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*Note*

# Ellagic Acid Formation from Galloylglucoses by a Crude Enzyme of *Cornus capitata* Adventitious Roots

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**The aqueous extract of acetone powder, which had been prepared from *Cornus capitata* 'Mountain Moon' adventitious roots, cultured in MS medium with a high concentration of $Cu^{2+}$ (10 $\mu$M), showed strong oxidative activity toward galloylglucoses. A compound formed from galloyglucoses, such as 1,2,3,4,6-penta-*O*-galloyl-$\beta$-D-glucose and tannic acid, by the reaction with the crude enzyme solution of the adventitious roots was isolated and characterized as ellagic acid by spectrometric analyses.**

**Key words:** *Cornus capitata*; adventitious root culture; tannic acid; 1,2,3,4,6-penta-*O*-galloyl-$\beta$-D-glucose; ellagic acid

*Cornus capitata* 'Mountain Moon' (Cornaceae), having four bright yellow bracts, is popular as a horticultural plant in Japan. To clarify the tannin metabolism of *C. capitata* adventitious roots, we have been investigating on the effects of macro- and microelements in the culture medium on the production of galloylglucoses, such as 1,2,3,6-tetra-*O*-galloyl-$\beta$-D-glucose and 1,2,3,4,6-penta-*O*-galloyl-$\beta$-D-glucose (PGG), in adventitious root cultures of *C. capitata*.[1,2] The production of galloylglucoses in the adventitious roots was regulated by the concentration of $Cu^{2+}$ in the culture medium. A high concentration of $Cu^{2+}$ (more than 1 $\mu$M) in the medium markedly inhibited the production of galloylglucoses.[2] The concentration of $Cu^{2+}$ in a culture medium has been reported to affect the activity of Cu enzymes such as polyphenoloxidases.[3] When PGG was mixed with acetone powder that had been prepared from adventitious roots cultured in the medium with a high concentration of $Cu^{2+}$, the amount of PGG decreased with time.[2] We have proposed that the acetone powder prepared from adventitious roots cultured in a medium with a high concentration of $Cu^{2+}$ might have high phenoloxidase activity which would cause the oxidative degradation of PGG in *C. capitata* adventitious roots. In this present study, we succeeded in determination of the oxidative derivative of PGG that had been treated with acetone powder prepared from *C. capitata* adventitious roots cultured in a medium supplemented with a high concentration of $Cu^{2+}$.

The adventitious root culture of *C. capitata* was established in Murashige-Skoog[4] (MS) liquid medium as described in the previous report.[2] The roots (*ca.* 0.8 g fresh weight) were transferred into MS liquid medium supplemented with different concentration of cupric sulfate (0.1 $\mu$M or 10 $\mu$M) containing 30 g/l maltose and 3.0 mg/l 3-indoleacetic acid and were cultured at 25°C for 6 weeks in the dark. The adventitious roots were subcultured for more than a year at 8-week intervals in the dark. To prepare the crude enzyme, adventitious roots cultured in MS medium containing 0.1 $\mu$M or 10 $\mu$M $CuSO_4$ for 4 weeks were washed twice with 100 ml of distilled water, and then homogenized with 150 ml of cold (−20°C) acetone for 3 min. The resulting slurry was filtered through a funnel under reduced pressure. The residue was washed twice with 50 ml of cold (4°C) acetone, dried at 4°C and stored at −20°C as acetone powder until needed. Two hundred milligrams of the acetone powder and 20 ml of water were mixed and shaken for 3 min, then filtrated by a filter paper. The filtrate prepared from the acetone powder was used as the crude enzyme solution. Ten microliter of this crude enzyme solution from the acetone powder, and 50 $\mu$l of PGG solution (1.0 mg/ml) were mixed with 440 $\mu$l of distilled water in a 1.5-ml Eppendorf tube. As a negative control, PGG was incubated with a crude enzyme solution that had been denatured by heating at 100°C for 10 min. The mixture was incubated at 37°C in the dark for 0.5, 1, 2, 3 or 24 hours, and after passing through a Millipore filter (0.45 $\mu$m), 5 $\mu$l of the filtrate was subjected to HPLC under the following conditions: column, TSK-gel ODS-120A (4.6 mm × 250 mm); mobile phase, 1 mM tetra-

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butylammonium chloride (TBA, adjusted to pH 2.9 with HOAc)-$CH_3CN$ (9:1→1:4, in 30 min); flow rate, 0.7 ml/min; column temperature, 40°C; detection, 254 nm (UV); Rt (min), PGG (20.1). When PGG was incubated with the aqueous extract from acetone powder of the adventitious roots cultured in MS liquid medium with 10 $\mu$M $Cu^{2+}$, it completely disappeared after three hours of incubation (Fig. 1). On the other hand, in the aqueous extract of acetone powder from the adventitious roots which had accumulated gallotannins, *i.e.* those cultured in MS medium containing 0.1 $\mu$M $Cu^{2+}$, the level of PGG decreased to 3/4 of its initial level after 24 hours of the reaction (Fig. 1). When incubated with the crude enzyme solution denatured by heating, the level of PGG hardly decreased during incubation. These results indicated that the decrease in the amount of PGG was caused by the enzyme contained in the acetone powder of *C. capitata* adventitious roots, and that the enzyme activity depended on the concentration of copper ions in the liquid medium. Fig. 2 shows HPLC profiles for the reaction mixture of PGG and the aqueous extract of acetone powder prepared from the adventitious roots cultured in the medium containing 10 $\mu$M $Cu^{2+}$. The crude enzyme solution of acetone powder prepared from adventitious roots cultured in MS medium with 0.1 $\mu$M or 10 $\mu$M $Cu^{2+}$ did not contain any detectable level of polyphenols before and after 3 hours of incubation (Fig. 2b). The negative control, after 3 hours of incubation, contained PGG that had not been converted into any other compound (Fig. 2a). On the other hand, when incubated with the native crude enzyme, a new peak marked by the arrow (Rt 18.9 min) was detected with a decrease in the PGG peak (Figs. 2d and 2e). It is presumed that this peak was a converted product from PGG.

To isolate the converted compound, a larger volume of the reaction mixture was prepared by mixing 25 ml of the aqueous extract prepared from acetone powder of the adventitious roots, which had been cultured in MS liquid medium with 10 $\mu$M $Cu^{2+}$, and 100 ml of the PGG solution (50 mg/100 ml). After 24 hours of incubation at 37°C, the reaction mixture was concentrated *in vacuo* at 40°C. The resulting precipitate was found by TLC and HPLC analyses to be the product formed from PGG. The precipitate was dissolved in 3 ml of DMSO and then purified on CHP 20P MCI-gel with $H_2O$–MeOH (1:0–0:1) to afford the formed compound (33.2 mg, off-yellow amorphous powder). The structure of the isolated compound from the reaction solution was elucidated by measuring the $^1$H-NMR and $^{13}$C-NMR

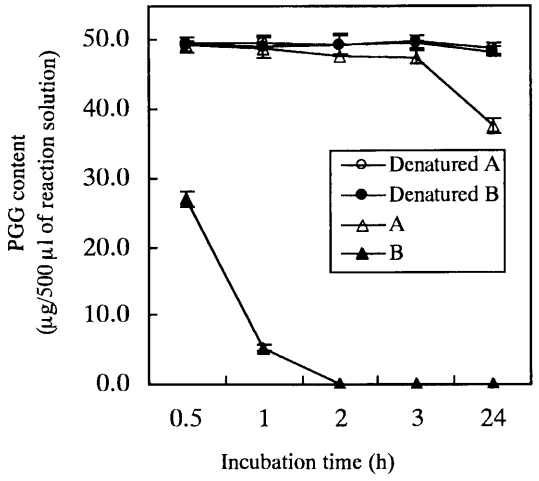


**Fig. 1.** Degradation of PGG by a Crude Enzyme Solution of Acetone Powder Prepared from *C. capitata* Adventitious Roots Cultured in MS Liquid Medium with 0.1 $\mu$M or 10 $\mu$M $CuSO_4$.

Denatured A and B: the denatured crude enzyme from adventitious roots respectively cultured in MS liquid medium with 0.1 $\mu$M and 10 $\mu$M $CuSO_4$ by heating at 100°C for 10 min; A: crude enzyme from adventitious roots cultured in MS liquid medium with 0.1 $\mu$M $CuSO_4$; B: crude enzyme from adventitious roots cultured in MS liquid medium with 10 $\mu$M $CuSO_4$. Bars indicate the standard error of the mean of three replicates.

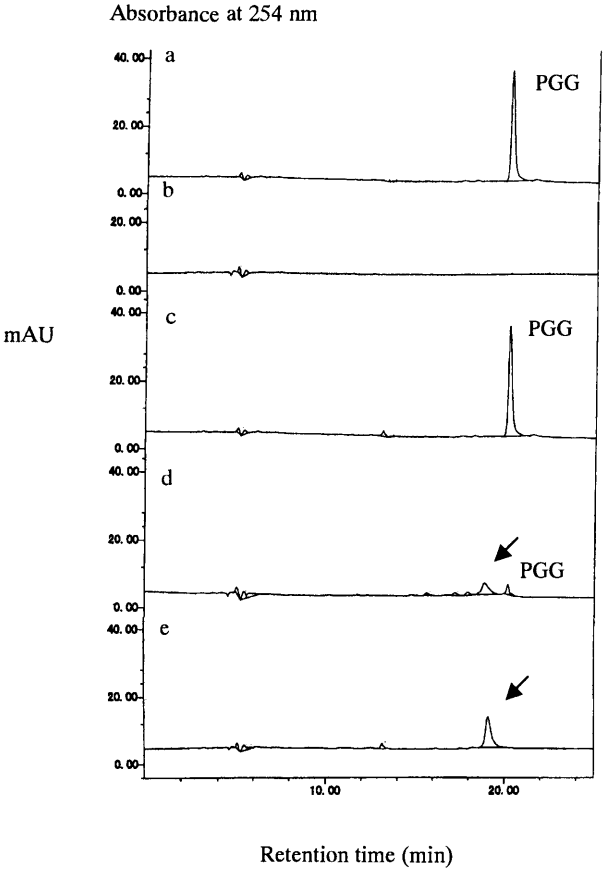


**Fig. 2.** HPLC Profiles of the Incubation of PGG and an Aqueous Extract of Acetone Powder Prepared from *C. capitata* Adventitious Roots Cultured in MS Liquid Medium with 10 $\mu$M $CuSO_4$.

a: after 3 h of incubating PGG and the crude enzyme denatured by heating; b: after 3 h of incubating the crude enzyme without PGG; c: after 0.2 h; d: 0.5 h; e: 3 h of incubation. The arrow shows the peak of a compound formed from PGG.

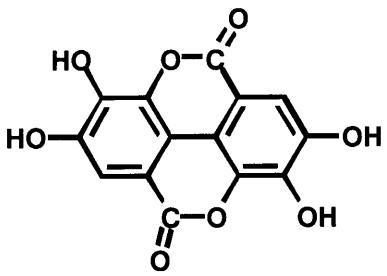


**Fig. 3.** Structure of Ellagic Acid.

spectra in DMSO-$d_6$ (TMS as internal standard) with a JNM-GSX spectrometer (JEOL, Japan). $^1$H-NMR, $\delta$ 7.24 (2H, *s*, H-5, H-5′) and $^{13}$C-NMR data, $\delta$ 107.4 (C-3, C-3′), 110.1 (C-2, C-2′), 112.3 (C-1, C-1′), 136.3 (C-5, C-5′), 139.8 (C-6, C-6′), 148.1 (C-4, C-4′), 159.1 (-COO-), of the compound were identical with those of ellagic acid (4,4′,5,5′,6,6′-hexahydroxydiphenolic acid 2,6,2′,6′-dilactone, Fig. 3) purchased from Sigma Chemical Company.

We also investigated whether ellagic acid had been formed from tannic acid (Katayama Chemical, Japan), *i.e.* crude gallotannin, by incubating with a crude enzyme solution from *C. capitata* adventitious roots. Twenty milliliter of a crude enzyme solution from acetone powder of *C. capitata* adventitious roots and 80 ml of a tannic acid aqueous solution (50 mg/80 ml) were mixed. Protein was determined by the method of Bradford,[5] using the Bio-Rad protein reagent (Bio-Rad Laboratories) with bovine serum albumin as the protein standard. After 24 hours of incubation at 37°C, the reaction mixture was passed through a filter paper, and the resulting filtrate was subjected to HPLC. In the reaction mixture from acetone powder of the adventitious roots in MS medium containing 10 $\mu$M $Cu^{2+}$, ellagic acid was formed at a higher level (0.029 mg/mg protein/h) than that in MS medium with 0.1 $\mu$M $Cu^{2+}$ (0.002 mg/mg protein/h).

On the other hand, when gallic acid as a substrate was incubated with the crude enzyme solution, ellagic acid was not formed (data not shown). Therefore, the foregoing results suggest that the biphenyl group of ellagic acid was formed by oxidative C-C coupling of the phenolic galloyl groups, and that the enzyme prepared from the *Cornus* adventitious roots did not directly react with gallic acid.

Quideau and Feldman have described ellagic acid being formed by lactonization *via* hydrolysis/esterification or transesterification of hexahydroxydiphenol (HHDP) unit of ellagitannin.[6] To prepare ellagic acid by the acidic hydrolysis of ellagitannins, the reaction must generally be performed at a high temperature with the addition of mineral acids such as HCl and $H_2SO_4$. The results of our investigation suggest that ellagic acid could easily be prepared from PGG or tannic acid without acid hydrolysis of ellagitannins by using acetone powder of *C. capitata* adventitious roots that had been cultured in the medium with a high concentration of $Cu^{2+}$.

Ellagic acid formation by the oxidative coupling of galloyl ester groups has also been reported in an experiment using horseradish peroxidase-$H_2O_2$.[7] In our investigation, the oxidative reactions we have described were conducted without additional $H_2O_2$. Zhentian *et al.* have recently reported that a high copper concentration in the culture medium increased the production of two C-glycosidic ellagitannins (castalagin and vescalagin) in callus cultures of white oak (*Quercus alba*).[8] They suggested that polyphenoloxidase (*e.g.* laccase) might have been involved in the biosynthesis of a galloyl biaryl linkage in white oak calli. The acetone powder prepared from *C. capitata* adventitious roots cultured in MS liquid medium with 10 $\mu$M $Cu^{2+}$, might also have contained polyphenoloxidase such as laccase. Purification and characterization of the polyphenol oxidases in *C. capitata* adventitious roots are currently in progress.

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