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Side Chain Oxidase of 4-Hydroxycinnamic Acid of *Phaseolus mungo*.—Purification and Characterization

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Introduction

4-Hydroxycinnamic acid is a precursor of lignin and flavonoides which are the secondary metabolites in higher plants[1,2]. 4-Hydroxycinnamic acid is converted to caffeic acid by 4-hydroxycinnamic acid 3-hydroxylase [3-6] in the biosynthetic pathway toward lignin.

4-Hydroxycinnamic acid coenzyme A ligase forms an intermediate of flavonoides from 4-hydroxycinnamic acid.[7,8] The acid is also metabolized to ubiquinone and platoquinon[9] by the reaction involving the side chain modification. Concerning the side chain modification of the acid, Stafford and Baldy[10] have reported that in sorghum peroxidase catalyzes lacking the double bond of the side chain. An enzyme decarboxylating the side chain of 4-hydroxycinnamic acid was found in Aerobacter.[11] It has been also reported that the side chain of the acid is decarboxylated by β-oxidase from the preparation from rat liver[12]. During the course of studies on the enzymatic hydroxylation of cinnamic acid and 4-hydroxycinnamic acid with the microsomal fraction from *Phaseolus mungo*, it was found that 4-hydroxycinnamic acid was further converted to an unknown compound by the fraction, when incubated in the presence of dithiothreitol.[13]

In the present study, we purified the enzyme from the post-mitocondrial fraction by ammonium sulfate fractionation and a column chromatography of Sephacryl S-200, and the purified enzyme was characterized.

Materials and Methods

Beans of *P. mungo* were obtained from Fuji Jiyo Sangyo Co. Ltd. (Tokyo). The following reagents were purchased from the source listed in parentheses: L-cysteine, ammonium sulfate (Wako Co.); 4-hydroxycinnamic acid (Tokyo Kasei Co.).

Preparation of post-mitocondrial fraction (PMF) from P. mungo hypocotyl.-The beans (300 g) were soaked in 900 m*l* 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 300 g of etiolated hypocotyls were harvested from seedlings and ground with 100 m*l* of 0.15 M potassium phosphate buffer (pH 7.5) containning 1 mM EDTA and 1 mM dithiothreitol. The homogenate was filterated and centrifuged at 12,000 g for 30 min. Supernatant (PMF) was stored at −20°C until use.

Enzyme assay.-The enzyme activity was assayed by determining the formation rate of the reaction product. The standard assay mixture (3.0 m*l*) contained the PMF (24.5 mg protein), 125 μM 4-hydroxycinnamic acid, 5 mM L-cysteine and 0.15 M potassium phosphate buffer (pH 7.5). The mixture was incubated at 30°C for 10 min. Side chain 4-hydroxycinnamic acid oxidase activity was essentially determined by decreas of absorbance at 280 nm from 2 to 5 min.

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Protein.-Protein was determined by the method of Lowry et al,[14] using bovine serum albumin as the standard reference.

Results and Discussion

Assay procedure for side chain oxidase of 4-hydroxycinnamic acid. Side chain oxidase of 4-hydroxycinnamic acid could be assayed using a procedure of absorption decrease at 280 nm. The enzyme activity in the post-mitochondrial fraction increased by the concentration of cysteine or 4-hydroxycinnamic acid. The activity depended on the incubation time. These results were almost similar to those obtained by a column chromatography method using the ^{14}C-labeled substrate.[15] Hereafter, we used the absorption procedure.

Purification of side chain oxidase of 4-hydroxycinnamic acid. The enzyme was solubilized from the membrane in post-mitochondrial fraction by freezing and thawing. The post-mitochondrial fraction treated by freezing and thawing was fractionated using ammonium sulfate (60—80%). The ammonium sulfate fraction was obtained by centrifugation. The pellet was

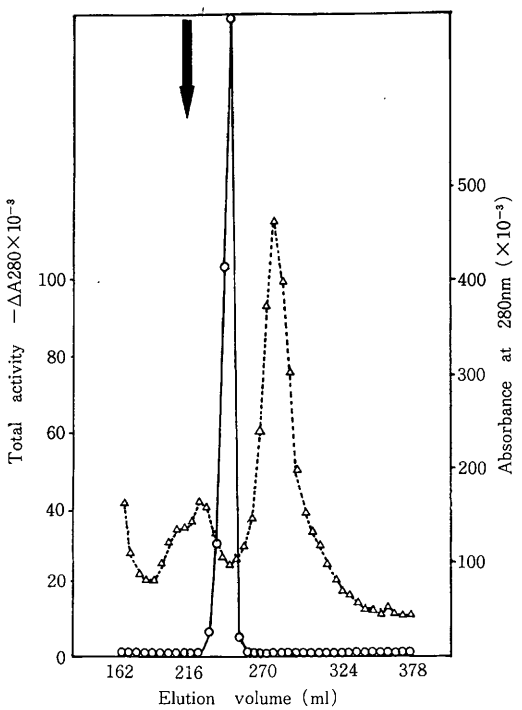

Fig. 1. A column chromatography of side chain oxidase of 4-hydroxycinnamic acid on Sephacryl S-200.
Ammonium sulfate fraction (60—80%, 56.2 mg protein) was applied to a Sephacryl S-200 column (2.5 × 86 cm) equilibrated with 0.15 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. Side chain oxidase of 4-hydroxycinnamic acid was eluted with 0.15 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. The enzyme activity was assayed by the procedure described in Materials and Methods.
——○—— the enzyme activity.
……△…… absorbance at 280 nm.
Arrow indicates the position of the peak of bovine serum albumin which was added as marker.

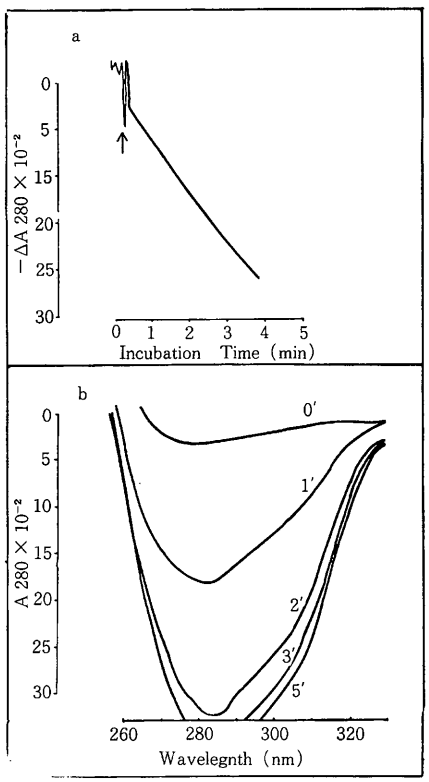

Fig. 2. Time course of the enzyme activity and change of spectrum with incubation time.
a : The enzyme activity was assayed by standard conditions with varied incubation times.
b : The eluate (1.431 g protein) from Sephacryl S-200 was incubated with 125 mM 4-hydroxycinnamic acid, 5 mM cysteine and 150 mM pottassium phosphate buffer (pH 7.5). Assay conditions were the same as described in the text.

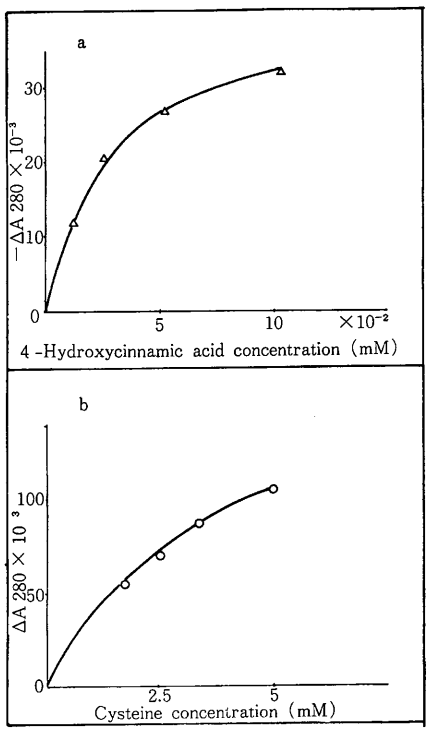

Fig. 3. Effect of 4-hydroxycinnamic acid and cysteine concentrations on the enzyme activity.
a : Effect of 4-hydroxycinnamic acid
b : Effect of cysteine

dissolved in 0.15 M potassium phosphate buffer (pH 7.5) and applied to a Sephacryl S-200 column. The enzyme activity was eluted near bovine serum albumin (Fig. 1). These results indicate that molecular weight of the enzyme is approximately 60,000.

Characterization on the enzyme activity.-Changes of spectra of thereaction mixture was observed. As shown in Fig. 2a, absorbance degrees at 280 nm had a lag phase for approximate one min. And the absorbance decreased linearly for five min thereafter. Fig. 2b, a peak spectrum at 280 nm decreased with the incubation time. The enzyme activity depended on cysteine concentration and it was plateau at 5 mM of cysteine (Fig. 3b). As shown in Fig. 3a, the enzyme was saturated by 125 mM of 4-hydroxycinnamic acid.

Concerning enzyme modification of the side chain of 4-hydroxycinnamic acid, several works have so far been reported. Stafford and Baldy[10] described that peroxidase from the soluble fraction of sorghum catalyzed oxidation of the side chain in the presence of sulfhydryl compounds such as 2-mercaptoethanol, dithiothreitol and glutathione, but the reaction was strongly inhibited by ascorbic acid. Although the double bond of the side chain of 4-hydroxycinnamic acid was reported to be eliminated in the reaction, the reaction product was not identified. Evolution of carbon dioxide was also undetectable in the reaction. A kind of decarboxylases catalyzing shortening the side chain of the acid in the absence of a reducing compound was found in *Aerobacter*.[11]

A preparation from rat liver also catalyzes shortening the side chain by β-oxidation.[12] These enzymes mentioned above apparently differ from the oxidase found in the microsomal fraction from *P. mungo* with respect to requirement for reducing substances and the mode of action. The enzyme is also different from

Table 1. Purification of Side chain Oxidase of 4-Hydroxycinnamic Acid from Post-Mitocondrial Fraction

Fraction	Total volume *ml*	Total protein mg	Total activity units	Specific activity units/mg protein	Yield %	Fold
Crude homogenate	200	1900.0	4.4	0.0023	100	1
PMF	200	1225.0	25.0	0.0200	568	9
Ammonium sulfate fraction (60—80%)	10	56.3	10.0	0.1777	227	77
Sephacryl S-200	21	1.4	7.9	5.5765	91	2425

Methods of preparation of each fraction and assay of side chain oxidase of 4-hydroxycinnamic acid were described in the text.

dopamine-β-hydroxylase which catalyzes the side chain hydroxylation of dopamine in the presence of ascorbic acid as an essential electron donor.[16]

Acknowledgements

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Summary

Side chain oxidase of 4-hydroxycinnamic acid was purified to approximately 2400-fold using ammonium sulfate fractionation and a column chromatography of Sephacryl S-200. The enzyme activity was assayed by decrease of absorbance at 280 nm. Time course, cystein dependency and 4-hydroxycinnamic acid dependency were almost similar to the previous report.

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ヤエナリ胚軸の *p*-クマル酸側鎖酸化酵素の精製とその性質

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ケイ皮酸水酸化酵素（C4H）のアッセイ法の検討の中で，ジチオスレイトールの存在によって*p*-クマル酸（4HCA）の側鎖が切断されることが，K. Suzuki により見出された．ミトコンドリア上清（PMF）を C4H と同様の方法により分画し，凍結融解により膜画分より本酵素を可溶化した。本酵素は硫安分画(60—80%)後，セファクリル S-200 のカラムクロマトグラフィーにより分子量約 60,000 の位置に単一のピークとして溶出され，約2400倍精製された。本酵素は基質として 4HCA，システインおよび分子状酸素が不可欠であった。