Research Articles

Inhibitor of the oxidation of catechin released from the roots of corn seedlings

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Summary. When the specific activities of the catechin oxidases (catechin as the substrate) which were released from the roots of the seedlings of alfalfa, tomato, wheat, lettuce and corn were compared, it was found that the oxidizing activity was absent from the root exudate of corn seedlings. A 6.3 kDa protein was purified from the root exudate of corn seedlings and in the presence of this protein, the oxidation of catechin was inhibited. This inhibitor is responsible for the inability of the root exudate of corn seedlings to oxidize catechin.
Key words. Zea mays; inhibitor of the oxidation of catechin; root release.

The roots of lettuce *(Lactuca sativa)* seedlings have been shown to release oxidases that were able to catalyze the oxidation of catechin[1], converting the substrate, catechin (maximum absorbance 270 nm), to a yellow-red oxidized product that absorbed maximally at 428 nm. The roots of the seedlings of alfalfa *(Medicago sativa)*, tomato *(Lycopersicon esculentum)* and wheat *(Triticum aestivum)* were also found to release catechin oxidases that catalyzed the oxidation of catechin[1]. However, when the root exudate of corn *(Zea mays)* seedlings was incubated with a freshly prepared aqueous solution of catechin, the catalysis of the oxidation of this flavonoid was not observed.

During our attempt to explain the apparent lack of catechin oxidases in the root exudates of corn seedlings, we found an inhibitor of the oxidation of catechin that was released from the roots of the seedlings of this monocotyledon. This inhibitor is described in this paper.

Materials and methods

Seeds: Corn, alfalfa and wheat seeds were purchased from the Carolina Biologicals Company (Oregon, USA). Lettuce and tomato seeds were from the Ferry Morse Seed Company (California, USA).

Catechin: Catechin was previously isolated from *Podocarpus nagi* which was collected from Nara, Japan[2].

Assay for catechin oxidase activity: Catechin oxidase activity was assayed by determining the amount of catechin oxidized at 30 °C, pH 8.2 in 10 min. The absorbance at 428 nm was monitored with a Hitachi 100–800 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that oxidizes 1 μmole of catechin per minute under these conditions.

Collection of root exudate: Corn seeds (200 g) were germinated on a tray for 60 h at 25 °C under continuous light (3000 lux). At the end of the incubation period, 100 ml of Tris buffer 20 mM, pH 7.3 were introduced into the tray and the mixture was shaken on a clinical rotatory shaker for 2 h. With a Pasteur pipette, the buffer containing the exudate was transferred to a storage flask.

Protein determination: The standard Bradford[3] method was employed; O.D. was monitored at 595 nm.

Protein purification: Gradient elution of the protein in the root exudate using anion exchange chromatography (DEAE Sepharose column). Column: Pharmacia SR 10/50. Bed height: 25 cm. Eluent: Buffer A was 20 mM Tris buffer, pH 7.3. Buffer B was Buffer A with NaCl added to give a concentration gradient. Flow rate: 0.7 ml/min. Sample: 1.0 ml corn root exudate (17 μg protein).

Gel filtration: The sample obtained in the previous step was applied to a preequilibrated Sephadex G-50 column and was eluted with 50 mM Tris buffer, pH 7.3

Results and discussion

Catechin, when dissolved in water and allowed to stand at 25 °C, becomes increasingly yellowish-red due to autoxidation[4]. In our earlier paper[1], we showed that when freshly prepared catechin solution (maximum absorbance 270 nm) was incubated with the root exudate of lettuce seedlings, catalysis of the oxidation of this flavonoid took place due to the presence of catechin oxidases released from the root.

When the root exudates of alfalfa, tomato, wheat, lettuce and corn seedlings (3 μg protein) were checked for the presence of oxidases that catalyze the oxidation of catechin, alfalfa had the highest specific activity and corn had no activity, as shown in figure 1, which shows the time-course of the oxidation. Heat treatment of these exudates at 90 °C for 10 min led to the concomitant loss of this catalytic activity, which indicated that it was enzymic. There was no apparent increase in the absorbance at 428 nm when catechin was incubated with the root exudate of corn seedlings (3 μg protein), suggesting the absence of catechin oxidases (fig. 1).

Purification of the proteins in the root exudate of corn seedlings by anion exchange chromatography using DEAE Sepharose as the anion exchanger resolved 4 major fractions, A, B, C and D as shown in figure 2. When

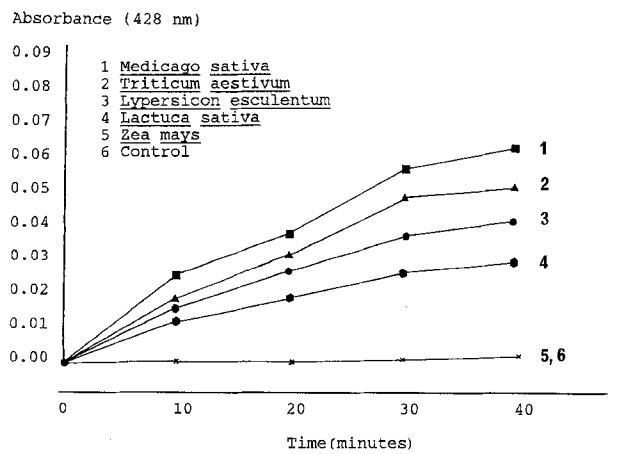

Figure 1. Exposure of catechin (final concentration = 0.95 mg/ml) to the root exudate of the plant seedlings of 1–4 (3 µg protein) causes a rapid increase in the optical density at 428 nm. However, this activity was not observed in 5. Heat treatment of the exudates (1–4) at 90 °C for 10 min led to the concomitant loss of the enzymatic activity.

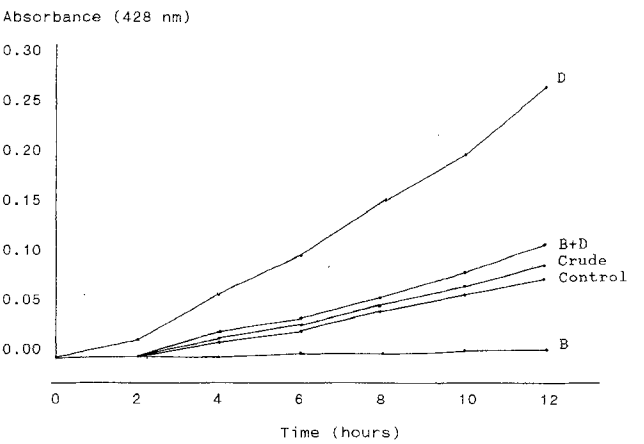

Figure 3. Fractions B and D (5 µg protein) were independently exposed to catechin and the O.D. was read at 428 nm. The O.D. of B is lower than the control due to the presence of an inhibitor of the oxidation of catechin. 'Crude' in the figure refers to the unseparated proteins in the root exudate. Final concentration of catechin in each solution was 0.3 mg/ml.

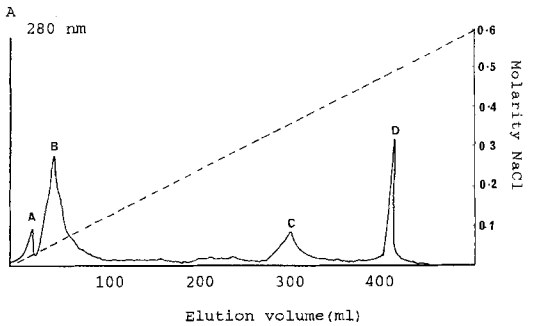

Figure 2. Gradient elution of the proteins in the root exudate of corn on DEAE Sepharose. 1.0 × 25 cm (column SR 10/50). Sample: 1.0 ml corn root exudate (17 µg protein). Eluent: 20 mM Tris buffer with NaCl, pH 7.3. Flow rate: 0.7 ml/min.

the fractions were independently assayed for the oxidase activity using catechin as the substrate, fraction D, which was eluted at approximately 0.5 M NaCl, contained oxidases that catalyzed the oxidation of this flavonoid and the products absorbed maximally at 428 nm. Heat treatment of fraction D at 90 °C for 10 min led to the concomitant loss of this activity.

The color of the solution in the control, which contained catechin and buffer, became increasingly red after 2 h due to the autoxidation of catechin[1] (fig. 3). However, when fraction B (5 µg protein), which was eluted at approximately 0.07 M NaCl, was incubated with catechin, the increase in O.D. 428 due to autoxidation was lower, indicating the presence of an inhibitor.

Hence, both catechin oxidase(s) (from fraction D) and an inhibitor of the oxidation of catechin (from fraction B) were shown to be released from the roots of corn seedlings. As shown in figure 3, when fraction B (5 µg protein) together with fraction D (5 µg protein) were incubated with catechin (final concentration 0.3 mg/ml), the oxidation was lower than with fraction D (5 µg protein) alone. This shows that the presence of fraction B inhibited the oxidation of catechin both in the presence and in the absence of the oxidase from fraction D. However, this inhibitor from the roots of corn seedlings was not able to prevent the oxidation of catechin when it was added to the catechin oxidases from the roots of alfalfa, wheat, tomato and lettuce.

When fraction B was further purified by size exclusion chromatography, 2 major fractions were resolved: 23.4 kDa and 6.4 kDa, respectively. When both the fractions were independently checked for the activity (inhibition of the oxidation of catechin), the inhibitor was found in the 6.3 kDa fraction.

For every 100 µg of protein in the root exudate of corn used for purification, 35 µg of B and 29 µg of D were obtained. Protein determination of the exudate by the Bradford method showed that for every gram of seeds germinated for 60 h, 4.3 µg of protein were released. At different stages of the germination of the corn, the ratio of B to D was checked and it was found to be constant. Hence, the inability of the corn root exudate to oxidize catechin is not due to the absence of the catechin oxidases but is due to the presence of an inhibitor of the oxidation of catechin released from the same plant. Plant roots secrete and release a range of products[5], primarily from the cap cells at the root tip[6]. It is interesting that these two proteins that have antagonistic effects on the same substrate were released from the roots of the same plant. We do not yet have sufficient information to assign specific roles for these proteins.

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