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Purification and Characterization of Dimethylallyl Pyrophosphate: Aspulvinone Dimethylallyltransferase from *Aspergillus terreus*†

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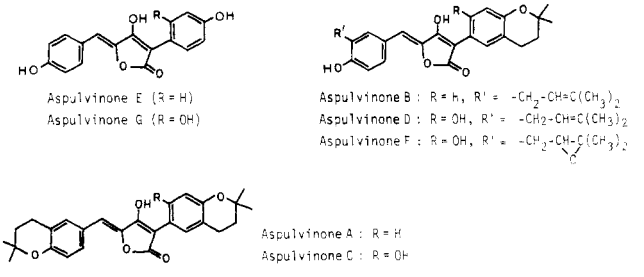
ABSTRACT: Dimethylallyl pyrophosphate:aspulvinone dimethylallyltransferase, the prenylation enzyme for the biosynthesis of aspulvinone pigments, has been purified from mycelia of *Aspergillus terreus*. The transferase catalyzed the transfer of the dimethylallyl moiety from dimethylallyl pyrophosphate to either of the two aromatic rings of aspulvinone E to give the mono- and diprenylated derivatives which were identified with the metabolites aspulvinone I and aspulvinone H, respectively. Aspulvinone G, another fundamental metabolite of this series, also acted as substrate to afford the corresponding diprenylated derivative, which is assumed to be a precursor for aspulvinone C, D, and F. The molecular weight of the enzyme was estimated to be 240 000–270 000 by gel filtration. Since the subunit molecular weight determined by NaDodSO$_4$–polyacrylamide disc gel electrophoresis was 45 000, the native enzyme appears to be a hexomeric protein composed of identical molecular weight subunits. The apparent K_m values for aspulvinone E, aspulvinone G, and dimethylallyl pyrophosphate were 13.7, 7.7, and 40.0 μM, respectively. The enzyme shows the maximum activity at pH 7.0, and no metal ion is necessary for the activation. Sulfhydryl blocking agents or mercaptoethanol has no effect. Bromophenol blue binds specifically to the transferase and strongly inhibits the enzyme activity.

A large number of phenolic compounds with prenyl groups attached to the aromatic ring occur widely in nature. Although it is generally conceived that enzymatic prenylation of an aromatic nucleus is involved as the juncture step in the biosynthesis of such mixed terpenoid molecules, only a few papers have appeared describing the prenylation enzyme. Recent works from this laboratory have shown that *Aspergillus terreus* produces several yellow pigments called aspulvinones (Ojima et al., 1973, 1975a, 1976). These compounds have provided us a chance to study a prenylation enzyme, because they are of a series of prenylated phenol derivatives which seem to be biogenetically related with one another. We communicated preliminarily that a crude extract of *A. terreus* catalyzed the transfer of the isoprene unit from dimethylallyl pyrophosphate to the aromatic rings of aspulvinone E, the fundamental



compound of this series of pigments (Ojima et al., 1975b). The present paper reports the purification and characterization of this new prenylating enzyme, dimethylallyl pyrophosphate: aspulvinone dimethylallyltransferase.

Materials and Methods

Cell Culture. Aspergillus terreus IAM 2054 (origin NRRL 1960 Raper ATCC 10020) were grown at 27 °C on Czapek Dox medium in stationary culture. Three-liter stationary culture flasks containing 1 L of medium were inoculated with 20 mL of a mycelial suspension from a 3-day-old shaking culture. After the culture was grown stationarily for 1 week,

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mycelia were harvested by filtration, washed with water, and used for the enzyme preparation.

[^{14}C]Aspulvinone E. [^{14}C]Aspulvinone E was prepared biosynthetically from [^{14}C]tyrosine according to the following procedure. A 1-mL solution of L-[U-^{14}C]tyrosine (50 μCi/mL, 0.103 μmol) was added to 100 mL of a 3-day-old culture of *A. terreus*. After 3 weeks of stationary culture, the medium was filtered and the filtrate was repeatedly extracted out at pH 7.0 with ether. The resulting aqueous layer was adjusted to pH 2–3 with hydrochloric acid and was extracted with ether until yellow material was no longer transferred into the ether layer. The ether layer was concentrated, and the extract was subjected to preparative silica gel thin-layer chromatography with a solvent system of ether–hexane–acetic acid (40:8:1, v/v). Radioactive regions of the developed plate were located with a radiochromatoscanner, and the section corresponding to aspulvinone E (R_f 0.28) was scraped and extracted with ether. The radioactive aspulvinone E thus obtained was further purified on a column (8 × 500 mm) packed with porous polymer (Hitachi Gel 3011) with methanol–hexane–acetic acid (180:20:1, v/v) at a flow rate of 1.5 mL/min with a Hitachi liquid chromatograph 635. The elution of aspulvinones was monitored by recording the absorbance at 370 nm. [^{14}C]-Aspulvinone E, 6.7 mg (specific activity, 5.3×10^5 dpm/μmol), was obtained in 11% yield based on [^{14}C]tyrosine added. The radioactive aspulvinone E was dissolved in 0.05 M Tris-HCl[1] buffer (pH 7.4) and used as substrate for the enzymatic reaction.

[^3H]Dimethylallyl Pyrophosphate. [1-^{3}H]Dimethylallyl pyrophosphate (specific activity, 1.12×10^7 dpm/μmol) was synthesized by the usual phosphorylation of 3-[1-^{3}H]methylbut-2-en-1-ol obtained by the reduction of methylcrotonic acid with [^{3}H]$LiAlH_4$, and the radioactive dimethylallyl pyrophosphate was purified by silica gel thin-layer chromatography with propanol–ammonia–water (6:3:1, v/v).

Aspulvinones were obtained from *A. terreus* as already described (Ojima et al., 1973). The methyl ether of aspulvinone E was prepared by treatment with diazomethane. Atromentic acid and pulvinone were synthesized according to the procedure described in the literature (Wikholm et al., 1972; Edwards et al., 1973; Ojima et al., 1973). Dimethylallyl pyrophosphate and its analogues were the same preparations as in the previous study (Nishino et al., 1972).

Enzyme Assay. The standard incubation mixture contained, in a final volume of 1.0 mL, 100 μmol of Tris-HCl buffer (pH 7.0), 40 nmol of [^{14}C]aspulvinone E, 200 nmol of dimethylallyl pyrophosphate, and an appropriate amount of enzyme protein (usually less than 50 μg). After the mixture was incubated at 37 °C for 1 h, unless otherwise stated, 1 mL of 1 M Tris-HCl buffer (pH 7.0) and 4.0 mL of ether were added, and the mixture was shaken. The ether layer was taken and washed with 1 mL of the same buffer containing 2 M NaCl, and an aliquot of the ether extract was counted for radioactivity. Neither aspulvinone E nor dimethylallyl pyrophosphate was extractable with ether under these conditions. The enzyme activity was expressed as radioactivity found in the ether extract. One unit of the enzyme is defined as the amount of enzyme which converts 1 nmol of aspulvinone E into the prenylated derivatives per minute. In some experiments, [^{3}H]-dimethylallyl pyrophosphate and nonlabeled aspulvinone G were used in place of dimethylallyl pyrophosphate plus [^{14}C]aspulvinone E. For the reaction with crude enzyme, 10 μmol of potassium fluoride was added to the above mixture to inhibit phosphatase activity.

Purification of Enzyme. All steps were carried out at 4 °C. Mycelia (200 g) of 7-day-old culture were ground in a mortar with HCl-washed sea sand (200 g) and 800 mL of 50 mM phosphate buffer (pH 6.8). The homogenate was centrifuged at 26 000*g* for 30 min, and then 108 000*g* for 30 min. The resulting supernatant was fractionated with ammonium sulfate. The fraction precipitating between 30 and 55% saturation was dissolved in a minimal volume of the same buffer, and the solution was filtered on a Sephadex G-25 column (2.0 × 41 cm) equilibrated with the same buffer. The resulting protein fraction is referred to as "crude enzyme". The crude enzyme was applied to a DEAE-Sephadex A-50 column (1.5 × 25 cm) with a linear gradient of KCl from 100 to 400 mM in 50 mM phosphate buffer (pH 6.8). Fractions containing the transferase activity were pooled, concentrated on Diaflow membrane UM-10, and chromatographed on a Sephadex G-200 column (2.5 × 38 cm) with 50 mM phosphate buffer (pH 6.8). Active fractions were collected and applied to a hydroxylapatite column (1.2 × 21 cm) previously equilibrated with the same buffer. Elution was performed with about 100 mL of the same buffer and then with a linear gradient of the buffer from 50 to 300 mM. The enzyme purified through the hydroxylapatite chromatography step can be stored frozen at −20 °C without loss of activity at least for 1 week, but 50% of the activity is lost when kept at 4 °C for 3 weeks. The addition of 2-mercaptoethanol did not affect the stability of the enzyme.

Electrophoresis and Molecular Weight Determination. Polyacrylamide disc gel electrophoresis was carried out usually with 7.5% gels at pH 8.3 as described by Davis (1964). When recovery of the enzyme activity was required, the addition of bromophenol blue as the tracking dye was omitted from the standard procedure, because the dye was bound strongly to the enzyme protein. Electrophoresis at varied gel concentrations was also carried out according to the procedure of Hedrick and Smith (1968) to see whether the two components observed on electrophoresis of the purified enzyme preparation are charge isomers or size isomers. The log relative mobility of protein was plotted against gel concentration. For repeated electrophoresis, the two components were separated from each other in the first electrophoresis as follows. A 1.6-mg sample of purified enzyme protein was divided and applied to three tubes containing gels, the bottoms of which had been covered with cellophane membrane equilibrated with the buffer solution. The electrophoresis was continued until each protein fraction came out of the gel to be trapped on the cellophane membrane. It was possible to follow the migration of the enzyme protein in the gels when the electrophoresis was carried out in the presence of bromophenol blue, because the enzyme was colored with this dye. However, bromophenol blue had to be omitted in the first electrophoresis, and so a reference run using this dye was also made. The protein fractions thus separated from the three gels were combined and concentrated by ultrafiltration with Minicon B-15 (Amicon Corp.) and subjected to the second electrophoresis. Gels were stained with amido black or Coomassie brilliant blue as usual.

For molecular weight determination, the enzyme was applied to Sephadex G-200 filtration with 50 mM phosphate buffer (pH 6.8). Ovalbumin (45 000), aldolase (158 000), and catalase (240 000) were used as the molecular weight standards. The molecular weight was determined from the linear plots of log molecular weight against the distribution coefficients for the standard proteins. The subunit molecular weight was estimated by $NaDodSO_4$–polyacrylamide gel electro-

[1] Abbreviations used are: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid.

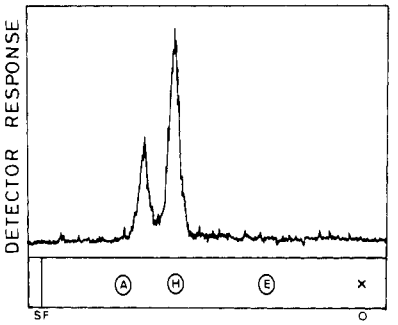


FIGURE 1: Thin-layer radiochromatogram of the ether extract of the reaction mixture of [^{14}C]aspulvinone E and dimethylallyl pyrophosphate. The incubation was carried out with crude enzyme as described under Materials and Methods. A silica gel plate (Merck) was used in ether–hexane–acetic acid (40:8:1, v/v). Spots of references: A, aspulvinone A; H, aspulvinone H; E, aspulvinone E.

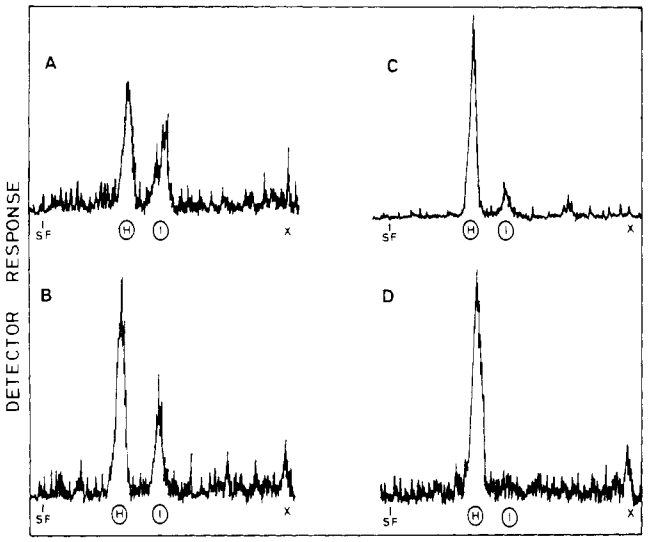


FIGURE 2: Thin-layer radiochromatograms of the enzymatic products obtained after various incubation times. The incubation times were 5 (A), 10 (B), 45 (C) and 60 min (D). The incubation mixture contained, in 1 mL of 1 mM Tris-HCl buffer (pH 7.0), 50 nmol of [^{3}H]dimethylallyl pyrophosphate, 1 μmol of aspulvinone E, 10 μmol of potassium fluoride, and crude enzyme (0.2 mg of protein). The chromatographic conditions were the same as described under Figure 1. Spots of references: H, aspulvinone H; I, aspulvinone I.

phoresis according to the method of Weber and Osborne (1969) from the log molecular weight against relative mobility plots with bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen (25 000), and cytochrome *c* (12 500) as standards. The standard proteins were products of Boehringer Mannheim.

Results

Enzymatic Reaction and Products. When the crude enzyme was incubated at pH 7.0 with [^{14}C]aspulvinone E and nonlabeled dimethylallyl pyrophosphate in the presence of potassium fluoride, radioactive material extractable with ether was formed. Since no radioactivity was found in the ether extract when either the enzyme or dimethylallyl pyrophosphate was omitted from the complete incubation mixture, the observation described above suggests that the enzymatic prenylation occurred. A similar result was obtained when ^{3}H-labeled dimethylallyl pyrophosphate and nonlabeled aspulvinone E were used as substrates. In this case, the formation of ether-extractable material depended on the enzyme or aspulvinone E. The thin-layer chromatography of the enzymatic product

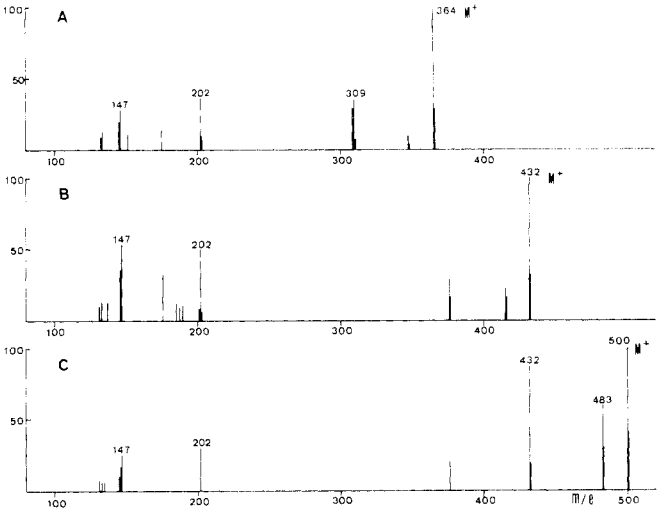


FIGURE 3: Mass spectra of enzymatic products. Enzymatic products derived from aspulvinone E and dimethylallyl pyrophosphate were purified by thin-layer chromatography and were introduced directly to the ion source. The electron beam potential was 70 eV. The intensities are given relative to the parent peak. Spots of references: A, monoprenyl derivative; B, diprenyl derivative; C, triprenyl derivative.

FIGURE 4: Thin-layer chromatogram of the ether extract of the reaction mixture of aspulvinone G and [^{3}H]dimethylallyl pyrophosphate. The chromatographic conditions were the same as described under Figure 1. Spots of references: C, aspulvinone C; D, aspulvinone D; G, aspulvinone G.

showed two radioactivity peaks at R_f values 0.59 and 0.70 (Figure 1). The mass spectra of the materials corresponding to these two peaks showed that they were di- and triprenyl derivatives of aspulvinone E (Figure 3). The diprenyl derivative was identified with an authentic sample of aspulvinone H isolated from *A. terreus*.

When a large excess of aspulvinone E relative to dimethylallyl pyrophosphate was subjected to the enzyme reaction for a short time, another material was formed along with aspulvine H (Figure 2). This material was shown to be a monoprenyl derivative by the mass spectrum and was cochromatographed with authentic aspulvinone I.[2] The amount of formation of aspulvinone H relative to aspulvinone I increased with incubation time, and the latter disappeared after 1 h, indicating that aspulvinone H is produced via aspulvinone I. It was also directly demonstrated that aspulvinone I could be a substrate to react with dimethylallyl pyrophosphate, affording aspulvinone H.

In order to see whether aspulvinone G, a hydroxyl derivative of aspulvinone E, can also be a substrate, the reaction of aspulvinone G was examined using [^{3}H]dimethylallyl pyro-

[2] The position of the prenyl group in aspulvinone I (Ojima et al., 1976) was determined by its cyclization followed by the methylation leading to the methyl ether with a chromane ring whose structure has been established by chemical synthesis (Knight and Pattenden, 1976).

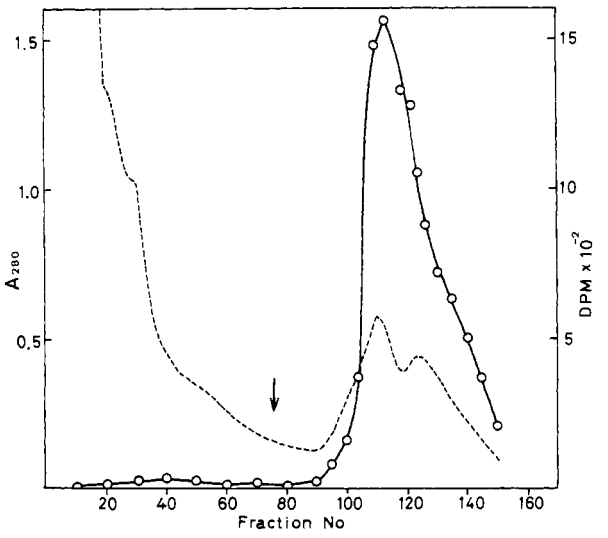


FIGURE 5: DEAE-Sephadex A-50 chromatography of a 30–55% ammonium sulfate fraction: (- - -) absorbance at 280 nm; (O–O) enzyme activity as assayed with [^{14}C]aspulvinone E and dimethylallyl pyrophosphate by the standard method described in the text. The arrow indicates the starting point of the linear gradient.

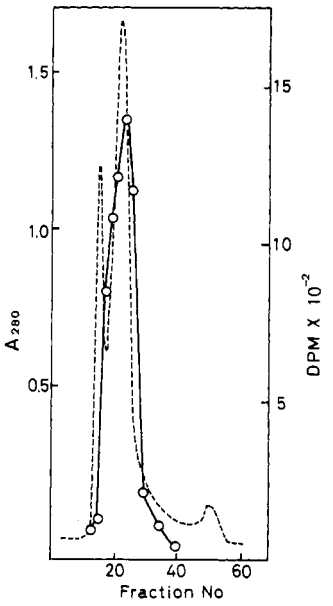
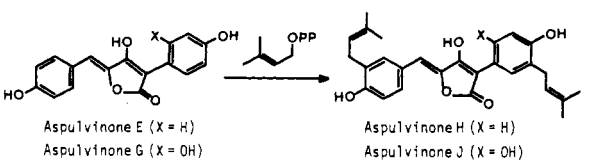


FIGURE 6: Sephadex G-200 chromatography of DEAE-Sephadex purified enzyme: (- - -) absorbance at 280 nm; (O–O) enzyme activity determined by the standard assay.

phosphate. Figure 4 shows the thin-layer radiochromatogram of the product. The major product (R_f 0.27) showed a mass spectrum (*m/e* 448, M^+) corresponding to a diprenyl derivative of aspulvinone G, and the minor one showed the same R_f value as that of aspulvinone D. Treatment of the enzymatic products with *p*-toluenesulfonic acid gave a radioactive material which was cochromatographed with authentic aspulvinone C. Consequently, the major product is aspulvinone J, whose occurrence in the fungus remains to be examined. The minor product seems to be aspulvinone D or its isomer having a chromane ring



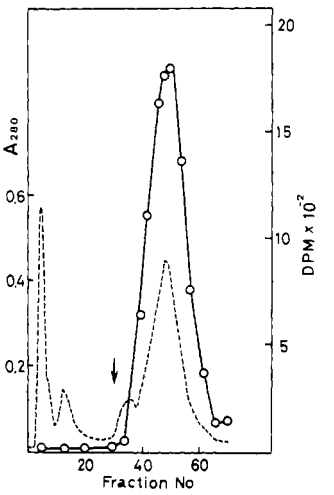


FIGURE 7: Hydroxylapatite chromatography of Sephadex G-200 purified enzyme: (- - -) absorbance at 280 nm; (O–O) enzyme activity. The arrow represents the starting point of the linear gradient.

on the other side, or a mixture of them, but at any rate this is a product derived secondarily from the major products. Therefore, these results indicate that the crude enzyme catalyzes at least the following reactions.

Purification of Enzyme. The prenylation enzyme was purified about 120-fold by chromatography on DEAE-Sephadex, Sephadex G-200, and hydroxylapatite. The purity was satisfactory as judged by polyacrylamide disc gel electrophoresis with and without $NaDodSO_4$. The results are given in Figures 5–7 and Table I.

Substrate Specificity. The purified enzyme was also active with dimethylallyl pyrophosphate and either aspulvinone E or G. Therefore, we call this enzyme dimethylallyl pyrophosphate:aspulvinone dimethylallyltransferase. Table II shows the relative reactivities of other aspulvinones and related compounds as assayed with ^{3}H-labeled dimethylallyl pyrophosphate as cosubstrate. The high reactivity of aspulvinone I is compatible with the observation that it is an intermediate in the formation of aspulvinone H from aspulvinone E. The lower but significant reactivity of aspulvinone H is also consistent with the formation of a triprenyl derivative as the minor product in the prenylation of aspulvinone E. Dimethylallyl pyrophosphate analogues were also examined for reactivity. Neither dimethylallyl monophosphate nor isopentenyl pyrophosphate (3-methylbut-3-enyl pyrophosphate) was active. The enzyme shows stringent specificity with respect to the prenyl structure. (*E*)-3-Methylpent-2-enyl pyrophosphate and a few cyclic compounds showed only weak activities (Table III).

Molecular Weight. The molecular weight of the transferase was estimated to be 240 000–270 000 by Sephadex G-200 filtration. $NaDodSO_4$–polyacrylamide electrophoresis showed a single protein band corresponding to molecular weight of 45 000. Therefore, the native enzyme seems to consist of six subunits of nearly identical molecular weight.

Behavior on Electrophoresis and Binding to Bromophenol Blue. When the enzyme purified through the hydroxylapatite chromatography step was subjected to polyacrylamide disc gel electrophoresis, two protein bands (fractions I and II) were observed, the major one always running faster (Figure 8). Interestingly, these two protein fractions were bound tightly to bromophenol blue used as the tracking dye, and as a result two greenish blue bands could be discerned without usual staining. When the gel was treated with amido black or coo-

TABLE I: Purification Scheme.

Step	Protein (mg)	Activity Unit	Activity Recovery (%)	Sp act. (unit/mg)	Purity (fold)
108 000*g* supernatant	4700	1140	100	0.24	1
30–55% ammonium sulfate fraction	658	617	54	0.96	4.0
Dialysis	517	911	80	1.85	7.7
DEAE-Sephadex	103	898	79	7.44	30.9
Sephadex G-200	71	978	86	13.8	57.1
Hydroxylapatite	36	1058	93	29.4	122

TABLE II: Reactivity of Aspulvinone Analogues as Substrates.

Compound[a]	Rel act. (%)	Compound	Rel act. (%)
Aspulvinone E	100	Aspulvinone B	0
Aspulvinone G	71	Aspulvinone D	0
Aspulvinone I	75	Aspulvinone E dimethyl ether	0
Aspulvinone I-c[b]	13		
Aspulvinone H	10	Atromentic acid[c]	7
		Pulvinone[d]	6

[a] All compounds were used at 40 μM. [b] An isomer derived from aspulvinone I by cyclizing the prenyl group to the chromane ring. [c] A carboxyl derivative of aspulvinone E (Kögl, 1928). [d] 3-Phenyl-4-hydroxy-5-benzylidene-2(5*H*)-furanone.

TABLE III: Reactivity of Dimethylallyl Pyrophosphate Analogues as Substrates.

Compound[a]	Rel act. (%)
Dimethylallyl PP[b]	100
(*E*)-Butenyl PP	10
(*Z*)-3-Methylpent-2-enyl PP	5
(*E*)-3-Methylpent-2-enyl PP	35
Ethylpent-2-enyl PP	0
(*E*)-3,4-Dimethylpent-2-enyl PP	0
(*Z*)-3-Methylhex-2-enyl PP	0
(*E*)-3-Methylhex-2-enyl PP	0
Cyclopentylideneethyl PP	35
Cyclohexylideneethyl PP	35
Cycloheptylideneethyl PP	12

[a] All compounds were used at 200 μM. [^{14}C]Aspulvinone E was the cosubstrate. [b] PP stands for pyrophosphate.

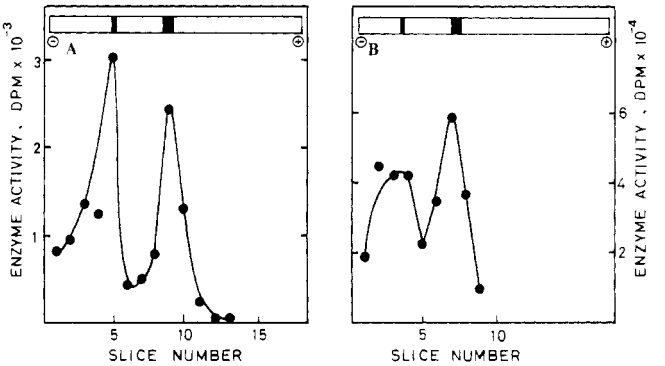


FIGURE 8: Polyacrylamide disc gel electrophoresis of purified enzyme. Electrophoresis was performed with 7.5% gels as described under Materials and Methods. After electrophoresis, the gel was sliced into 5-mm sections, and the slices were crushed in Tris buffer (pH 7.0) and assayed for the transferase activity with [^{14}C]aspulvinone E and dimethylallyl pyrophosphate (A) and with aspulvinone G and [^{3}H]dimethylallyl pyrophosphate (B). The assay conditions were the same as described in the text, except that the incubation time was extended to 2 h.

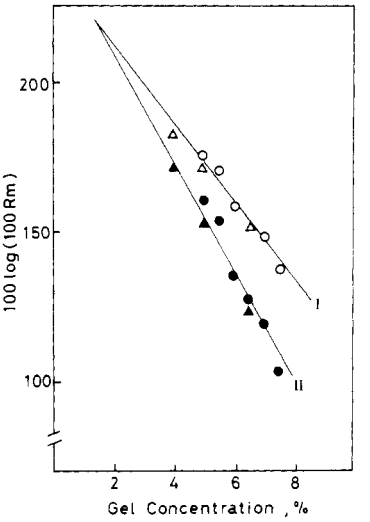


FIGURE 9: Effect of different gel concentrations on the mobilities of fractions I and II. Experiments were duplicated. The open and closed symbols correspond to fractions I and II, respectively, and the circles and triangles correspond to different runs.

masie brilliant blue, two bands were stained, being superimposed on the bands colored with bromophenol blue. The electrophoresis of the crude enzyme, 30–55% ammonium sulfate fraction, showed also a band colored with bromophenol blue at the same distance as that for the major band (fraction I) of the purified enzyme, indicating that the coloration with the dye is due to a specific binding to the transferase. This fact led us to examine whether the purified enzyme might have been separated by electrophoresis into two enzymes responsible for the prenylations of aspulvinone E and of aspulvinone G. However, both of these two fractions were active to prenylate either aspulvinone E or aspulvinone G (Figure 8). The products obtained by the action of these two enzyme fractions were also found to be identical with each other.

In order to see the interconvertibility of the two fractions, they were isolated from each other and subjected again to electrophoresis. The reelectrophoresis of fraction I showed two protein bands in a similar manner to the first electrophoresis, indicating that fraction II was derived from fraction I. However, the reelectrophoresis of fraction II showed only one band at the original distance. These fractions appear to be size isomers as judged by the criteria of Hedrick and Smith (1968), since the log of relative mobility vs. gel concentration showed nonparallel lines extrapolating to a point near 0% gel concentration (Figure 9). Therefore, fraction II may be an aggregated form of fraction I. The molecular weights of fractions I and II were estimated to be approximately 280 000 and 470 000, respectively, according to the procedure of Hedrick and Smith (1968).

TABLE IV: Effect of Bromophenol Blue and Substrate Analogues and Their Related Compounds.

Compound	Concn (μM)	Enzyme act. (%)[a]
None		100
Bromophenol blue	20	45
	40	31
	100	15
	200	4
Bromopyrogallol red	20	98
	40	94
Toluenesulfonate	100	97
	200	94
Aspulvinone E dimethyl ether	20	77
	100	63
	200	50
Isopentenyl pyrophosphate	1000	59
Inorganic pyrophosphate	20	72
	50	60
	100	43
	200	31

[a] [^{14}C]Aspulvinone E and dimethylallyl pyrophosphate were the substrates.

Inhibitory Effect. The binding of bromophenol blue to the transferase led us to examine the effect of this dye and its related compounds on the enzyme activity. Bromophenol blue inhibited the enzyme reaction more strongly than even substrate analogues (Table IV), and the inhibition was of mixed type when aspulvinone E was the varied substrate. Although bromopyrogallol red is structurally close to bromophenol blue, it has little effect. Inorganic pyrophosphate, which is a product of the transferase reaction, shows strong inhibitory effect. The reciprocal plots of initial rates against the reciprocal concentrations of dimethylallyl pyrophosphate or aspulvinone E showed that the inhibition of inorganic pyrophosphate was of mixed type against dimethylallyl pyrophosphate (K_i = 17.8 μM, K_i' = 37.4 μM) and noncompetitive against aspulvinone E (K_i = 50 μM).

The enzyme activity was not affected by the addition of Mg^{2+} ion or EDTA, and other divalent metal ions showed some inhibitory effect.

The transferase was activated 2.3 times by 0.025% Tween 80, and 1.5 times by 0.025% Triton X-100, but was 69% inhibited by lecithin of the same concentration.

Effect of Substrate Concentration. The apparent K_m values obtained from Lineweaver-Burk plots were 40.0, 13.7, and 7.7 μM for dimethylallyl pyrophosphate, aspulvinone E, and aspulvinone G, respectively.

pH Optimum. The transferase showed the maximum activity at pH 7.0 of Tris-HCl buffer. In phosphate buffer at the same pH, the enzyme showed ca. 70% of the activity in Tris buffer (Figure 10).

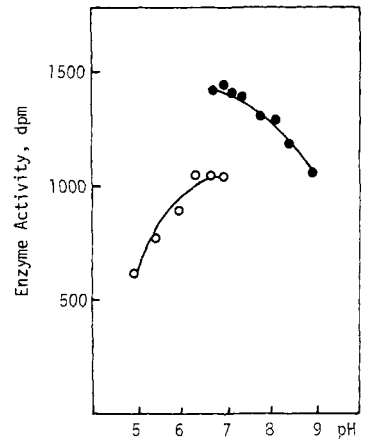


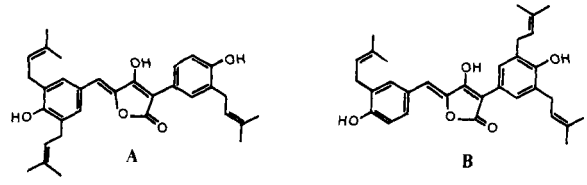
FIGURE 10: Effect of pH on the enzyme activity. Each incubation mixture contained 100 mM Tris-HCl (●) and phosphate buffer (○).

Discussion

A great number of prenylation steps are involved in the biosynthesis of natural products, since a variety of prenylated aromatic compounds occur widely in nature. Several cell-free enzyme systems catalyzing the C_5 unit transfer have so far been obtained. They are the enzymes for the prenylation of indole ring in the formation of echinulin (Allen, 1972), cyclopiazonic acid (McGrath et al., 1973), and tryptophan (Lee et al., 1976) and the prenylation of umbelliferone for the biosynthesis of psoralen (Dhillon and Brown, 1976). Each of them transfers only one C_5 unit to an aromatic ring to give a monoprenylated derivative.

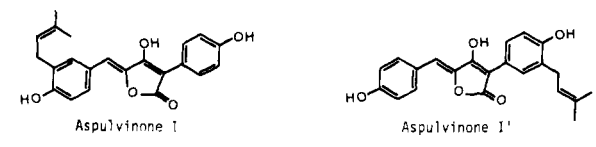
The present enzyme, dimethylallyl pyrophosphate:aspulvinone dimethylallyltransferase, is an example belonging to this class in terms of the prenylation of an aromatic compound but is unique in that this enzyme catalyzes the transfer of more than one prenyl unit to give even a triprenylated derivative. Furthermore, either aspulvinone E or G can be prenylated as substrate. It is also in contrast to the other prenylation enzymes in that the present enzyme is not activated by any divalent metal ions.

Although a third prenyl group is introduced, the rate of the prenylation is much slower than those of the first or the second prenylation. The structure of the triprenylated product is assumed to be A or B, but there is so far no indication of its natural occurrence. Aspulvinone I and H, mono- and diprenyl



derivatives, which have been found as metabolites in *A. terreus* must be precursors for aspulvinone A and B. Although neither the mono- nor the diprenyl derivative of aspulvinone G has yet been found in the fungus, they are conceivably the precursors for aspulvinone C, D, and F.

We have no concrete answer to the question—which of the two aromatic rings of aspulvinone E or G is first prenylated? It is true that the prenylation of aspulvinone E gives a monoprenylated derivative whose chromatographic and mass spectrometric properties are identical with those of authentic aspulvinone I and that aspulvinone I is highly reactive as substrate to be prenylated to give aspulvinone H. However, this alone cannot exclude the possibility that the enzymatically derived monoprenyl product might be an isomer (aspulvinone I′) of aspulvinone I or a mixture of them, since they would not



be distinguishable from each other by chromatographic or mass spectrometric analysis. Preliminary experiments suggest

that aspulvinone I′ also occurs in an early culture of this fungus (Takahashi, unpublished). There is no reason to believe that the enzyme capable of prenylating both of the aryl rings must transfer the prenyl units in a specific sequence. Further studies would answer this question.

It would also be of interest to see whether the active site of the enzyme accommodates one molecule of dimethylallyl pyrophosphate and one aspulvinone molecule, or two molecules of dimethylallyl pyrophosphate and one aspulvinone molecule. In the former case, the two aromatic rings of aspulvinone cannot be prenylated at the same time, but the aspulvinone substrate, when once prenylated, must turn itself over to fit the enzyme so that it can be further prenylated. In the latter case, however, the active site has two separate catalytic positions responsible for the prenylation, and the two rings of aspulvinone can be prenylated in situ.

The transferase shows novel properties of binding to bromophenol blue, and consequently the enzyme protein can be easily located on electrophoresis or gel filtration in the presence of this dye. Since the dye can be extracted with ether from an acidified solution of the bromophenol blue bound enzyme, the nature of the binding is not covalent. The 590-nm absorption of the dye is shifted to 615 nm when it is bound to the enzyme, suggesting that the surrounding of the dye may be hydrophobic rather than ionic. A similar behavior of bromophenol blue has been reported for human serum albumin (Kragh-Hansen et al., 1974). The strong inhibitory effect of this dye suggests that the binding may occur at a hydrophobic cavity of the active site of enzyme.

The significance of the two active species separated by the electrophoresis of the transferase is now under investigation.

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