms and properties of some terpene cyclases (10), but little is known about their structure.

Because of evidence indicating that aristolochene is an intermediate in PR-toxin biosynthesis, we have purified aristolochene synthase from *P. roqueforti*. In this paper, we describe the purification of aristolochene synthase and some of its properties.

## MATERIALS AND METHODS

*Organism and culture conditions.* *P. roqueforti* NRRL 849 was maintained on a V-8 agar (15) slant at 4°C. Plates of V-8 agar were inoculated with 2-mm squares of agar from a stock slant and incubated 7–10 days at 28°C. Conidial suspensions were obtained by flooding plates with 9.0 ml of sterile water and rubbing with a bent glass rod. The conidia in these suspensions were counted by means of a hemocytometer. Six Erlenmeyer flasks (2 liter), each containing 1.0 liter of GYEP medium (16), 5% glucose (autoclaved separately), 0.1% peptone (Difco), and 0.1% yeast extract (Difco), were inoculated to a final concentration of $1 \times 10^6$ conidia/ml. These flasks were incubated for 50 h at 28°C and 180 rpm on a gyratory shaker.

*Preparation of cell homogenates.* Cultures were harvested by filtration and homogenized in the Bead Beater (Biospec Products) as described (14). The homogenization buffer consisted of 10 mM Tris, pH 7.8, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 15% glycerol (buffer T). Cell homogenates were centrifuged at 8500*g*, 4°C, for 10 min (Sorvall SS-34 rotor, Dupont), and supernatants were decanted. This material was then centrifuged at 175,000*g*, 4°C, for 65 min (Beckman 70 Ti rotor, Beckman Instruments, Inc.). The supernatants were either used immediately or stored at −70°C.

*Enzyme purification–ammonium sulfate concentration.* The 175,000*g* supernatant from 5 liters of culture (approximately 350 ml) was made 75% saturated by the gradual addition of solid $(NH_4)_2SO_4$ with stirring in an ice bath. The precipitated protein was collected by centrifugation at 15,000*g*, 4°C, for 15 min in the SS-34 rotor. The pellets were resuspended in buffer T, pooled (total volume = 32 ml), and stored at −70°C in 8-ml aliquots.

*Sephacryl 200 gel filtration chromatography.* All column chromatography steps were performed with an FPLC system (Pharmacia, Inc.) at 4°C. Aliquots (8 ml) of $(NH_4)_2SO_4$-concentrated homogenate were then applied individually to a Sephacryl 200 (Pharmacia, Inc.) column equilibrated in buffer T. Enzyme activity eluted in a broad peak within the included volume of the column well separated from material eluting in the void volume. The activity pools from four S-200 columns were combined and then divided into five aliquots which were stored at −70°C.

*Mono Q anion-exchange chromatography (KCl elution).* The S-200 aliquots were made 100 mM in KCl and applied to a Mono Q column (0.5 × 5 cm; Pharmacia, Inc.). The column was washed with 6.5 ml of 125 mM KCl in buffer T, and eluted with a linear gradient (22.5 ml) of 125–350 mM KCl in buffer T. Aristolochene synthase activity eluted between 190 and 215 mM KCl.

*Mono Q anion-exchange chromatography (pyrophosphate, KCl elution).* The activity peaks from the KCl elution of five columns were pooled and diluted twofold with buffer T. This material was reapplied to the Mono Q column, washed with 2.0 ml of buffer T, washed with a linear gradient (15.0 ml) of 0–18.5 mM tetrasodium pyrophosphate (Sigma Chemical Co.) in buffer T, and eluted with a linear gradient (40.0 ml) of increasing KCl (0–300 mM) and decreasing (18.5–13 mM) tetrasodium pyrophosphate, both in buffer T. Enzyme activity eluted between 0 and 40 mM KCl in a volume of 4–6 ml.

*Superose 6 gel filtration chromatography.* The activity peak from the Mono Q column above was concentrated by ultrafiltration in a Centricon microconcentrator (Amicon) and centrifuged at 5000*g* for 2 h (final volume, 100–250 μl). Concentrated enzyme (200 μl) was then applied to a Superose 6 column (1.0 × 30.0 cm; Pharmacia, Inc.), equilibrated in buffer H (10 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and 10% glycerol), and eluted with a flow rate of 0.2 ml/min.

*Enzyme assay.* Aristolochene synthase was assayed by the method described for trichodiene syn-

thase (14) except that the incubation time was 10 min. The standard assay conditions employed a reaction mixture consisting of 10 mM Hepes pH 7.5, 3 mM $MgCl_2$, 1 mM DTT, and 10 μM [1-$^3$H]FPP in a total volume of 150 μl. Aristolochene synthase preparations were added in volumes of 0–15 μl, and the assay was performed in transparent 1.5-ml polypropylene microcentrifuge tubes (Tekmar). In assays employing purified preparations of aristolochene synthase, the PrepSep-silica column (Fisher Scientific) step was omitted, and the hexane extracts were assayed directly by liquid scintillation spectrometry. The [1-$^3$H]-FPP, a gift from Professor D. E. Cane of Brown University, had a radiospecific activity of 775 Ci/mol.

*Native molecular weight.* The native molecular weight of purified aristolochene synthase was determined by gel filtration chromatography using a Superose 6 column (1 × 30 cm, Pharmacia). The column was equilibrated with a buffer composed of 50 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 10% glycerol, and run at a flow rate of 0.25 ml/min. Under these conditions aristolochene synthase elutes as a single peak. Proteins employed for column calibration were chymotrypsinogen (bovine pancreas), $M_r$ 25,000; ovalbumin (chicken egg), $M_r$ 45,000; bovine serum albumin, $M_r$ 68,000; aldolase (rabbit muscle), $M_r$ 158,000; and pyruvate kinase (rabbit muscle), $M_r$ 235,000.

*Gel electrophoresis.* SDS–PAGE in slab gels (0.75 mm × 18 cm × 16 cm) was performed by the method of Laemmli (18). Molecular weights were determined in 11% acrylamide separating gels with trypsinogen (bovine pancreas), $M_r$ 24,000; glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), $M_r$ 36,000; ovalbumin, $M_r$ 45,000; bovine serum albumin, $M_r$ 68,000; and phosphorylase *b* (rabbit muscle), $M_r$ 97,400; as standards. Protein was detected by staining with Coomasie brilliant blue R-250 in a methanolic acetic acid solution.

*Protein determinations.* Protein concentrations were determined by the method of Bradford (19) with the Bio-Rad protein assay (Bio-Rad Laboratories), and bovine γ globulin as standard.

*GC/MS analysis of product.* The purified enzyme was used to prepare an amount of product sufficient for GC/MS analysis. The enzyme assay conditions described above were employed except that the incubation time was 2 h, and the hexane extract was not passed over a PrepSep-silica column. The hexane extract was concentrated under a stream of $N_2$ to a concentration of 15 ng/μl of $^3$H-labeled olefin, and then 3 μl was injected onto a Finnegan 4535/TSQ GC/MS. The mass spectrometer source was operated in either the electron ionization (EI) or the chemical ionization (CI) mode with isobutane (0.3 Torr) as the CI gas. A 15 m × 0.25 mm fused silica capillary DB-1 column (J&W Scientific) was used with the outlet directly coupled into the source of a quadrupole tandem mass spectrometer. Column conditions were the same as those previously described (20). Comparison with a semisynthetic preparation of (−)-aristolochene, provided by Professor D. E. Cane (3), revealed the presence of a peak with identical GC retention time and EI and CI mass spectra. The amount of material detected as aristolochene synthase was ±20% of that predicted based on the amount of radioactivity analyzed and the radiospecific activity of the substrate. It was also possible to detect a peak with identical GC retention time and mass spectrum as aristolochene in ethyl acetate extracts of *P. roqueforti* cultures. The presence of the aristolochene peak in cultures harvested at various times corresponded with the detection of aristolochene synthase activity (T. M. Hohn, R. D. Plattner, and D. E. Cane, unpublished).

## RESULTS AND DISCUSSION

*Purification.* The growth conditions required for high level expression of aristolochene synthase, described under Materials and Methods, were determined in a preliminary study. It was observed that sesquiterpene cyclase activity appeared at 40 h post inoculation, increased to maximum levels at 50 h, and was undetectable by 90 h. On the basis of these results, 50-h cultures were used for all enzyme isolations.

The purification procedure is outlined in Table I. This procedure differs from that used in the purification of trichodiene synthase from *Fusarium sporotrichioides* (14) in the utilization of $(NH_4)_2SO_4$ to concentrate the 175,000*g* supernatant and in the substitution of a Sephacryl 200 column for the octyl-Sepharose step. Elution of the enzyme from the Mono Q column with a combined pyrophosphate/KCl gradient provided substantial purification (Fig. 1). Recovery data were not obtained for this step, but the $A_{280}$ elution profile of the following step (Superose 6) in the purification (Fig. 2B) indicates that aristolochene synthase represented 20–30% of total protein. The application of this step to the purification of both trichodiene and aristolochene synthases suggests that it may be useful for the isolation of other terpene cyclases.

The critical step in this procedure proved to be the elution of aristolochene synthase from the Superose 6 column. A peak consisting only of aristolochene synthase eluted from this column ahead of all

TABLE I

PURIFICATION OF ARISTOLOCHENE SYNTHASE FROM *P. roqueforti*

| Step | Total protein (mg) | Total activity (units)[a] | Specific activity (units/mg) | Recovery (%) |
|---|---|---|---|---|
| 175,000*g* supernatant | 918 | 422 | 0.46 | 100 |
| Sephacryl-200 | 100.8 | 243 | 2.41 | 57.5 |
| Mono Q | | | | |
| KCl | 7.0 | 33.3 | 4.8 | 7.9 |
| Pyrophosphate, KCl | — | — | — | — |
| Superose 6 | 0.40 | 28.0 | 70 | 6.6 |

[a] A unit is defined as nanomoles of product (aristolochene) per minute under the assay conditions described under Materials and Methods.

other proteins (Fig. 2B). The peaks of both enzyme activity and polypeptide (data not shown) trailed significantly. This result was unexpected since the gel filtration step (Sephacryl-200) utilized earlier in the purification should have selected a single size class of proteins. In order to better characterize the altered elution behavior of the enzyme, a portion of the activity pool from the Mono Q (KCl) step was concentrated and chromatographed on a Superose 6 column. The resulting activity elution profile was complex (Fig. 2A), but most of the activity eluted later than was observed in Fig. 2B. This suggested that sample composition was important in producing the

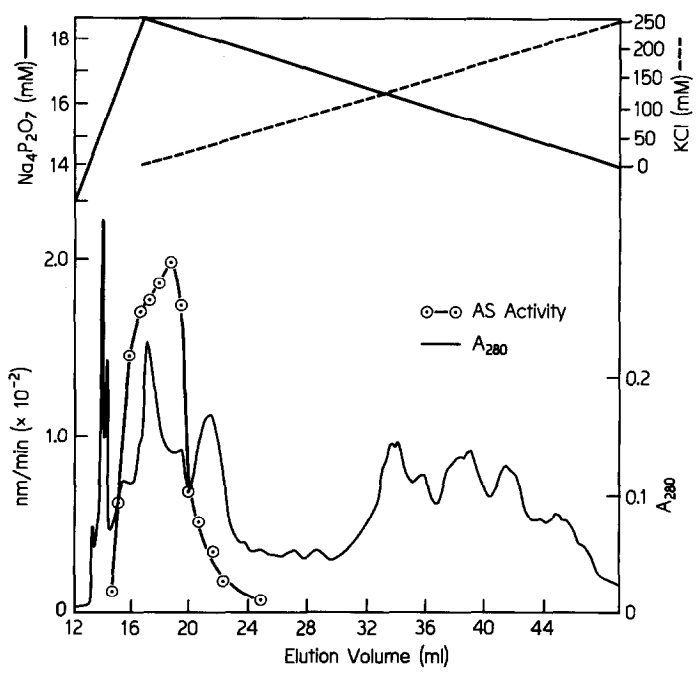


FIG. 1. Anion-exchange chromatography of aristolochene synthase on a Mono Q column (KCl/$Na_4P_2O_5$ elution). The enzyme activity pool from the Mono Q (KCl elution) step in the purification was applied to the column.

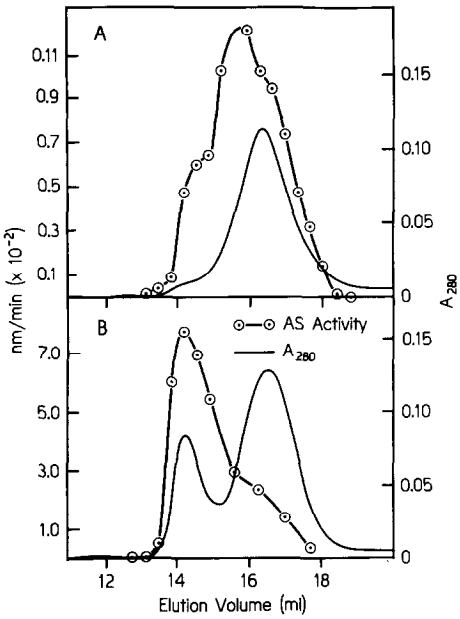


FIG. 2. Gel filtration chromatography of aristolochene synthase on Superose 6. The samples applied to the column were: (A) concentrated enzyme activity pool from the Mono Q (KCl elution) step in the purification; (B) concentrated enzyme activity pool from the Mono Q (KCl/pyrophosphate elution) step in the purification.

early elution of aristolochene synthase from the Superose 6 column. The major differences between the two samples analyzed in Fig. 2 were the presence or absence of pyrophosphate and the concentration of aristolochene synthase. The addition of pyrophosphate (15 mM) to the sample chromatographed in Fig. 2A had no effect on the activity elution profile thus indicating that enzyme concentration may play a role. In the concentrated Mono Q (pyrophosphate/KCl) activity pool, aristolochene synthase was present at a 50- to 75-fold higher concentration (approx 1.2 mg/ml) than in the other samples chromatographed on gel filtration columns. This relatively high concentration may have produced an aggregated or higher oligomeric form of the enzyme, although other explanations, such as nonspecific interactions with the gel filtration matrices employed, cannot be ruled out.

*Molecular weight determinations.* Analysis of aristolochene synthase preparations by SDS-PAGE indicated that their purity was ≥95% (Fig. 3). The molecular weight determined by SDS-PAGE was found to be $M_r$ 37,000. This is smaller than the molecular weight determined by SDS-PAGE for trichodiene synthase of 45,000 (14). The native molecular weight obtained from analysis on a calibrated Superose 6 column was $M_r$ 48,000 and is similar to the $M_r$ 57,000 reported for three other sesquiterpene cyclases (11, 12). Comparison of the molecular weights obtained by SDS-PAGE and gel filtration analysis suggests aristolo-

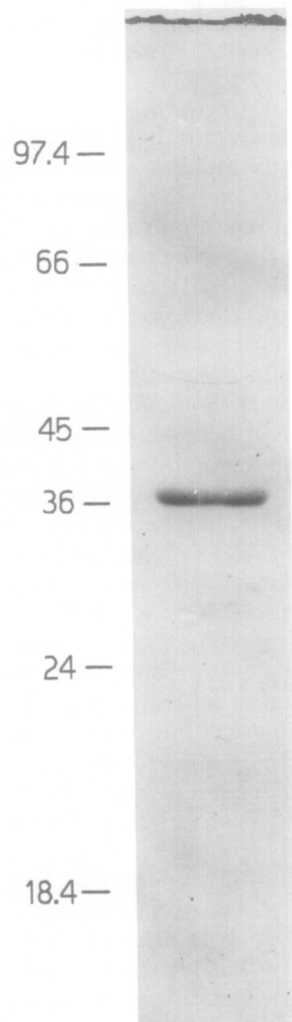


FIG. 3. SDS-PAGE of purified aristolochene synthase (2 μg protein). The migration of protein standards is indicated as $M_r \times 10^3$.

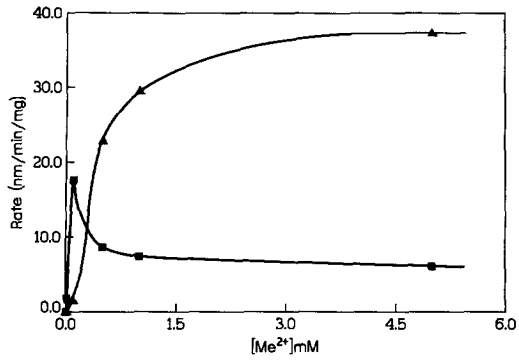


FIG. 4. Effect of $Mg^{2+}$ and $Mn^{2+}$ on aristolochene synthase activity. Aristolochene synthase was diluted in buffer T without $MgCl_2$ and assayed in the presence of various amounts of $Mg^{2+}$ (▲) and $Mn^{2+}$ (■) as described under Experimental Procedures.

chene synthase may be a monomer. However, the unpredictable elution behavior of this enzyme from gel filtration columns indicates this technique may be unreliable for estimating the native molecular weight. Determination of the native molecular weight by other methods will be necessary before a probable subunit composition can be assigned.

*Effect of metal ions, pH, FPP, and pyrophosphate.* All terpene cyclases previously examined require a divalent metal ion for activity (10). When aristolochene synthase was assayed in the presence of a low concentration of $Mg^{2+}$ (2 $\mu$M), no activity was observed (Fig. 4). Enzyme activity was restored by the addition of $Mg^{2+}$ with maximum activity occurring at 3 mM. Partial activity could be restored with $Mn^{2+}$ at a concentration of 0.01 mM, but higher concentrations were inhibitory. Other enzymes also show a preference for $Mg^{2+}$ as cofactor and are inhibited by $Mn^{2+}$ (11–13). The effect of pH on aristolochene synthase activity was analyzed as described previously (14) using Mes, Mopso, Hepes, and Tris buffers. A broad peak of activity was observed between pH 5.5 and pH 8.5. Maximum activity was observed between pH 6.25 and pH 7.5. Differences of less than 15% occurred at pH values where buffers overlapped (data not shown). These results are in agreement with the reports for a number of terpene cyclases (12, 21–24).

Plotting the rate of aristolochene formation as a function of FPP concentration gave a typical hyperbolic saturation curve. A double reciprocal plot yielded a $K_m$ for FPP of 0.55 ± 0.06 $\mu$M. This value is ninefold higher than that reported for trichodiene synthase, but within the range of values reported for other sesquiterpene cyclases (11, 12).

A distinguishing feature of aristolochene synthase is the failure of pyrophosphate, a product of the reaction, to inhibit activity. Pyrophosphate is a competitive inhibitor of other terpene cyclases (11, 12, 24). No inhibition of aristolochene synthase was observed in reactions containing 0.1 $\mu$M FPP, 15 mM $MgCl_2$, and 5 mM pyrophosphate.

Terpene cyclases from both microbial and plant sources appear similar based on a number of properties. However, no information exists on the relationship between these enzymes at the structural level. The availability of purified preparations of aristolochene synthase will facilitate studies of its structure. Preliminary results from immunoblotting experiments employing antiserum specific for trichodiene synthase indicate no detectable cross-reactivity with aristolochene synthase (data not shown). This is consistent with other differences observed between these two enzymes such as molecular weight, $K_m$ for FPP, and inhibition by pyrophosphate (T. M. Hohn, J. Pawlak, and D. E. Cane, unpublished). Further studies on the mechanism and structure of aristolochene synthase are in progress.

## ACKNOWLEDGMENTS

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