OXIDASES INVOLVED IN BIOSYNTHESIS OF MACROLIDE ANTIBIOTIC PATULOLIDES FROM *PENICILLIUM URTICAE* S11R59

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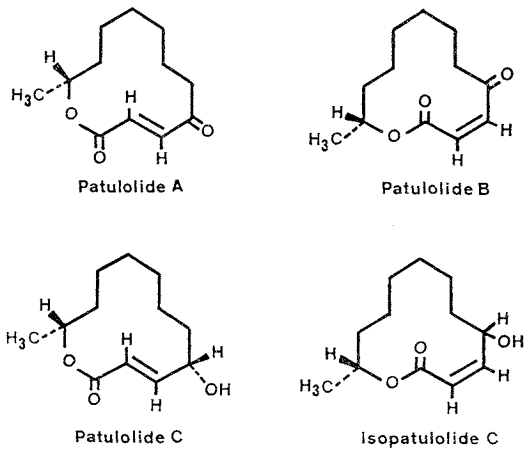
(Received for publication December 13, 1988)

Patulolides are a group of 12-membered macrolide antibiotics produced by *Penicillium urticae* S11R59. An enzyme involved in the conversion of patulolide C to patulolide A was purified from *P. urticae* S11R59 and characterized. The enzyme showed a single band on SDS-PAGE and molecular sieve HPLC both of which indicated a M_r of 86,000, indicating that the enzyme is monomeric. However, the enzyme was separated into two bands of very similar pI's (pI 4.2 and 4.3) by isoelectric focusing. Both bands catalyzed the conversion of patulolide C to patulolide A, as demonstrated by activity staining. The two isoenzymes were proved to be oxidases by the simultaneous production of H_2O_2 during the conversion of patulolide C to patulolide A. The molar ratio for patulolides C, A and H_2O_2 was determined to be 1 : 1 : 1. The optimum pH and temperature were determined to be 7 and 35~40°C, respectively, and the enzymes were stable at pH 6~9 and 4~40°C. The oxidases showed characteristic absorption at 345 and 450 nm, indicating the presence of flavin as coenzyme. Among several analogues of patulolide C tested, the oxidases showed very narrow substrate-specificity; only patulolide C was oxidized to patulolide A. No enzyme activity for the reverse reaction, *i.e.* from patulolide A to patulolide C, was present in the cell-free extract of *P. urticae* S11R59. Patulolide C oxidases therefore play a key role in the biosynthesis of patulolides.

Patulolides are a group of structurally related macrolide antibiotics which are produced by *Penicillium urticae* S11R59. So far, four novel patulolides have been isolated and purified from the culture broth of *P. urticae* S11R59. They were named patulolides A[1], B, C[2] and isopatulolide C (Fig. 1). All of them share a common 12-membered lactone structure with minor differences with respect to geometrical isomerism of a double bond (*trans* or *cis*) and a group at C-4 (a carbonyl or hydroxyl group). Among macrolide antibiotics, 12-membered macrolides such as patulolides are the simplest and can serve as the easiest model for understanding the biosynthetic pathway of more complicated macrolides. From our previous study, the patulolides were shown to be pure acetogenic hexaketides derived from the head-to-tail condensation of 6 acetate units[3]. Patulolide C was suspected to be the parent of the other patulolides because of its higher production at the early production phase as well as

Fig. 1. Native patulolides isolated from the culture broth of *Penicillium urticae* S11R59.



a higher incorporation of ^{13}C-labeled acetate in its molecule than the other patulolides. To explain the biosynthetic relationship among the patulolides, we have investigated plausible enzymes involved in the biosynthesis of patulolides, and found an enzyme which could convert patulolide C to patulolide A. This report describes the isolation and characterization of the enzyme and its relationship with the biosynthesis of the patulolides.

Materials and Methods

Cultivation Conditions and Preparation of Crude Cell-free Extract

P. urticae S11R59 was maintained as a spore suspension at −80°C in a medium consisting of glucose 40 g, peptone 10 g, yeast extract 5 g, $MgSO_4 \cdot 7H_2O$ 2 g, KH_2PO_4 5 g and $CaCl_2 \cdot H_2O$ 2 g, per liter, pH 6.5. Glucose - yeast extract medium[3] was used as a seed culture medium. For the inoculum preparation, 250 ml of glucose - yeast extract medium in a 1-liter Erlenmeyer flask was inoculated with the spore suspension to yield a final concentration of 5.4×10^5 spores/ml, and incubated at 28°C on a reciprocating shaker (100 strokes/minute) for 48 hours. The main cultivation was done with 6-liter of the glucose - yeast extract medium inoculated at 48-hour and cultivated at 28°C for 66 hours with agitation speed of 300 rpm and aeration rate of 1 v/v/minute. The cells were harvested by suction filtration, washed three times with cold water, and stored at −80°C until use. Approximately, 17 g of cells (wet weight) were obtained from 1 liter of the medium.

Enzyme Activity and Protein Assay

The oxidase activity was assayed by measuring either the amount of patulolide A formed from patulolide C or the amount of H_2O_2 formed along with the conversion of patulolide C. For H_2O_2 determination, a reaction mixture contained 0.4 μmol of 4-aminoantipyrine, 0.5 μmol of 2,4-dichlorophenol, 1 μmol of patulolide C, 1.54 μmol of sodium azide, 5 U of horseradish peroxidase and the enzyme in 1 ml total volume of 0.1 M potassium phosphate buffer (pH 7.0). The reaction was carried out at 28°C for 20 minutes and stopped by the addition of 1 ml of ethanol. The absorbance at 505 nm was measured and the amount of H_2O_2 was calculated from a standard curve of H_2O_2. One U of the enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of H_2O_2 per minute under the assay conditions described.

For patulolide A determination, a reaction mixture contained 1 μmol of patulolide C and the enzyme in 1 ml total volume of 0.1 M potassium phosphate buffer (pH 7.0). After 20 minutes reaction at 28°C, the reaction was stopped by the addition of 1 ml of ethyl acetate containing 1 mM *N,N'*-dimethyldodecamide as an internal standard. The mixture was vigorously mixed, centrifuged to separate the water and ethyl acetate layers, and 2 μl of the ethyl acetate layer was analyzed by Silica gel HPLC (Cosmosil 5SL, 4.6×250 mm, Nacalai Tesque, Inc., Kyoto, Japan) using *n*-hexane - 2-propanol (9.8 : 0.2) as solvent. The amount of patulolide A was determined from the peak ratio of patulolide A to the internal standard. One U of the enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of patulolide A per minute under the assay condition described.

Protein concentration was determined by the method of LOWRY *et al.*[4] using bovine serum albumin as a standard protein. Absorbance at 280 nm was measured in chromatographic procedures.

Purification Procedure

All operations were carried out at 0~5°C, unless otherwise specified.

Step 1. Preparation of Cell-free Extract: The cell paste (300 g) was suspended in 0.02 M potassium phosphate buffer (pH 6.5) containing 10% glycerol to give a suspension of about 1 g/10 ml. The suspension was subjected to Dyno-Mill homogenizer (type KDL, Willy A Bachofen AG., Basel, Switzerland) at flow rate of 20 ml/minute. Cell debris was removed by centrifugation at $16,000 \times g$ for 30 minutes.

Step 2. Protamine Sulfate Treatment: A solution of protamine sulfate (2%) was added dropwise to the cell-free extract to give final concentration of 40 μg/mg protein. After standing for 10 minutes,

the precipitate formed was removed by centrifugation at 16,000×*g* for 30 minutes.

Step 3. Ammonium Sulfate Fractionation: Solid ammonium sulfate was added to the suspension to give 0.5 saturation. After standing for 8 hours, the precipitation was removed by centrifugation at 16,000×*g* for 30 minutes. The ammonium sulfate concentration was then increased to 0.8 saturation by the addition of solid ammonium sulfate. After standing for 8 hours, the percipitation was collected by centrifugation at 16,000×*g* for 30 minutes and dissolved in 0.02 M potassium phosphate buffer (pH 6.5) containing 10% glycerol. The solution was dialyzed for 18 hours against 3 changes of 3 liters of the same buffer.

Step 4. DEAE-Toyopearl Column Chromatography: The dialyzed enzyme solution was subjected to DEAE-Toyopearl column chromatography. The gel was packed in a column (5.5×45 cm) and equilibrated with 0.02 M potassium phosphate buffer (pH 6.5). The enzyme solution was passed through the column which was then washed with 2.1 liters of the same buffer. The enzyme was subsequently eluted with 2.8 liters of a linear gradient of sodium chloride from 0 to 0.5 M in 0.02 M potassium phosphate buffer (pH 6.5) in fractions of 15 ml. This step removed a contaminating protein and 5-fold purification was attained. Active fractions were combined and the solution was concentrated by ultrafiltration.

Step 5. Hydrophobic Chromatography on a Octyl Sepharose CL-4B Column: To the concentrated enzyme solution, solid ammonium sulfate was added to a final concentration of 2 M. The solution was then applied to an Octyl Sepharose CL-4B column (3×28.5 cm) preequilibrated with 0.02 M potassium phosphate buffer (pH 6.5) containing 2 M ammonium sulfate. After washing with 0.5 liter of the buffer, the enzyme was eluted with 1.5 liters of a linear gradient of ammonium sulfate from 2 to 0 M in 0.02 M potassium phosphate buffer (pH 6.5) in fractions of 10 ml. Active fractions were combined and concentrated to 10.6 ml by ultrafiltration.

Step 6. Sephadex G-150 Column Chromatography: The concentrated enzyme solution was applied onto a Sephadex G-150 (superfine) column (3×61 cm) preequilibrated with 0.02 M potassium phosphate buffer (pH 6.5) containing 10% glycerol (Fig. 2). By gel filtration, a contaminating protein of high molecular weight was removed and active fraction number 45 to 51 were combined.

Step 7. DEAE-Sephadex A-50 Column Chromatography: To remove a trace amount of impurities, the combined solution was applied to a DEAE-Sephadex A-50 column (1.5×16.0 cm) pre-

Fig. 2. Chromatography of patulolide C oxidases on a Sephadex G-150 column.
● Absorbance, ○ activity.

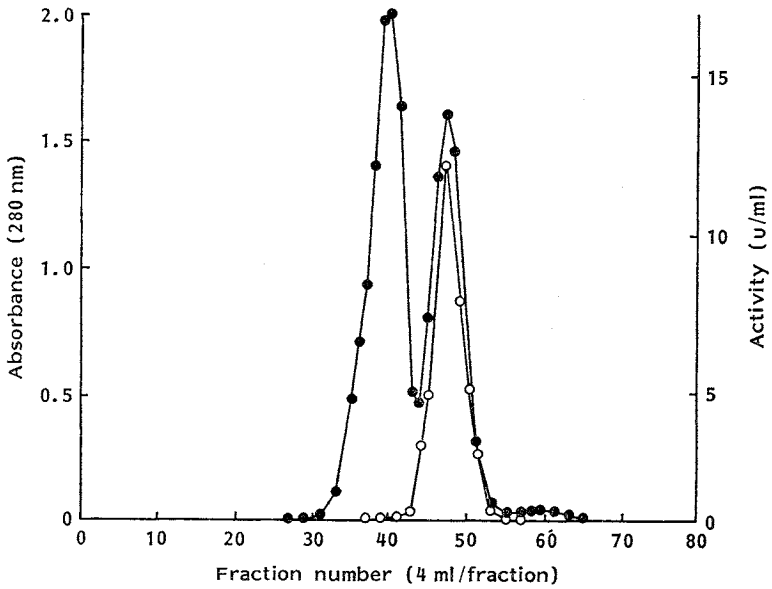


Fig. 3. Chromatography of patulolide C oxidases on a DEAE-Sephadex A-50 column.
● Absorbance, ○ activity, --- NaCl.

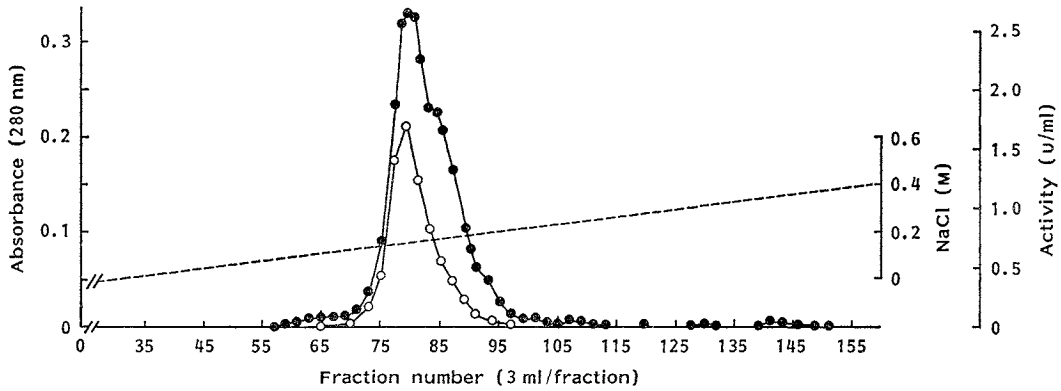


Table 1. Purification of patulolide C oxidases from *Penicillium urticae* S11R59.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification (fold)	Recovery (%)
Cell-free extract	13,103.2	728.5	0.06	1	100
Protamine sulfate	7,663.5	679.9	0.09	1.59	93.3
Ammonium sulfate (50~80%)	2,246.7	617.2	0.27	4.91	84.7
DEAE-Toyopearl	303.4	380.9	1.26	22.43	52.3
Octyl Sepharose CL-4B	56.2	169.6	3.02	53.91	23.3
Sephadex G-150 (superfine)	18.1	100.7	5.55	99.11	13.8
DEAE-Sephadex A-50	9.5	32.6	4.48	80.00	4.5

equilibrated with 0.02 M potassium phosphate buffer (pH 6.5) containing 10% glycerol. After washing with 81 ml of the buffer, the enzyme was eluted with 0.4 liter of a linear gradient of sodium chloride from 0 to 0.4 M in the same buffer in fractions consisting of 3 ml (Fig. 3). The specific activity of the enzyme was increased from 0.06 to 4.48 U per mg protein and approximately 80-fold purification was achieved with an overall yield of 4.5%. The purification procedure is summerized in Table 1.

Molecular Sieve HPLC

Moleculer weight of the native enzyme was determined by molecular sieve HPLC on a TSK-gel G2000SW$_{XL}$ column (0.75×60 cm, Tosoh Manufacturing Co., Ltd., Japan) equilibrated with 50 mM potassium phosphate buffer (pH 6.5) containing $MgCl_2$ 1 mM, 2-mercaptoethanol 5 mM and NaCl 0.2 M. Glutamate dehydrogenase (M_r 290,000), lactate dehydrogenase (M_r 142,000), enolase (M_r 67,000), adenylate kinase (M_r 32,000) and cytochrome C (M_r 12,400) were used as molecular weight markers.

Electrophoresis

SDS-PAGE was done on a slab gel (8.4×9.0×0.1 cm) with 4~20% polyacrylamide gradient according mainly to the method of LAEMMLI[5], and protein was stained with comassie brilliant blue G-250. Phosphorylase b (M_r 130,000), bovine serum albumin (M_r 75,000), ovalbumin (M_r 50,000), carbonic anhydrase (M_r 17,000) were used as molecular weight markers.

Analytical isoelectric focusing was done as described by WRIGLEY[6] on a 7.5%-disc gel containing 2% of ampholine (pH 4~6) at 4°C for 6 hours at 300 V. After the focusing, the gel was sliced into 2 mm pieces, and soaked in 0.2 ml of distilled water, and the pH of the supernatant was measured. Protein was stained by 0.04% comassie brilliant blue G-250 in 3.5% perchloric acid as described by

REISNER *et al.*[7]. For activity staining, the gel was soaked in a mixture of peroxidases and 3,3'-diaminobenzidine or peroxidase and *o*-dianisidine containing 1 mM patulolide C as substrate at 28°C for 20 minutes. This procedure is a modification for H_2O_2 staining described by TSUGE and MITSUDA, TSUGE and NAKANISHI[8,9].

Stoichiometric Conversion of Patulolide C to Patulolide A and H_2O_2

Stoichiometric conversion of patulolide C to patulolide A was investigated in order to confirm the activity of the enzyme. Patulolide A production and patulolide C consumption were determined by HPLC analysis of the reaction mixture containing 0.36 ml of enzyme preparation from DEAE-Sephadex A-50 in 5.64 ml of 1 mM of patulolide C in 0.1 M potassium phosphate buffer at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 minutes of reaction at 28°C. The concentration of patulolides A and C were calculated from calibration curve of each compound. H_2O_2 production was determined by the enzymatic method. Enzyme preparation (0.36 ml) was added into 5.64 ml of substrate mixture. The H_2O_2 concentration was determined after 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 minutes of reaction.

The Effect of Patulolide C Concentration on Oxidases Activity

The effect of patulolide C concentration on enzyme activity was observed by using enzyme preparation from DEAE-Sephadex A-50 column. Fifty μl of the enzyme was separately added to patulolide C solution in 0.1 M potassium phosphate buffer (pH 6.5) at several concentration. The reaction mixture was incubated at 28°C for 20 minutes. Patulolide A produced from the reaction was determined by HPLC analysis.

Substrate-specificity of Patulolide C Oxidases

Substrate-specificity of the enzyme was determined by using various types of derivatives which have similar structure to patulolide C (Fig. 11). Isopatulolide C (Fig. 1) is the native patulolide which was purified from the culture broth of *P. urticae* S11R59. Each compound was added, instead of patulolide C, into the enzyme activity assay mixture as described and the enzyme activity was determined by the production of H_2O_2 from the reaction after incubation at 28°C for 30 minutes.

Materials

DEAE Toyopearl was purchased from Tosoh Manufacturing Co., Ltd. (Japan). Octyl Sepharose CL-4B, Sephadex G-150 (superfine) and DEAE-Sephadex A-50 were products of Pharmacia Fine Chemicals (Sweden). 2,4-Dichlorophenol, 4-aminoantipyrine, 3,3'-diaminobenzidine were obtained from Nacalai Tesque, Inc. (Japan), H_2O_2 from Wako Pure Chemical Industries, Ltd. (Japan) and *o*-dianisidine from Sigma Chemical Company (U.S.A.). *N,N'*-Dimethyldodecamide was synthesized from dimethylamine and dodecanoyl chloride. Patulolides A, B, C and isopatulolide C were isolated and purified from the culture broth of *P. urticae* S11R59. Epipatulolide C and reduced patulolide B were synthesized by the reduction of patulolides A and B, respectively, with (*tert*-BuO)$_3$-LiAlH[2]. Dihydropatulolide C and dihydroisopatulolide C were obtained by the hydrogenation of patulolide C and isopatulolide C, respectively, on Pd - C (5%) under atmospheric pressure of H_2. Hydrolyzed patulolide C was synthesized by hydrolysis of patulolide C by LiOH in methanol - H_2O at room temperature for 45 hours. Standard proteins for molecular sieve HPLC and SDS-PAGE were obtained from Oriental Yeast Co., Ltd. (Japan) and Bio-Rad Laboratory (U.S.A.), respectively.

Results and Discussion

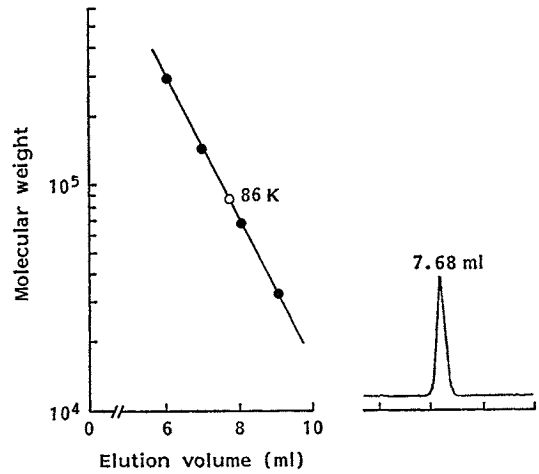
We found that a crude cell-free extract of *P. urticae* S11R59 could convert patulolide C to patulolide A, which was detected on the TLC plate of an extract of the reaction mixture. The presence of the activity in the cell-free extract was further confirmed by the conversion of [^{14}C]patulolide C to [^{14}C]patulolide A as well as the patulolide C-dependent appearance of patulolide A by HPLC analysis (data not shown). Because it does not require any coenzymes for dehydrogenation even after extensive dialysis and H_2O_2 seems to be produced during the conversion, this enzyme was suspected

as a kind of oxidase. To study the character of this enzyme and the function of it in the biosynthesis of patulolides, the enzyme was purified and characterized as described here.

Homogeneity

The enzyme preparation from the DEAE-Sephadex A-50 chromatography showed a single and symmetrical peak on molecular sieve HPLC (Fig. 4) and a single band on SDS-PAGE (Fig. 5). The molecular weights of the enzyme under the denatured and native condition were identical (M_r 86,000), indicating that the enzyme is monomeric. However, the enzyme preparation was separated into two bands by isoelectric focusing (Fig. 6): One band showed pI value of 4.2 and the other of 4.3. Both bands showed patulolide C-dependent H_2O_2 production as evident from the activity staining. Therefore, the two enzymes appear to be isozymes of almost the same molecular weight. We observed two more bands (totally 4) by activity staining when the crude cell-free extract was applied on isoelectric focusing. One showed a pI value a little lower than 4.2 and the other a little higher than 4.3. Therefore, *P. urticae* S11R59 may have at least 4 isozymes catalyzing the conversion of patulolide C to patulolide A, two of which were purified as described in this report. Although we cannot exclude the possibility that these isozymes may come from proteolysis during the purification procedures, it seems unlikely from the constant ratio between the two major bands during the purification and the presence of four isozymes even in the crude extracts.

Fig. 4. Molecular weight of native patulolide C oxidases by molecular sieve HPLC on TSK-gel G2000SW$_{XL}$.



Catalytic Properties of the Enzymes

Since the enzymes did not require any

Fig. 5. Gel concentration gradient SDS-PAGE of patulolide C oxidases.
Lane 1: Purified patulolide C oxidases from DEAE-Sephadex A-50. Lane 2: M_r marker proteins.

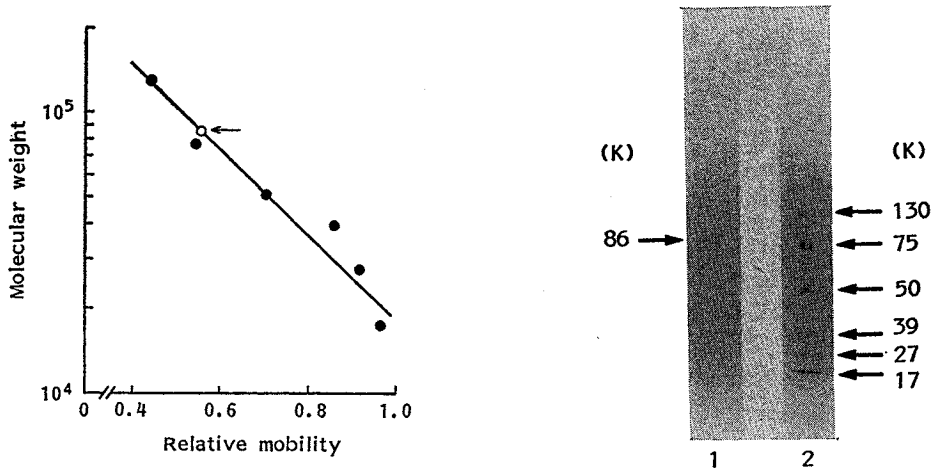


Fig. 6. Analytical isoelectric focusing of patulolide C oxidases.

Lane 1: Protein staining with comassie brilliant blue. Lane 2: Activity staining for oxidases with 2,4-diaminobenzidine. Lane 3: Activity staining for oxidases with *o*-dianisidine.

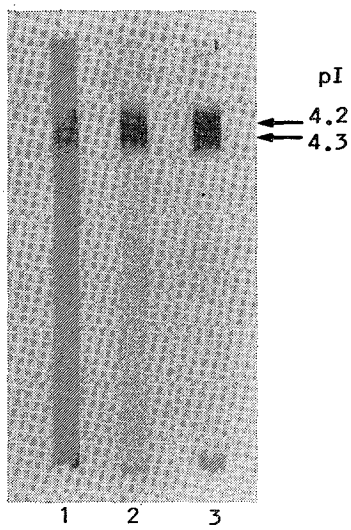


Fig. 7. Stoichiometric conversion of patulolide C to patulolide A and H_2O_2 by patulolide C oxidases.

□ H_2O_2 production, ○ patulolide A production, ● patulolide C consumption. Patulolide A/patulolide C=1.00.

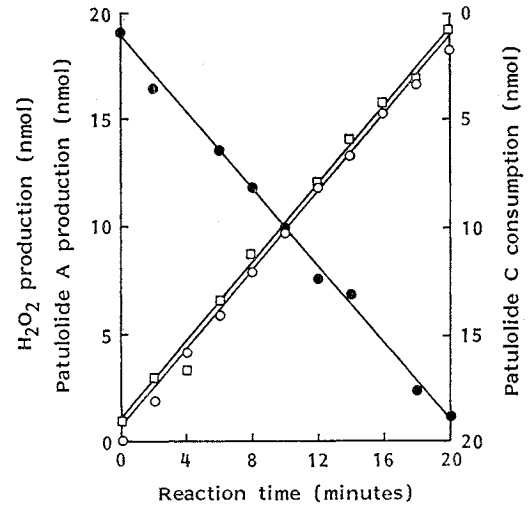
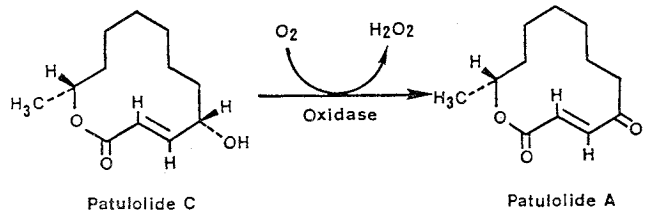
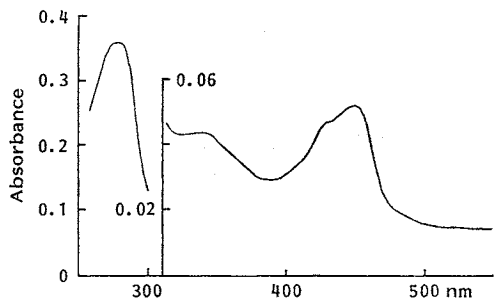


Fig. 8. Enzymatic conversion of patulolide C to patulolide A and H_2O_2 by patulolide C oxidases.



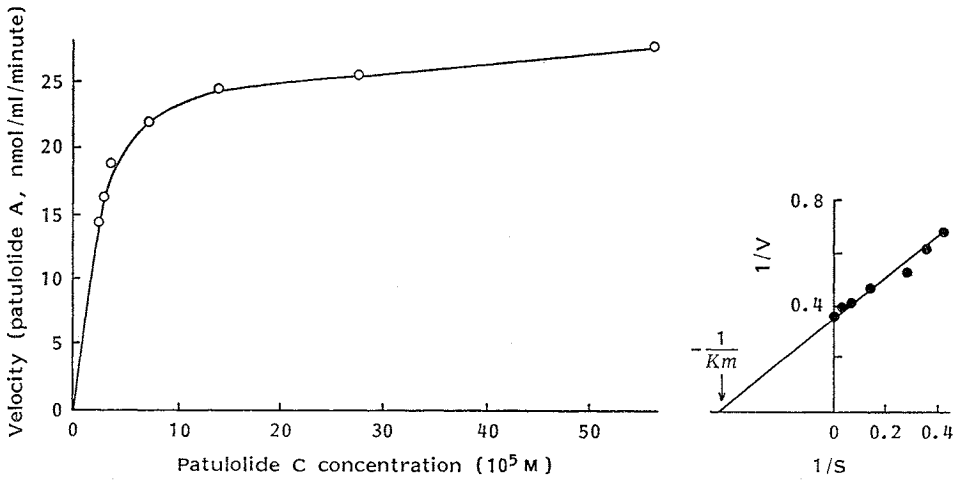
possible coenzymes such as NAD^+ or $NADP^+$ for the conversion of patulolide C to patulolide A and H_2O_2 production was dependent on the presence of patulolide C, the enzymes seemed to be a kind of oxidase. To examine this point further, the stoichiometry for patulolide C, patulolide A and H_2O_2 were determined and the ratio for patulolide C - patulolide A - H_2O_2 found was 1:1:1 (Fig. 7). Therefore, the enzymatic conversion of patulolide C to patulolide A must be as shown in Fig. 8. Since the absorption spectrum of the enzyme (Fig. 9) showed absorption maxima at 345 and 450 nm, which is a characteristic for flavin containing enzymes, the enzyme is a flavin containing oxidases and was

Fig. 9. Optical absorption spectrum of patulolide C oxidases isolated from *Penicillium urticae* S11R59.



The absorption of the enzyme was recorded by using a Hitachi 220A spectrometer (Japan). The enzyme preparation from DEAE-Sephadex A-50 was scanned from 250 to 600 nm using 0.02 M potassium phosphate buffer (pH 6.5) containing 10% glycerol as a blank.

Fig. 10. The effect of patulolide C concentration on the activity of patulolide C oxidases.
Velocity, Vmax=84.4 nmol/minute/mg protein, Km=23 μM.



named patulolide C oxidases. *Km* and Vmax values were determined by conventional Lineweaver-Burk plot (Fig. 10) to be 23 μM and 84.4 nmol/minute/mg protein, respectively, using patulolide C as substrate. The small value for the *Km* indicated that the enzymes can bind patulolide C tightly.

Table 2. Substrate-specificity of patulolide C oxidases.

Substrates	Relative activity (%)
Patulolide C	100.00
Isopatulolide C	0.003
Epipatulolide C	0.003
Dihydropatulolide C	0.007
Dihydroisopatulolide C	0.010
Hydrolyzed patulolide C	0.037
Reduced patulolide B	0.004

Effect of pH and Temperature

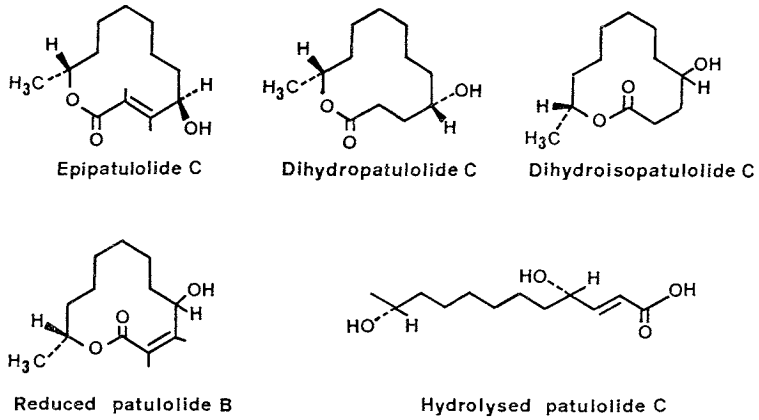
Patulolide C oxidase had an optimum pH for activity at around 7 and was stable at pH 6~9 in citrate, potassium phosphate or glycine-NaOH buffers. The oxidase seemed rather unstable at acidic pH as evident from around 50% loss of the activity at pH 3.0.

Optimum temperature was determined to be around 35~40°C. Lower or higher temperature resulted in a loss of enzyme activity. The oxidases were found stable up to 50°C, but lost almost all activity at 60°C or higher.

Substrate-specificity

Various kinds of structurally related patulolide C analogues were examined as a potential substrate for patulolide C oxidases (Table 2 and Fig. 11). All compounds except isopatulolide C were synthesized by chemical modification of native patulolides A, B and C as described in Materials and Methods. From Table 2, the enzymes utilized patulolide C as almost a sole substrate. Hydrolyzed patulolide C which is a linear form of patulolide C could be a substrate but with only 0.04% reactivity. Surprisingly, neither a *cis* isomer of patulolide C (isopatulolide C and reduced patulolide B) nor a C-4 epimer of patulolide C (epipatulolide C) served as substrate, indicating that the enzyme has severe stereospecificity toward the *trans* geometrical isomerism of the C-2 double bond and the *S*-configuration of C-4 hydroxyl group. Furthermore, the presence of the C-2 double bond seemed to be very important for enzyme activity because dihydropatulolide C which was derived from patulolide C did

Fig. 11. Structurally related patulolide C analogues obtained from chemical modification of native patulolide A, B or C.



not act as substrate. From these lines of evidence, patulolide C oxidases are very specific enzymes in converting patulolide C to patulolide A. During jar fermentation, specific activity of patulolide C oxidases became highest at around 60~72 hours when patulolide C concentration reached a plateau and patulolide A concentration was increasing. This fact together with the description of the enzyme above supports that patulolide C should be a parent compound for the other patulolides and that patulolide C oxidases play an important role in the early step of patulolides biosynthesis.

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