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Author(s)	SHIMADA, Mikio; HIGUCHI, Takayoshi
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Some Properties of *O*-Methyltransferase from Bamboo Shoot

Mikio SHIMADA* and Takayoshi HIGUCHI*

Abstract——The incubation of CA, 5-HFA and TCA with sliced bamboo shoot in the presence of methionine-$^{14}CH_3$ gave radioactive FA and SA. On the other hand, the incubation of those compounds with sliced gingko shoot yielded only FA from CA and did not SA from 5-HFA and TCA. The enzyme preparation obtained from bamboo shoot was fractionated by $(NH_4)_2SO_4$- and iso-electric point precipitations and column chromatographies on DEAE-cellulose. Variation of the ratios (SA/FA) which were obtained by assay of *O*-methyltransferase are indicative of possible presence of two enzymes in the bamboo enzyme preparation. The results are discussed in relation to the biochemical differences of the methoxyl patterns in lignins between angiosperms and gymnosperms.

Introduction

In plant kingdom occur a number of methoxylated phenolic compounds such as lignins, lignans[1], flavonoids[2], and coumarins[3], which are sometimes characteristic constituents of higher plants. However, it is not clear how *O*-methyltransferase participates in formation of the methoxyl groups of those plant phenolics. As for lignins, it is well-known that angiosperm lignins consist of guaiacyl-, syringyl- and *p*-hydroxyphenyl propane units, whereas gymnosperm lignins largely contain guaiacyl units[4~6]. Although formation of the syringyl units is assumed to correlate to metabolic differences in lignification between angiosperms and gymnosperms, the mechanism of the syringyl unit formation has not yet clearly been elucidated. HIGUCHI and BROWN[7] showed that labeled ferulic acid (FA) and 5-hydroxyferulic acid (5-HFA) were efficiently incorporated into the syringyl units of wheat lignin, indicating that sinapic acid (SA) was formed from FA after hydroxylation at 5-position of FA and subsequent methylation of 5-HFA. Hess demonstrated the transformation of FA-$O^{14}CH_3$ into SA-$O^{14}CH_3$ in seedlings of red cabbage[8]. However, evidence for the occurrence of 5-HFA and "FA-5-hydroxylase" in plants has not yet been obtained.

In the previous paper[9] it was reported that both 3, 4, 5-trihydroxycinnamic acid (TCA) and 5-HFA were transformed to SA in the presence of S-adenosylmethionine by both bamboo and poplar *O*-methyltransferases. On the other hand, neither of them was utilized for formation of SA in the sliced shoot of *Gingko biloba* which

* Division of Lignin Chemistry.

is one of gymnosperms. In relation to biosynthesis of lignins in various higher plants, it is of interest to elucidate biochemical differences of methoxylation patterns between gymnosperm and angiosperm lignins. Present paper describes differences of substrate specificity of bamboo and gingko *O*-methyltransferases and some properties of the enzyme from bamboo shoot concerning a possibility that bamboo *O*-methyltransferase may consist of at least, two methylating enzymes.

Experimental

Samples and Preparation of Labeled Compounds

Young shoots of a maidenhair tree (*Gingko biloba*) and bamboo shoots (*Phyllostachys reticulata*), about 1.5 m tall were sampled as plant materials during early summer. These plants were growing near the Institute in the suburbs of Kyoto City.

Methionine-$^{14}CH_3$ (9.3 mCi/mM) and malonic acid-2-^{14}C (9.7 mCi/mM) were procured from Japan Radioisotope Society. Caffeic acid-2-^{14}C (266 μCi/mM) and 5-hydroxyferulic acid-2-^{14}C (90 μCi/mM) were prepared from the corresponding benzaldehydes and malonic acid-2-^{14}C according to the method of NEISH[10].

Radiochromatography of Radioactive Compounds

Bamboo shoot (5 g) or gingko shoot (5 g) was cut into small pieces (about $2\times 2\times 3$ mm) with a razor. The sliced tissue was put into a beaker, to which 2 ml of water was added which contained 1 mg of non-labeled CA, 5-HFA, or TCA with methionine-$^{14}CH_3$ (5 μCi). The reaction mixtures were incubated for 90 min. at room temperature. After adding non-labeled FA and SA (0.2 mg each) as carriers, the incubation was stopped by the addition of 50 ml of hot ethanol into the beaker. The tissue in the beaker was homogenized and the homogenate was filtered through celite filter. Then, the filtrate was evaporated to dryness *in vacuo* at 35°C. The residue was dissolved in 2 ml of 5 % $NaHCO_3$ and the solution was washed thoroughly with ether. After acidification of this solution by the addition of 5 % HCl, the acid fraction was extracted three times with 5 ml portion of ether. The ether was evaporated to dryness *in vacuo*. The residue was dissolved in a small amount of ethanol and submitted to descending paper chromatography with a solvent system of toluene-acetic acid-water (4 : 1 : 5, upper layer). FA and SA on the chromatogram were located in u.v. light. After cutting the strip at 1 cm intervals, radioactivity contained in each piece was determined by a usual paper strip method with a Beckman liquid scintillation counter (LS-100).

Extraction of O-Methyltransferase from Bamboo Shoot

In order to protect the enzyme against inactivation the extraction procedures

described in the previous paper[11] were more or less modified by addition of 2 mM of NaN_3, EDTA, cysteine and iso-ascorbic acid, and 0.1 % of bovine serum albumine into 0.1M phosphate buffer solution (pH 7.5), with which plant tissue was homogenized.

Bamboo shoots (1.4 kg) were homogenized with an Ultraturax homogenizer in the above-mentioned buffer solution at 4°C. The homogenate was filtered through gauze and the filtrate was centrifuged at 8,000 g for 30 min. at 4°C. The precipitates were discarded and the supernatant solution containing the enzyme was fractionated into three fractions by salting out with $(NH_4)_2SO_4$ as follows. Solid $(NH_4)_2SO_4$ was added to the supernatant solution to 0.4 saturation. After 10 min. of stirring, the resulting precipitate was collected by centrifugation at 10,000 g (Fraction I). To the supernatant solution obtained after the first fractionation, $(NH_4)_2SO_4$ was again added to 0.65 saturation. The resulting precipitate was obtained by centrifugation (Fraction II). Similarly, Fraction III was obtained by collection of the precipitate resulting after addition of $(NH_4)_2SO_4$ to 0.80 saturation. These three enzyme fractions were stored at −20°C. A portion of each fraction was taken out and used for further experiments.

Assay of O-Methyltransferase Activity

The assay conditions described in the previous paper were modified by the addition of NaN_3 and iso-ascorbic acid into the reaction mixture and in this case labeled CA and 5-HFA were used as substrates.

The enzymatic reaction mixtures usually contained the following components: 0.1 ml of 1 M Tris-buffer (pH 8.0), 0.1 M $MgCl_2$, 0.1 M NaN_3, 0.1 M iso-ascorbic acid, S-adenosylmethionine (0.25 μmole), 0.2 ml of CA-2-^{14}C (0.5 μmole) or 5-HFA-2-^{14}C (0.5 μmole) and 1.0 ml of the enzyme solution. The reaction mixtures were incubated in duplicate for 30 min. at 30°C; one test tube contained CA-2-^{14}C and the other, 5-HFA-2-^{14}C as a substrate. After the reaction was stopped by the adition of 0.5 ml of 10 % HCl, 0.2 mg of non-labeled FA or SA was added as carriers into the respective test tubes. Then, a pair of reaction mixtures were combined into one. The combined mixtures were extracted three times with 5 ml portion of ether. The ether was evaporated and the residue was submitted to paper chromatography as described above. Two spots on the chromatogram containing FA and SA were separately cut into pieces and transferred into vial tubes, to each of which 1 ml of dioxane was added in order to elute the compounds. After 30 min. of incubation at room temperature, 10 ml of toluene scintillator solution containing 40 mg of PPO and 0.2 mg of POPOP was added and then the radioactivities of FA and SA were determined with a Beckman scintillation counter. The amounts of enzymatically formed FA and SA were calculated from the ratio-

activities determined.

Iso-Electric Point Fractionation of the Enzyme Preparation

The enzyme preparation (Fraction II) which was passed through Sephadex G-25 column (2.8×40 cm) was acidified at 4°C with N acetic acid to pH 5.5. The resulting precipitate was collected by centrifiugation at 10,000 g for 20 min. (Enzyme I) and from the supernatant solution Enzymes II and III were obtained similarly by precipitation at pHs 5.0 and 4.5, respectively. These three enzyme preparations were used for assay of *O*-methyltransferase activity after dissolving each of them in 5.0 ml of 0.2 M Tris-buffer (pH 8.0).

Stepwise and Gradient Elution Column Chromatographies on DEAE-Cellulose

After passing a portion of Fraction II through Sephadex G-25, the eluate (50 ml) containing 570 mg protein was applied onto the top of DEAE-cellulose column (1.6×13 cm). Unadsorbed proteins were washed down with 0.02 M phosphate buffer solution (pH 7.4) containing 1 mM of cysteine. The adsorbed proteins were then eluted stepwisely with the same buffer solutions containing 0.1 M, 0.2 M, 0.3 M and 0.6 M KCl. The washings and the eluate were collected in 5 ml fraction with a fraction collector following the absorbances at 280 nm with a UV-Auto (Ohtake). Several fractions with greater absorbances were chosen and dialyzed against 0.02 M phosphate buffer solution (pH 7.4) containing 1 mM of cysteine for 5 hrs. at 0°C. Then, *O*-methyltransferase activities of the fractions were assayed as described above.

Alternatively, Fraction II (450 mg protein) was applied on DEAE-cellulose column (1.6×15). The adsorbed proteins were eluted by a gradient elution method. A mixing chamber and a reservoir contained 0.02 M phosphate buffer (200 ml) and the same buffer with 0.5 M KCl (200 ml), respectively. The eluate was collected and *O*-methyltransferase was assayed in the same way as described above.

Results and Discussion

Radiochromatography of the Radioactive Compounds

Fig. 1 shows the radiochromatographical patterns of FA-$O^{14}CH_3$ and SA-$O^{14}CH_3$ which were formed after incubation of the corresponding substrates with sliced bamboo or gingko shoots in the presence of methionine-$^{14}CH_3$. Since it was confirmed that the methyl group of methionine was transferred to the hydroxyl groups CA and 5-HFA via S-adenosylmethionine[11], the radioactivities of both FA and SA formed were believed to be exclusively derived from the labeled methyl group of the methionine. The sliced tissue of bamboo shoot was found to transfer the methyl group to CA and 5-HFA administered and the areas of the two peaks for

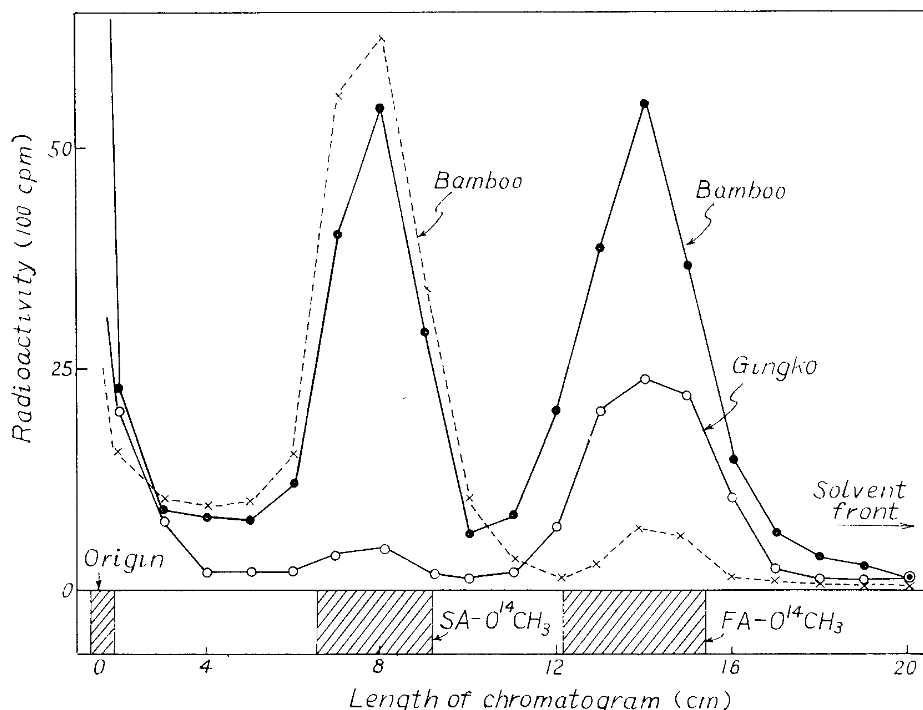

Fig. 1. Radiochromatgraphic patterns of hydroxycinnamic acids methylated by sliced tissue of bamboo shoot and young shoot of *Gingko biloba* in the presence of methionine-$^{14}CH_3$. —●—, A mixture of CA and 5-HFA was incubated with bamboo tissue. ···×···, TCA was incubated with the tissue. —○—, A mixture of CA and 5-HFA was incubated with gingko tissue.

radioactivities of FA and SA on the chromatogram showed that both acids were formed in nearly equal amounts during the incubation. On the other hand, gingko shoot transferred the methy group mostly to CA but to 5-HFA in a neglible amount. It was also found that TCA was transformed to SA only in bamboo shoot. These results are quite in accordance with those previously obtained with CA-2-^{14}C and 5-HFA-2-^{14}C[9]. When bamboo tissue was incubated with labeled methionine alone, no radioactive SA but only a small amount of FA was formed. This result shows that pool size of a substrate for SA was much smaller than that of a substrate for FA.

Thus, the radiochromatographical data show that *O*-methyltransferase of bamboo shoot clearly differs from that of gingko shoot in that the former can catalyze the methylation of CA, 5-HFA and TCA, whereas the latter can methylate only CA.

Stabilization of Bamboo O-Methyltransferase and Protection of Some Hydroxycinnamic Acids against Oxidative Decomposition

In order to find a clue for the question whether or not bamboo *O*-methyltransferase consists of more than two enzymes, it must be purified. However, successful

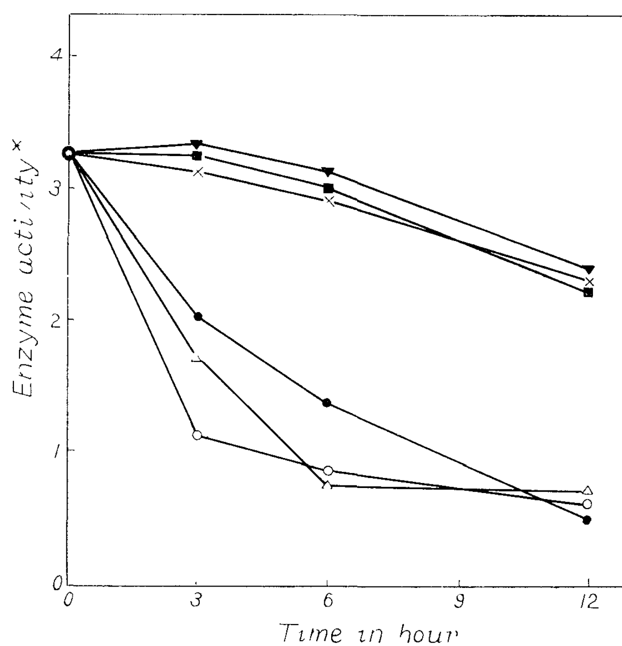

Fig. 2. Stability of enzyme at 15°C.

▼—▼ Enzyme+Cysteine ■—■ Enzyme+Clerond's reagent
×—× Enzyme+Mercapto-ethanol ●—● Enzyme+Na-Ascorbate
△—△ Enzyme+Caffeic acid ○—○ Enzyme+Glycine

* Enzyme activity is expressed as the amount of ferulic acid formed (10 mμ mole).

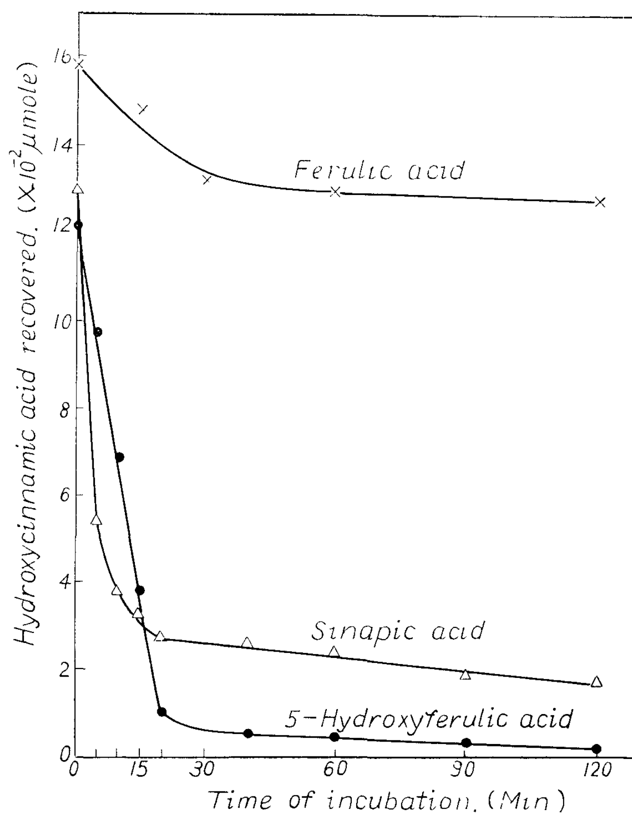

Fig. 3. Stability of hydroxycinnamic acids.

purification was not achieved because the enzyme was quite unstable and most enzyme activity was lost during the purification. Besides, more than 70 % of the enzyme was inactivated on $(NH_4)_2SO_4$ precipitation. However, it was found that the loss of the enzyme activity was effectively reduced by addition into the enzyme solution of SH-reagents such as cysteine, mercaptoethanol and dithiothreitol (Fig. 2). EDTA was also effective to prevent from the inactivation of the enzyme on $(NH_4)_2SO_4$ precipitation.

As shown in Fig. 3, 5-HFA and SA were very labile compounds on incubation with enzyme solutions, whereas FA was relatively stable. After the incubation of the hydroxycinnamic acids with the enzyme solutions, FA was recovered in the nearly original amount. On the other hand, more than 80 % of SA and 5-HFA disappeared probably because they were oxydatively decomposed by action of phenoloxidases coexisting in the crude enzyme preparation. Such oxidative decomposition was considerably prevented with NaN_3 and iso-ascorbic acid added into the reaction mixture.

Changes in Ratio of the Methylated Products after Several Treatment of Fraction II

1) *$(NH_4)_2SO_4$ Fraction*—When there is an enzyme apprenation acting upon two substrates yielding two respective products, it is useful to check the presence of isozymes in terms of varying ratios of the two products as reported on phenylalanine ammonia-lyase[12] which consists of two components[13,14]. Table 1 shows the changes in the ratio (SA/FA) after $(NH_4)_2SO_4$ fractionations. Before the fractionation the ratio was 1.09. However, the ratio varied as the original enzyme preparation was fractionated into three preparations. Fraction I gave 1.60 in average, which is a considerably high value compared with the original one. Fractions II and III gave 1.0 and 1.11, respectively which are almost equal to the

Table 1. Changes in ratio of the methylated products after ammonium sulfate fractionation of the enzyme.

Enzyme	Incubation time in Min	Products (mμmole)		Ratio (SA/FA)	mg protein
		FA	SA		
Buffer Ex.	60	55.0	60.0	1.09	62.5
Fraction I	0	0	0	—	4.0
	20	2.1	3.2	1.52	〃
	45	4.9	8.3	1.70	〃
	60	6.7	10.7	1.60	〃
Fraction II	30	60.0	59.4	0.99	14.3
	60	65.6	67.0	1.10	〃
	90	62.0	56.4	0.91	〃
Fraction III	60	4.5	5.0	1.11	9.0

original value. The changes in the ratio described above indicate that *O*-methyltransferase activities yielding FA and SA were contained in the equal amounts in both Fractions II and III and that Fraction I contained more or less a larger amount of the SA yielding activity.

Table 2. Changes in ratio of the methylated products after iso-electric point precipitation of the enzyme.

Enzyme preparation*	Products (mμmole)		Ratio (SA/FA)
	FA	SA	
I	47.7	46.3	0.97
II	10.2	13.0	1.27
III	10.0	10.0	1.00
IV	6.4	5.1	0.80

* I, Enzyme preparation precipitated between 0.40 and 0.65 saturation with ammonium sulphate. The enzyme II, III and IV are fractions obtained by precipitation of I at pH 5.5, 5.0 and 4.5, respectively.

2) *Iso-Electric Point Precipitation*—Table 2 shows the changes in the ratio after iso-electric point precipitation of the enzyme. The value of SA/FA is found to decrease in the order of Enzymes II, III and IV, which suggests that the ratio of enzyme activities for SA and FA varied with this fractionation. However, the

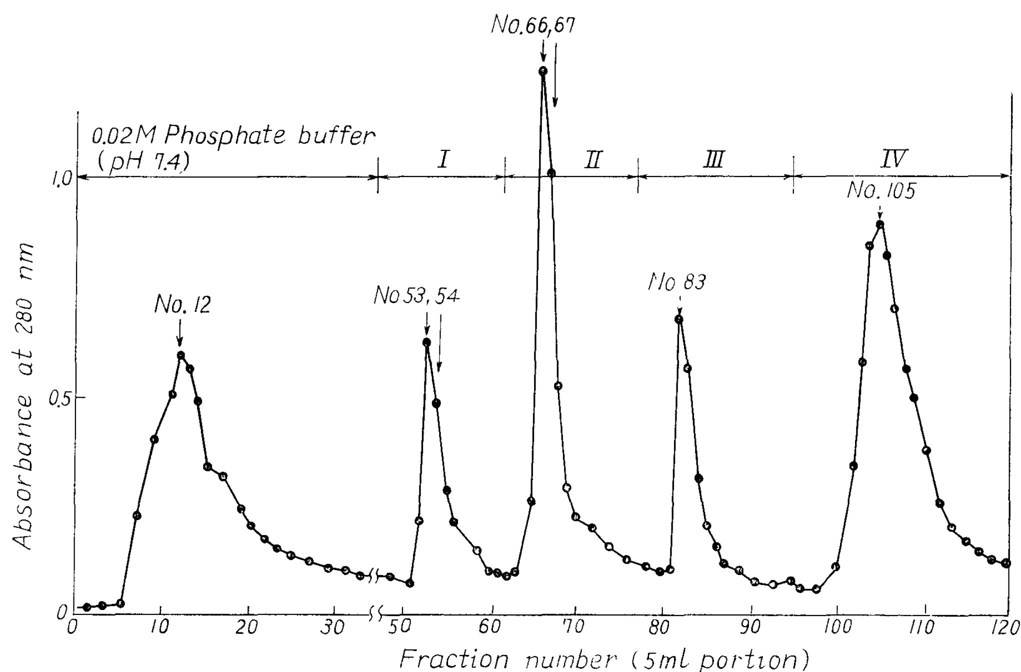

Fig. 4. Stepwise elution of bamboo *O*-methyltransferase on DEAE-cellulose colume. I, II, III and IV are 0.02M phosphate buffer solutions containing 0.1M, 0.2M, 0.3M and 0.6M KCl, respectively. Numbers shown with arrows mean fractions employed for the enzyme assay.

changes were not so great as those observed on $(NH_4)_2SO_4$ fractionation.

3) *Stepwise and Gradient Elutions on DEAE-Cellulose*—Fig. 4 shows the stepwise elution pattern of Fraction II on DEAE-cellulose. The fractions marked with arrows were employed for the assay of *O*-methyltransferase activities. The amounts of the products and the ratios obtained are given in Table 3. The fact that no enzyme activity was observed from fraction No. 12 means that no methyltransferase was present in the first eluate from the ion exchanger column. Fractions No. 53 and 54 gave rather low values, 0.84 in average and the ratios obtained with fractions No. 66 and 67 were nearly equal to the original ratio (1.09).

Table 3. Changes in ratio of the methylated products after the stepwise elution through DEAE-cellulose.

Fraction No.	Products (mμmole)		Ratio (SA/FA)
	FA	SA	
12	0	0	—
53	14.8	13.4	0.91
54	25.2	19.5	0.77
66	41.0	43.0	1.05
67	44.0	44.0	1.00
83	24.8	30.3	1.22
105	3.0	2.9	0.97

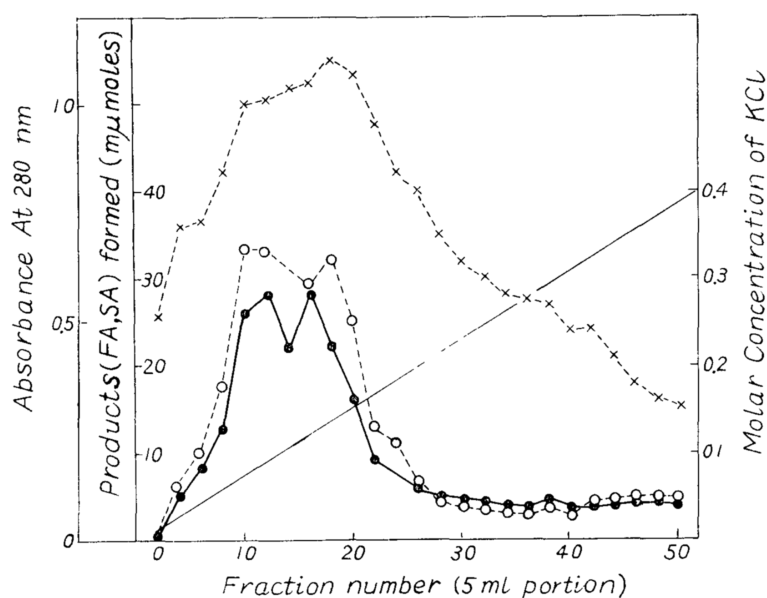

Fig. 5. Chromatographic pattern of *O*-methyltransferase on DEAE-cellulose colume. —●—, FA formed by the enzyme. —○—, SA formed. —×—, Absorbance of the eluate at 280 nm. The elution procedure and the assay method of the *O*-methyltransferase are described in the text.

On the other hand, fraction No. 83 showed rather high value of 1.22. These results indicate that the values of SA/FA varied with a slight increase, as the enzyme proteins were eluted with the buffer containing greater ionic strength.

Fig. 5 shows a linear gradient elution pattern of the enzyme preparation. All fractions collected were employed for the assay of the enzyme activities. The amounts of FA and SA formed were plotted against the numbers of fractions. The purpose of the gradient elution was to make it clear whether more than two peaks would be observed in the curves of the activities. If the two peaks are found, there is a possibility that two isozymes are present in the original enzyme preparation. The fact that the activity curves gave two peaks between fractions No. 10 and 20 suggests the presence of isozymes in bamboo *O*-methyltransferase in connection with the varying ratios (SA/FA). However, the peaks shown in Fig. 5 are very adjacent each other, so more separated and clearer chromatographic peaks must be obtained. Further experiments are being carried out to establish the presence of isozymes.

In conclusion, one of the reasons for the different methylation of the hydroxycinnamic acids by bamboo and gingko *O*-methyltransferases can be explained in terms of differences in substrate specificity between the two heteroenzymes. Therefore, *O*-methyltransferase involved in biosynthesis of lignin precursors such as FA and SA is considered as one of the key enzymes which regulate formation of the guaiacyl- and syringyl units of lignins in higher plants. However, more conclusive data are needed to declare that bamboo *O*-methyltransferase consists of two components.

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