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Further Purification of Side Chain Oxidase of 4-Hydroxycinnamic Acid from *Phaseolus mungo*

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Summary

Side chain oxidase of 4-hydroxycinnamic acid was found and isolated by ammonium sulfate fractionation, a colum chromatography on Con A Sepharose and gel filtration on Sephacryl S-200. The final preparations were homogeneous by multiple cristeria. The molecular weight determined by SDS electrophoresis to be about 40,000 and 30,000. This suggests the enzyme is hetorodimer. The optimal pH of the enzyme activity existed at pH 7.5 and the stability was at pH 4.0. The enzyme activity was decreased by treatment with acid protease, pepsin, or thermolysin. *o*-phenanthroline, di-pyridyl and dithiocarbamate inhibited the enzyme acrivity. These results suggest that the enzyme contains iron and copper.

Introduction

Lignin biosynthesis is believed to regulate the growth of higher plants.[1),2)] Metabolism of phenylpropanoid regulates biosynthesis of lignin, because lignin is synthesized from phenylpropanoid.[3-12)] The metabolism of phenylpropanoid with growth of plants has been studied.[13)] Already, it was known that the lignin biosynthesis pathway, at the first, amino group of phenylalanine is separated, the amino acid is converted to cinnamic acid, and hydroxylated to be trans-4-hydroxycinnamic acid, caffeic acid, and lignin.[14-17)] On the other hand, Suzuki[18)] sugested that the side chain oxidase of 4-hydroxycinnamic acid is activated in the presence of hydrosulfate group and regulates of phenylpropanoid metabolism. It is important that a role of the oxidase in the metabolism of phenylpropanoid is investigated to determine growth of plant. We previously reported[19)] the partial purification of side chain oxidase of 4-hydroxycinnamic acid from *P. mungo*. In this paper, we will report the further purification and characterization of side chain oxidase of 4-hydroxycinnamic acid.

Materials and Methods

Materials: The following chemicals were purchased from the sources; L-cysteine, 4-hydroxycinnamic acid from Tokyo Kasei Co., α-methyl-D-mannoside from Nakarai Co., Con A Sepharose 4B and Sephacryl S-200 from Pharmacia Fine Chemicals.

Reaction mixture: This enzyme activity was determined by the method that was previously reported by Miyoshi and Suzuki.[19)] The standard assay mixture (3 ml) contained the PMF (24.5 mg protein), 125 μM 4-hydroxycinnamic acid, 5 mM L-cysteine and 0.15 M posassium phosphate buffer (pH 7.5). The mixture was incubated at 25°C for 10 min. Side chain oxidase of 4-hydroxycinnamic acid activity was measured by decrement of absorbance at 290 nm from 2 to 5 min by a Gilford UV spectrophotometer. One unit of the enzyme activity was decreasing rate of 0.0001 per 1 min at 290 nm (Table 1).

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Table 1. Reaction mixture

Temperature; 25°C
1 unit = −A290/min = 0.0001

	Sample	Reference	Final conc.
4-Hydroxycinnamic Acid	30	30	125 μM
Enzyme	100	0	
L-Cysteine	30	30	5.0 mM
150 mM K-P buffer (pH 7.5)	1850	1950	
Distilled Water	990	990	
Total	3000 μl	3000 μl	

Determination of protein: Protein was determined by the method of Lowry et al.[20] or by the method estimating absorbance at 230 nm using bovine serum albumin (BSA) as standard.

Purification procedure: The beans (3 kg) were soaked in 1,000 ml of distilled water for 24 hr. at 25°C and transfered to a tray seated with a filter paper. They germinated at 25°C in the dark for 3.5 days. Approximately 3 kg of etiolated hypocotyls were harvested from seedlings and ground in 1,000 ml of 0.15 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM dithiothreitol at 0°C with motor and pestle. The homogenate was filtered by four layers guaze. Crude homogenate was centrifuged at 10,000 X *g* for 30 min at 4°C. The supernatant (PMF) was stored at −80°C until use (Fig. 1).

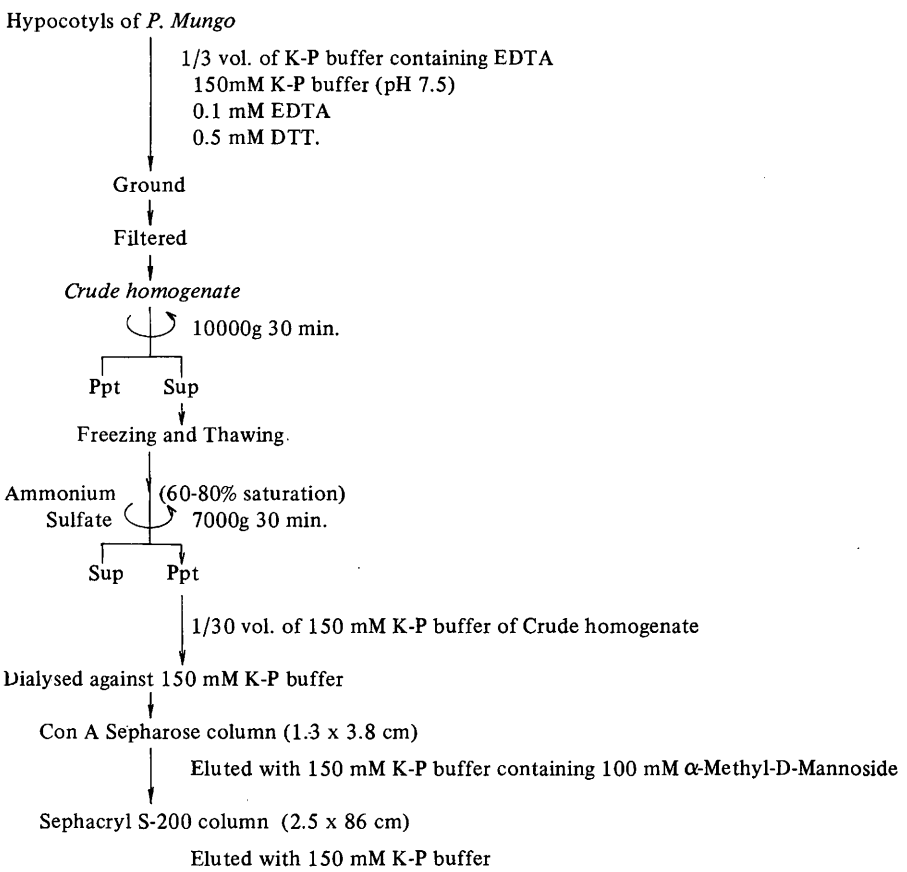

Fig. 1 Purification Procedures
This preparation was prepared from 300g dry weight of *P. mungo*

Results

Ammonium sulfate fractionation and Con A column chromatography: Fig. 1 shows that the enzyme was solibilized from the membrane in PMF by freezing and thawing. The solibilized enzyme was fractionated in 60~80% saturation of ammonium sulfate (AS) at 0°C. Each AS fraction was obtained by centrifugation at 9,000 × *g* 30 min at 4°C. The pellet was dissolved with 60 ml of 0.15 M potassium phosphate buffer (pH 7.5) and dialyzed against 9 liters of the same buffer overnight at 4°C. The enzyme was applied to a Con A sepharose 4B column (1 × 5 cm) at 4°C. After the column was washed by 60 ml of 0.15 M potassium phosphate buffer, the fractions involving enzyme activity were eluted by 0.15 M potassium phosphate buffer containing 0.1 M α-methyl-D-mannoside. The elution profile is shown in Fig. 2. Line shows enzyme activity, and broken line shows absorbance at 230 nm. 180% of the enzyme activity and 14% of protein were recovered.

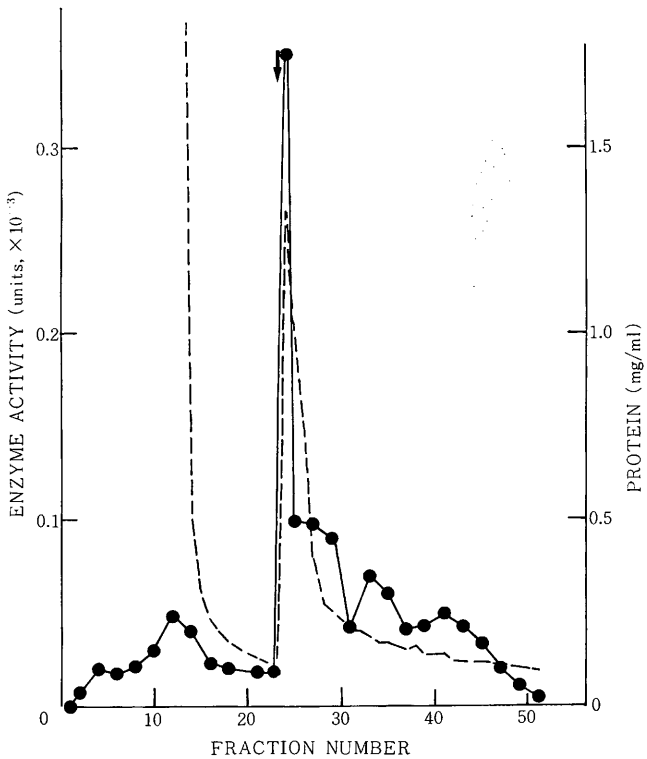

Fig. 2 Elution profile of the Side Chain Oxidase of 4-Hydroxycinnamic Acid from a Con A Sepharose 4B column.
The column was washed with equlibration buffer at 4°C It was eluted with 150mM potassium phosphate buffer (pH 7.5) containing 100 mM α – methyl – D-mannoside. Line shows the enzyme activity and broken line shows absorbance at 230 nm.

Chromatography on Sephacryl S-200: Fractions containing enzyme activity were combined and concentrated by the Millipore CX-10 filter at 4°C, the concentrate mixture was applied on a Sephacryl S-200 column (2.5 × 85 cm). The enzyme activity was eluted with 0.15 M potassium phosphate buffer into fractions 47~54. The profile is shown in Fig. 3. Determination of an apparent molecular weight by gel filtration through a Sephacryl S-200 column using BSA (68,000). The enzyme activity was eluted near BSA.

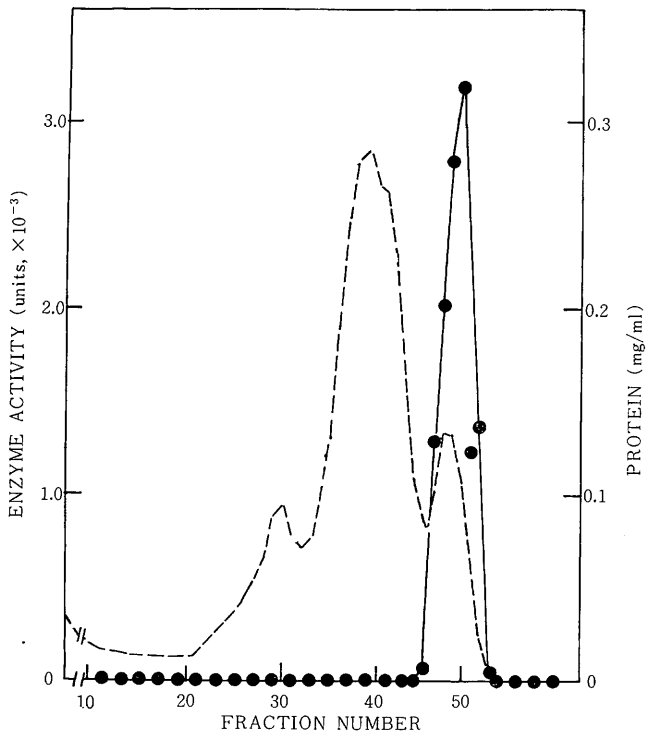

Fig. 3 Elution profile of the Side Chain Oxidase of 4-Hydroxycinnamic Acid from a Sephacryl S-200 column chromatography.
Line shows the enzyme activity and broken line shows absorbance at 230 nm.

Summary of the purification of the enzyme: Purification table is shown in Table 2. Total protein was 94.88 mg in the initial to 0.95 mg in the final. The recovery was 28%. The activity in AS fraction was 140 times compared to that in crude homogenate. Samely, the enzyme activity in Con A Sepharose 4B column fraction was 5850 times. Purify in each step were determined by SDS electrophoresis by the method of Laemmli[21] (Fig. 4). Two bands were observed in the profile of SDS electrophoresis in sample of Sephacryl S-200 step, whereas on the Davis method[22] it was one band. These results suggest that the enzyme consists of heterodimer.

Table 2. Isolution of the side chain oxides of 4-hydroxycinnamic acid

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg pro.)	Yield (%)	Fold
Crude	9488	224	0.023	100	1
PMF	5200	141	0.027	63	1.2
Ammonium Sulfate (60-80%)	79.1	253	3.2	113	140
Con A Sepharose	11.1	443	40.0	197	1740
Sephacryl S-200	0.95	128	134.7	28	5850

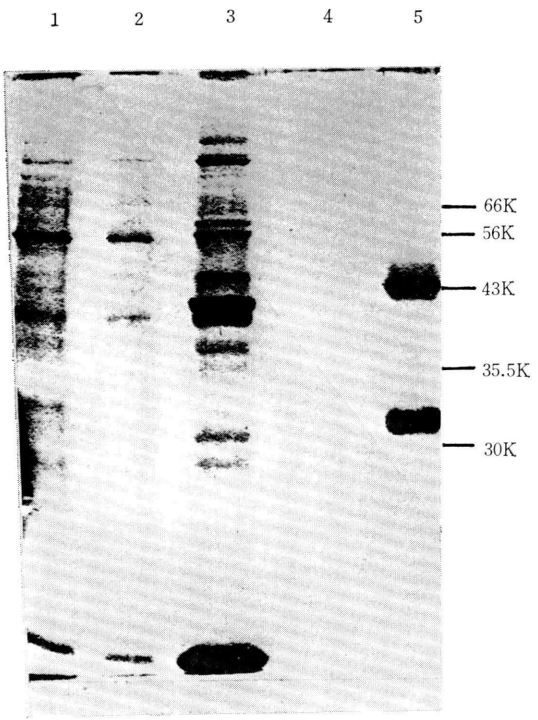

Fig. 4 Electrophoresis by Laemmli's method (0.1% SDS) constant power 1W for 4 h.
Running gel; 16.5% acrylamide (pH 8.8)
Stacking gel; 3% acrylamide (pH 6.8)
Electrode buffer; pH 8.3 Sample sol.; pH 6.8
1 Crude homogenate 2 PMF
3 Ammonium sulfate (60-80%)
4 Con A Sepharose 5 Sephacryl S-200

Characterization of the purified enzyme: 1) pH treatment – The enzyme was treated with pH 7.5, 4.0 and 1.0 leave in cold room for 20 hours. The enzyme activity was assayed at pH 7.5. The enzyme treated pH 7.5 was inactivate about 50%, treated pH 1.0 was inactivate 8% but treated pH 4.0 was scarcely inactivate. 2) Heat stability – The enzymes were treated with 30°C, 45°C, 55°C, 60°C and 70°C for 1 hour. The enzyme activity increased by treatment at 30°C for 1 hour. However, the enzyme activity was inactivated by treatment at 70°C 1 hour. Half life of the enzyme, $T_{1/2}$ (65°C) was 30 min. 3) Protease effect – To examine the protease effect on the enzyme activity pepsin and thermolysin (5 μg per 100 μg protein in the sample) were added respectively. After left at 4°C for 20 hours, enzymes activity were measured. As shown in Table 3, the enzyme treated with pepsin was almost inactivated. Even if strong electrolyte effect considered, the enzyme activities were inactivated. The enzyme activity treated at pH 7.5 at 4°C for 20 hours decreased to 43%. The enzyme activity that was treated with urea and left at 4°C for 20 hours decreased to 55%. 4) Chlater effect – As the enzyme was oxidase, it was considered enzyme containing metal. *o*-phenanthroline and diphyridyl chlate iron and dithiocarbamate chlates cupper. As shown Table 4, these chlators completely inhibited the enzyme activity at concentration of 0.15~0.2 mM. EDTA inhibited slightly. These results suggest that the enzyme is a metalic enzyme containing iron or copper. 5) Kinetics – As shown in Fig. 5, increase of the enzyme activity was plateau at over 10 mM of cysteine. Similarly, the activity depended upon 4-hydroxycinnamic acid concentration. The enzyme was saturated by 25 μM of 4-hydroxycinnamic acid which was another substrate. It was showed 15 μM of Km value.

Table 3. Change of the enzyme activity under several conditions

Treatment	Condition	%Activity
Control	25°C, 10 min	100
Heat	30°C, 60 min	157
	45°C, 60 min	95
	55°C, 60 min	74
	60°C, 60 min	74
	70°C, 60 min	5
pH	7.5, 20 hr	43
	4.0, 20 hr	97
	1.0, 20 hr	8 (100)
Protease (5μg/100μg pr.)	Pepsin, pH 1.0, 20 hr	1 (13)
	Thermolysin, pH 7.5 20 hr	37
Urea	6M, 20 hr	55

Table 4. Chelator effects on the enzyme activity

Chelator	Conc (mM)	%Inhibition
o-Phenanthroline	0.1	33
	0.15	85
	0.2	100
2, 2'-Dipyridyl	0.1	25
	0.15	92
	0.2	100
Diethyldithio-carbamate	0.1	24
	0.15	100
EDTA	1.0	17
	5.0	21
	10.0	51

Discussion

Yield of the enzyme activity in PMF decreased to 63%, but in ammonium fraction increased to 113% and in Con A Sephasose fraction to 197%. These results suggest that an inhibitor of the enzyme occurs in extracts. Finally, the enzyme activity yielded 28% in Sephacryl S-200 fraction and the enzyme was purified 5,850 folds. The purity of enzyme was determined by SDS-electrophoresis by method of Laemmli [21]. Four bands were detected in Con A Sepharose fraction and two bands in Sephacryl S-200 fraction. Molecular weight of the bands in the final step was 40,000 and 30,000. The enzyme was strongly adsorbed to Con A Sepharose column. Also, the fraction contained sugars which were

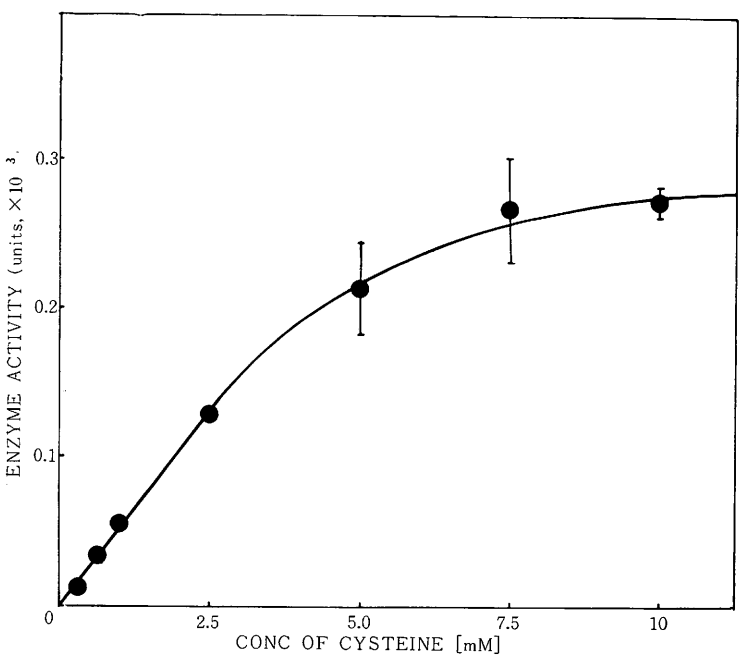

Fig. 5 The effect of cysteine concentration on Side Chain Oxidase of 4-Hydroxycinnamic Acid

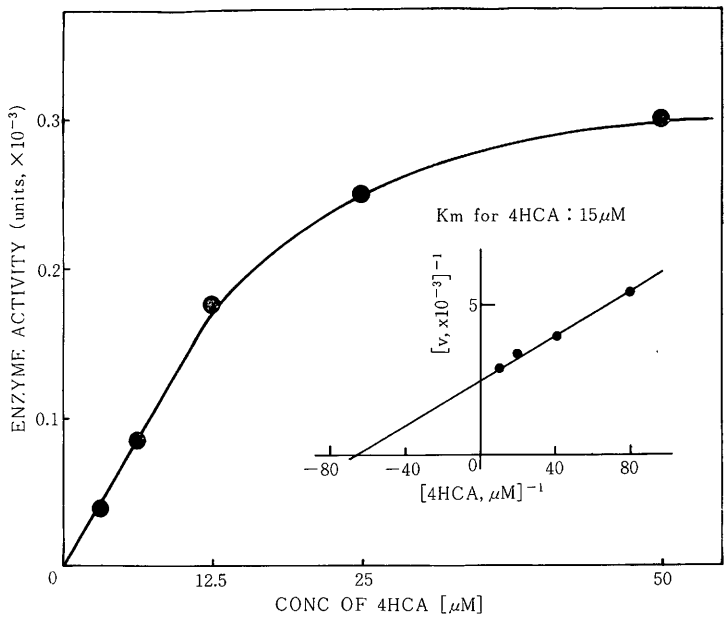

Fig. 6 The effect of 4-Hydroxycinnamic acid concentration on Side Chain Oxidase of 4-Hydroxycinnamic Acid
Km for 4HCA and the plotting by Lineweaver and Burk's equation were inserted.

determined by PASS staining. These results strongly suggest that the enzyme contains sugar moiety.

The enzyme activity was completely lost by treatment with pH 1.0 for 20 hours and decreased to 43% by treatment with pH 7.5 for 20 min compared to the control. However, the enzyme activity recovered to 97% when the enzyme was treated with pH 4.0 for 20 hours. These results suggest that an inhibitor was inactivated by treatment with pH 4.0. The enzyme activity was increased by incubation at 30°C for 60 min, indicating activation of the activity. Half life of the enzyme at 65°C was 30 min. The enzyme activity was inhibited by 0.2 mM *o*-phenanthroline, 0.2 mM dipyridyl and 0.15 mM diethyldithiocarbamate. This suggest that the enzyme contains iron and copper.

Apparent Km for cysteine was less than 5 mM. Apparent Km for 4HCA was 15 μM. These concentrations are considerable to be reasonable *in vivo*.

Side chain of 4HCA was decarboxylated by this enzyme in the presence of cysteine or SH group, and the product is no longer precusor of lignin. Therefore, the enzyme could regulate lignin synthesis and metabolism of phenylpropanoids in the presence of cysteine or SH group.

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ヤエナリ胚軸のp-クマル酸側鎖酸化酵素の高度精製

越尾淑子　鈴木和男

（昭和57年9月30日受理）

p-クマル酸側鎖酸化酸素の存在が、Suzukiにより見出され、Miyoshi, Suzukiにより部分精製されたが、今回、硫安分画（60—80%），Con A Sepharose及びSephacryl S-200カラムクロマトグラフィーによるゲルろ過法により高純度に単離精製された。SDS電気泳動法により分子量は約4万と3万のヘテロダイマーであると思われる。至適pHはpH 7.5，pH 4.0で安定であり、半減期は65℃，30分であった。本酵素は、アシドプロテアーゼのペプシンや、サーモライシン及びキレーターである*o*-フエナンスロリン，ジピリジル又はジチオカルバメイトなどにより阻害され、これらの結果から鉄と銅を含む金属酵素であることが示唆された。