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Epoxidation of styrenes with the peroxidase from the cultured cells of *Nicotiana tabacum*

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Abstract: An enzyme concerned with the epoxidation of styrenes was isolated from cultured cells of *Nicotiana tabacum*. The enzyme had peroxidase activity as well as epoxidation activity, and its amino acid sequence showed 89% homology in their 9 amino acid overlap with horseradish peroxidase. In the enzymatic reaction, hydrogen peroxide and *p*-cresol were necessary and molecular oxygen was the source of the oxygen atom of the epoxide. The enzymatic reaction using a spin trap reagent and monitoring of the reaction with ESR indicated that the epoxidation reaction of styrenes proceeded by a radical mechanism with peroxidase.

Keywords: Enzymes and enzyme reactions; Epoxidation; Radicals and radical reactions

Chiral introduction of oxygenated functional groups into alkenes is one of the important reactions for the syntheses of chiral compounds. Many studies have been reported on the specific oxidation of alkenes by use of biocatalysts.[1-3] We have reported that the cultured cell suspensions of *Nicotiana tabacum* have the ability to introduce

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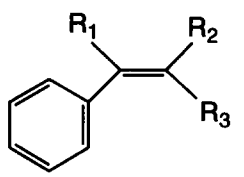
enantioselectively an epoxy and/or a hydroxyl group into monoterpenes.[4-7] Recently we observed that, when exogenous substrate was administered to the cultured plant cells, hydrogen peroxide and some enzymes were secreted into the cultured medium.[8,9] In continuation of the studies on the oxidation of alkenes with the cultured plant cells, we have now isolated the enzyme responsible for the epoxidation of styrenes and examined the reaction mechanism of the reaction.

Results and Discussion

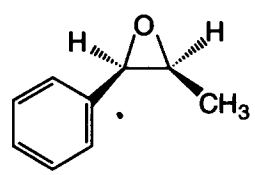
Epoxidation of styrenes with the cultured cells of N. tabacum

Styrenes, such as *cis*-2-methylstyrene (**1**), *trans*-2-methylstyrene (**2**), styrene (**3**), and 1-methylstyrene (**4**), were administered to the cell suspension of *N. tabacum*. As shown in Table 1, styrenes **1-3** were converted into their corresponding epoxides, but 1-methylstyrene (**4**) was not.

In order to clarify the relationship between epoxidation of the substrates and H_2O_2 generation in the cells, the amount of H_2O_2 generated was examined in the biotransformation of the substrates. H_2O_2 generation was observed (2.5 - 4.9 μM) when **1** -**3** were administered. In contrast, when **4** was administered, the H_2O_2 generation was not observed. This indicates that H_2O_2 generation was necessary for the epoxidation with these cultures.



1: R_1=R_2=H, R_3=CH_3
2: R_1=R_3=H, R_2=CH_3
3: R_1=R_2=R_3=H
4: R_1=CH_3, R_2=R_3=H



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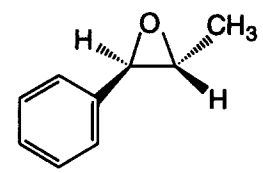

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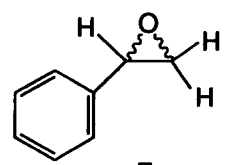

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Table 1. Formation of epoxides and H_2O_2 generation in the biotransformation of styrenes **1**~**4** with the cultured cells of *N. tabacum*

Substrates	Products	Yield of epoxides[a]	H_2O_2 generation[b]
		%	μM
1	**5**	7.5	3.2
	6	0.3	
2	**5**	0.4	4.9
	6	4.5	
3	**7**	4.0	2.5
4	---	nd[c]	< 0.3

a) Yield of epoxides for 1-day incubation.
b) H_2O_2 concentration in the cultured medium of 15-hr incubation mixtures.
c) nd denotes 'not detected'.

Isolation of epoxidase from the cultured cells of N. tabacum

The crude enzyme preparation, which was prepared from the cultured medium of the suspension cultures of *N. tabacum,* was purified by chromatography on CM-TOYOPEARL column, as shown in Table 2. The SDS gel electrophoresis of the purified enzyme preparation showed a single band at *ca.* 38 kDa. The epoxidase activity of the purified enzyme preparation (p38) was up about 30 times against the crude enzyme preparation. Furthermore, p38 was found to have peroxidase activity,[10,11] as shown in Table 2.

Table 2. Purification of the epoxidase from the culture medium of the *N. tabacum* cultures

Steps	Total protein	Epoxidation activity		Peroxidase activity	
	μg	μg / mg protein	Fold	U / mg protein	Fold
Crude prep.	189	1.1	1	26	1
CM-Toyopearl (1st)	24	8.5	7.7	56	2.2
CM-Toyopearl (2nd)	3.8	33.5	30	232	8.9

To clarify the amino acid sequence, p38 was electroblotted from the SDS-polyacrylamide gel to a PVDF membrane was applied to a protein sequencer. However,

no amino acid sequence was identified because the *N*-terminus of the protein was blocked for Edman degradation.[12] A deblocking reaction for a protein having an *N*-terminal pyroglutamic acid with pyroglutamyl peptidase was effective. This suggests that the *N*-terminal amino acid was glutamine and that the glutamine was cyclized to pyroglutamic acid. The amino acid sequence of the deblocked protein, therefore was found to be Glu-Leu-Ala-Pro-Thr-Phe-Tyr-Asp-Asn from its *N*-terminus. The amino acid sequence showed 89% identity in its 9 amino acid overlap against horseradish peroxidase (EC 1.11.1.7).[13] These observations show that p38 is a kind of peroxidase and that the epoxidation reaction of alkenes in *N. tabacum* may proceed by action of the peroxidase.

Epoxidation with the enzyme preparation from the cultured cells of N. tabacum

Epoxidation of *cis*-2-methylstyrene (**1**) with the p38 preparation was carried out under various conditions, as shown in Table 3. A complete enzymatic reaction with *p*-cresol and H_2O_2 was found to give not only the epoxides but also 4-methylcatecol and polyphenols. In the absence of H_2O_2 and *p*-cresol, the yield of epoxides decreased strikingly. Particularly, in the absence of *p*-cresol, the epoxidation did not occur at all. Thus it was demonstrated that H_2O_2 and *p*-cresol were necessary for enzymatic epoxidation as co-factors.

Table 3. Effects of *p*-cresol and H_2O_2 in the enzymatic epoxidation of **1**

Conditions	Relative activity %
p-Cresol + H_2O_2	100
p-Cresol	17
H_2O_2	trace
None	trace

When the epoxidation reaction was carried out under an N_2 atmosphere, the yield of epoxide decreased about 12% compared with the reaction in air. The result suggests that molecular oxygen participates in the epoxidation. Participation of molecular oxygen was

confirmed by the epoxidation of **1** under $^{18}O_2$ (99.2 atom %) atmosphere. The MS spectrum of the epoxide obtained from the enzyme reaction showed a molecular ion peak at m/z 136; this indicates the incorporation of ^{18}O (61% incorporation on the basis of the peak intensity).

The foregoing results, particularly that H_2O_2 and *p*-cresol were necessary and peroxidase was involved in the epoxidation, suggested that the enzymatic reaction occurred with the participation of radicals, such as hydroxyl radical and/or *p*-cresol radical. In order to clarify the participation of a radical reaction, the following experiments were carried out. In the enzymatic epoxidation in the presence of a spin trap reagent, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), the yield of the product decreased. This shows that a portion of the radicals generated in the epoxidation reaction was trapped by DMPO and therefore, the reaction stopped halfway. This was confirmed by monitoring the ESR spectrum of enzymatic reaction. As shown in Fig. 1, an ESR signal was observed in the incubation mixture with the active enzyme preparation, whereas no signals were observed

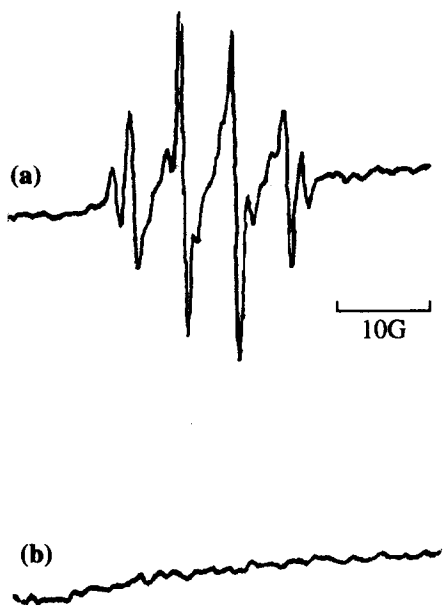
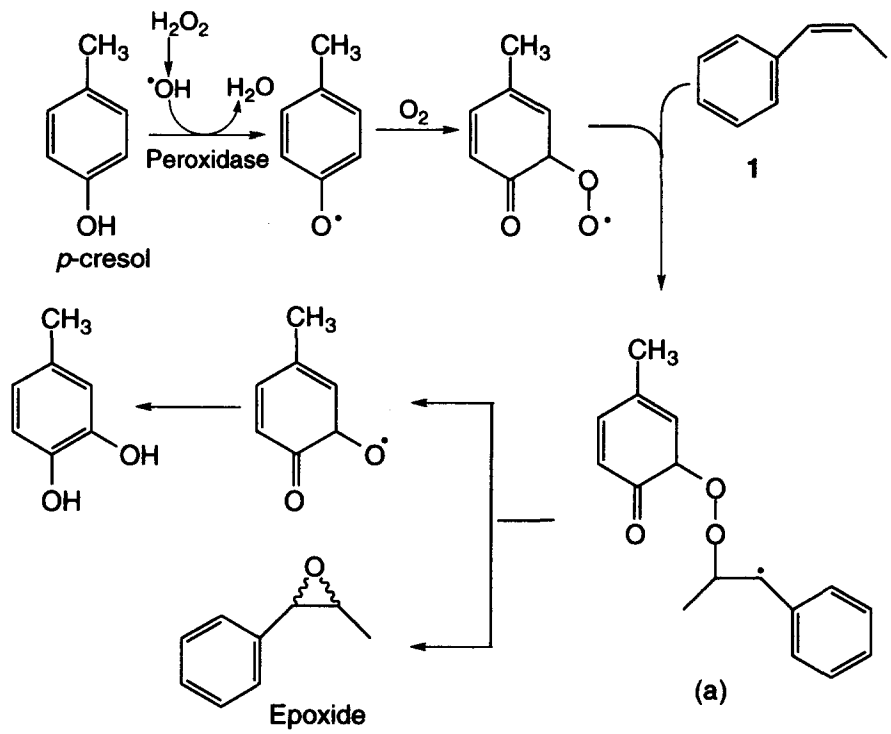


Fig. 1. ESR spectra in the enzymatic epoxidation of **1** with (a) active enzyme and (b) heated enzyme preparation.

in the incubation mixture when the enzyme was heated at 90 °C for 30 min. This observation confirmed that some radicals participated in the enzymatic epoxidation.

Thus, it was found that: (1) presence of H_2O_2 and *p*-cresol was essential for the epoxidation reaction as co-factors, (2) the origin of the oxygen atom in the epoxidation was molecular oxygen, (3) the protein participating to the epoxidation was a peroxidase, and (4) the radicals, probably hydroxyl and *p*-cresol radicals, were involved with the epoxidation reaction. These observations bear resemblance to the reported results for epoxidation of styrene with horseradish peroxidase.[14] Considering these results, a reasonable mechanism in the enzymatic epoxidation of styrenes may be proposed, as shown in Scheme 1. Hydroxyl radicals produced from H_2O_2 with peroxidase attack



Scheme 1. Proposed mechanism for the epoxidation of styrenes with the peroxidase from the cultured cells of *N. tabacum*

phenols such as *p*-cresol to produce a corresponding radical, and then the radical bonds with molecular oxygen. The oxygenated cresol radical oxidizes the C=C bond of the

substrate such as **1** to give a intermediate (a) in Scheme 1. The intermediate (a) falls apart to the epoxide product and a semidione radical, which may be terminated by the formation of 4-methylcatecol and/or polyphenols. If the α-position of the styrenes is substituted by any functional groups, e.g. the 1-methyl group of **4**, the intermediate become a *tert*-radical, which is more unstable than a *sec*-radical; this may be a reason why no epoxide was obtained in the biotransformation of **4** by the cultured cells of *N. tabacum*.

Experimental

Analytical Procedures

GLC analyses were performed on an instrument equipped with a capillary column (0.25 mm x 30 m, CP-cyclodextrine-β-236M-19). The column, injector and detector temperatures were held at 120 °C, 180 °C and 200 °C, respectively. A standard curve for quantitative analyses was obtained by carrying a range of concentrations of authentic *cis*-2-methylstyrene epoxide (**5**). GC-MS analyses were taken on a mass spectrometer which was installed with an EI ion source (70 eV) and a GC equipped with a capillary column (0.25 mm x 30 m, OV-17) programmed to rise from 70 °C to 170 °C at 5 °C / min.

Protein sequencing was carried out by a protein sequencer (ABI 473A, Applied Biosystems) as follows. The protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[15] and electroblotted onto the polyvinylidene difluoride (PVDF) membrane,[16] by standard methods. The membrane carrying the protein was cut out and analyzed by the protein sequencer.

Monitor of the enzymatic reaction by ESR measurements

The mixture containing **1** (80 mM), H_2O_2 (8.0 mM), *p*-cresol (8.0 mM), $Zn(OAc)_2$ (0.7 M) and enzyme preparation in 0.2 M acetate buffer solution (pH 5.5) was incubated at 37 °C for 60 min and then subjected to ESR analysis. In order to obtain the fine ESR spectra, DMPO was added to incubation mixture for trapping radicals.[14]

Biotransformation of styrenes with the cultured cells of N. tabacum

The suspension cells of *N. tabacum* were prepared as reported previously.[17] To the flask containing the suspension cells, each of the substrates **1-4** (30 mg) was administered and incubated at 25 °C on a rotary shaker. After incubation, the culture medium collected by filtration was extracted with *n*-hexane. The hexane layer was evaporated and then subjected to short-column chromatography on silica gel with ether to give a crude product. Yield of the epoxide was determined by GLC analyses of the crude product.

Measurement of H_2O_2 amount

The H_2O_2 concentration in the culture medium was by a reported procedure[18] using scopoletin and horseradish peroxidase (HRP). HRP (12 μM), scopoletin (25 μM) and cultured medium (2 cm^3) were added to cuvette and measured fluorescent intensity. The wave length for activation was used at 365 nm and that for emission at 450 nm. The H_2O_2 concentration was calculated on the basis of the standard curve.

Assay of the epoxidation activity

To the solution containing **1** (4.2 mM), H_2O_2 (8.8 mM) and *p*-cresol (9.3 mM) in 0.1 M potassium phosphate buffer (1 cm^3, pH 7.4),[14] the enzyme solution (1 cm^3) was added and then the mixture was incubated at 37 °C for 6 h. The incubation mixture was extracted with ether and analyzed by GLC for determining of the yield of epoxide.

Assay of peroxidase activity

Peroxidase activity was assayed with guaiacol (*o*-methoxyphenol) according to the reported procedure.[10,11] The enzyme solution (0.01 cm^3) added to a solution of guaiacol (20 mM) and H_2O_2 (6 mM) in 50 mM sodium phosphate buffer (1 cm^3, pH 6.0). The rate of tetraguaiacol formation was monitored at 470 nm. The background rate for the oxidation of guaiacol in the absence of the enzyme solution was negligible. Peroxidase activity was calculated using the molar absorptivity of tetraguaiacol (ε = 26,600 M^{-1} cm^{-1}).

Isolation and Purification of Epoxidase

To the culture medium, $(NH_4)_2SO_4$ (767 g / dm^3 of medium) was added and the

mixture was stirred at 4 °C over night. The protein precipitated was collected by centrifugation at 12,000 *g* for 60 min. After dissolved in a minimum volume of 5 mM sodium phosphate buffer (pH 6.0), the protein solution was dialyzed against H_2O for 12 h. The protein fraction was loaded on a CM-TOYOPEARL column (1.9 x 38 cm) equilibrated with 5 mM sodium phosphate buffer (pH 6.0) and then the CM-TOYOPEARL column was eluted with linear gradient of 0--0.4 M NaCl in the buffer solution. The active enzyme fraction was subsequently subjected to further purification on a CM-TOYOPEARL column with linear gradient of 0.2--0.4 M NaCl in the buffer.

Deblocking of proteins having the N-terminal pyroglutamic Acid

The PVDF membrane with p38 spot was dried up and moist with acetonitrile. The membrane was treated with 200 μl of 0.5% (w/v) polyvinylpyrrolidone (PVP)-40 in 100 mM acetic acid at 37 °C for 30 min and washed with water. The membrane in 0.1 M phosphate buffer solution (0.01 cm^3, pH 8.0) containing 5 mM dithiothreitol, 10 mM EDTA, 5 % (w/v) glycerol and 5 μg of pyroglutamyl peptidase (EC 3.4.19.3; Boehringer Mannheim Co. Ltd.) was incubated at 30 °C for 24 h, and then the membrane was washed with water, dried up, and applied to sequencer.[19,20]

Epoxidation of cis-2-methylstyrene (1) with the p38 preparation

To the solution containing **1** (5 mM), H_2O_2 (9 mM) and *p*-cresol (9 mM) in 0.1 M potassium phosphate buffer (20 cm^3, pH 7.4), the enzyme solution (5 cm^3) was added and then the mixture was incubated at 37 °C for 3 h. The incubation mixture was extracted with ether and analyzed by preparative TLC to give epoxide **5** [^{1}H NMR ($CDCl_3$) δ 1.08 (d, 3H, J=5.4 Hz), 3.34 (m, 1H), 4.06 (d, 1H, J=4.4 Hz), and 7.3 (m, 5H)] and 4-methylcatecol [^{1}H NMR ($CDCl_3$) δ 2.37 (s, 3H), 6.60 (d, 1H, J=7.5 Hz), 6.69 (s, 1H), and 6.75 (d, 1H, J=7.5 Hz).

Enzymatic epoxidation under $^{18}O_2$ atmosphere

Prior to the incubation, the enzyme solution and the reaction buffer containing **1** (8.8 mM) and *p*-cresol (9.3 mM) were evacuated for 1 h and purged with N_2 to remove

dissolved oxygen. The reaction buffer (1 cm^3) and enzyme solution (1 cm^3) were put to a reaction tube, and then the solution was evacuated and purged with $^{18}O_2$. After three time evacuating and purging, H_2O_2 (0.01 cm^3; 1 mmol) was added by syringe through septum without leaking gases. The mixture was incubated at 37 °C for 30 min. After usual work-up, the crude product was analyzed by GC-MS.

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