Release of oxidases from the roots of plants

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Summary. In this paper, we report on the isolation and purification of the oxidase released from the roots of *Lactuca sativa* seedlings. This is the first time that the release of oxidases from the roots of plants into their rhizosphere has been reported.

Key words. Lactuca sativa; catechin; oxidase; root release; rhizosphere.

As part of our continuing search for plant growth regulators from natural sources [1], we isolated a number of compounds from crude extracts [2], which were then tested for their bioactivity. The isolated compounds were screened by the Kamisaka [3] method for growth assay, in which lettuce seedlings were employed.

In the isolation of bioactive compounds from *Podocarpus nagi* (Podocarpaceae), a norditerpene dilactone, nagilactone E, catechin and epicatechin were identified as components of the root bark extract [4]. During the course of the bioassay, we observed that when catechin (fig. 1) was present in the test solution, the roots and the solution surrounding the roots of lettuce seedlings were stained yellow red (plate). Further studies of this phenomenon led us to the discovery that oxidases were released from the roots of lettuce seedlings into the rhizosphere, which is reported in this paper. This is the first time that such a report has been made.

Materials and methods

Lettuce germination and growth assay: Lettuce seeds (*Lactuca sativa* cv Grand Rapids MT Lot 14620-10521) were placed on a layer of filter paper in a 9-cm petri dish

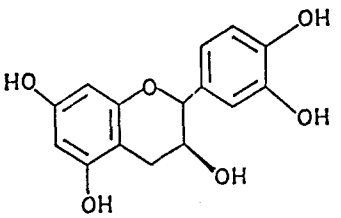

Figure 1. Catechin.

containing 2 ml of the test solution. The lettuce seeds were allowed to germinate and grow under continuous fluorescent light (3000 lux at plant level) at 25 °C. The lengths of the roots and hypocotyl were checked daily.

Seeds: Lettuce seeds were purchased from Ferry Morse Seed Company, (California); corn, alfalfa and tomato seeds from Carolina Biologicals (Carolina); rice was donated by M. Taniguchi (Japan).

Isolation of natural substances: Catechin, epicatechin and nagilactone E were previously isolated from *P. nagi* which was collected from Nara, Japan [4].

Assay for enzymatic activity: Catechin oxidase activity was assayed by determining the amount of catechin oxidized at 30 °C, pH 8.2 in 15 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: Lettuce seeds (10 g) were germinated on a tray for 36 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 was 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 method [5] was employed; optical density was monitored at 595 nm.

Antioxidant competition assay: 1 ml of catechin (400 µM), together with 1 ml of the root exudate (10 µg protein), was added to various concentrations of the antioxidants (ascorbic acid and gallic acid independently), in the range from 0 to 400 µM. Optical density was read at 428 nm.

Enzyme purification: All enzyme purification procedures were carried out below 5 °C. The proteins in the root exudate were fractionated using anion exchange chromatography (DEAE Sepharose column) and stepwise elution. Column: Pharmacia SR 10/50. Bed height: 25 cm. Eluents: Buffer A was 20 mM Tris buffer, pH 7.3. Buffer B was Buffer A with NaCl added to give a stepwise gradient; concentrations 0.1, 0.4 and 1.0 M. Flow rate: 1.2 ml/min. Sample: 1.0 ml lettuce root exudate (30 µg protein/ml). The enzyme solution was concentrated with a Diaflow membrane PM 10.

Gel filtration: The sample obtained in the previous step was applied to a pre-equilibrated Sephacryl S-300 column.

SDS-polyacrylamide gel electrophoresis: The characterization of the oxidase was carried out on a 12.5% SDS-PAGE gel according to the Laemmli method [5]; standard molecular weight markers were used. The gel was stained with silver stain [8].

Enzyme kinetics: The value for the Michaelis-Menten constant, K_M, was obtained from the Lineweaver-Burk plot.

pH optimum and stability at varying pH values: The pH optimum of the purified enzyme were determined by assaying the catechin oxidation activity (428 nm) over the pH range 4–10 using various buffers in the reaction mixture. To test pH stability, the enzyme was treated in each buffer (pH 4–12) for 15 min at 40 °C. After the pH had been adjusted to 8.2 with HCl or NaOH solution, the residual activity was measured by the standard assay method. The buffer systems were as follows: 0.1 M CH_3COOH-CH_3COONa (pH 4.0–5.0), 0.1 M HEPES-KOH (pH 6.0–8.0), 0.1 M $KHCO_3$-K_2CO_3 (pH 8.7–10.4) and K_2CO_3-KOH (pH 11.0–12.8).

Heat stability: The enzyme was treated at each temperature (30–60 °C with 10 °C intervals) for 15 min in buffer and then the residual activity was measured by the standard assay method.

Results and discussion

Freshly prepared catechin solution is colorless (maximum absorbance 270 nm). When dissolved in water and allowed to stand for 16 h at 25 °C, catechin is converted to a yellow-red solution (maximum absorbance 428 nm) due to the oxidation of the flavonoid in air. However, in the lettuce germination assay, the change of color of the roots and of the surrounding solution required only 10 min for the same intensity of color development (fig. 2). This rapid color development was suggestive of an enzyme-catalyzed reaction, and led us to postulate that the root secreted enzymes with the ability to oxidize catechin. To confirm our postulate, root exudate of 36-h-old lettuce seedlings was collected and enzymatic activity in it investigated.

When the collected root exudate was mixed with freshly prepared catechin, the mixture rapidly turned red, to give a solution with an absorption maximum at 428 nm (fig. 3). However, when catechin was mixed with exudate that had been previously heated at 90 °C for 15 min, there was a loss of activity (fig. 2). The rapid rate of catechin

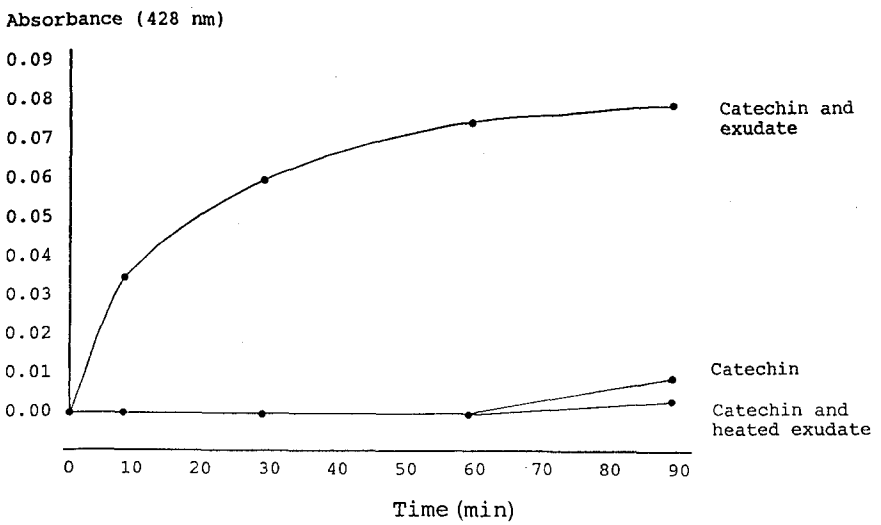

Figure 2. Exposure of catechin (final concentration = 0.95 mg/ml) to the root exudate of 36-h-old lettuce seedlings (containing 10 µg protein) causes a rapid increase in the optical density (O.D.). Lambda max. 428 nm. Heat treatment of the exudate at 90 °C for 15 min led to the concomitant loss of the enzymatic activity.

oxidation by the exudate, and the loss of this activity due to heat inactivation, strongly suggested the involvement of one or more enzymes in the oxidation process.

When oxygen was removed by passing nitrogen through the solution containing catechin and the exudate, no oxidation was observed. To further confirm that the enzyme is an oxidase, catechin-antioxidant competition assays were conducted. Ascorbic acid and gallic acid, both of which are water-soluble antioxidants, were independently able to prevent the oxidation of catechin by being oxidized preferentially, as shown in figures 4 and 5. The preferential oxidation of these antioxidants confirms that the enzyme is an oxidase.

Protein determination in the exudate by the Bradford [5] method showed that for every gram of seeds germinated for 36 h, 84.1 µg of proteins were released. In order to isolate the oxidase from the exudate, enzyme purification was conducted by anion exchange chromatography using a DEAE Sepharose column with NaCl as the ion exchanger in stepwise elution (fig. 6) monitored at 280 nm. The fraction corresponding to peak 0.1b was the fraction that contained the oxidase, as demonstrated by the rapid increase in the O.D. at 428 nm when catechin was used as the substrate (fig. 7). Gel filtration on a Sephacryl S-300 column was performed and the enzyme was eluted at a position corresponding to a molecular weight of 20 kDa.

Characterization of this oxidase with SDS-polyacrylamide gel electrophoresis [6] showed that the protein was approximately 85% homogeneous and its molecular weight was approximately 20 kDa. The K_M value of the oxidase using catechin as the substrate was 1.82 mM. The optimum temperature was approximately 30 °C, and the enzyme was stable up to 40 °C. The optimum pH was 8.2, and the enzyme lost its stability above pH 9.

Plant root exudates from other families were also checked for the presence of the oxidase. Seedlings of

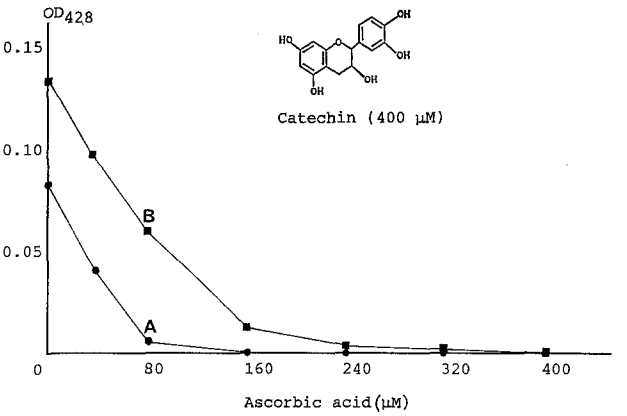

Figure 4. Antioxidant activity of ascorbic acid in the presence of catechin (400 µM) and the root exudate (10 µg protein). $A = 30$, $B = 90$ min.

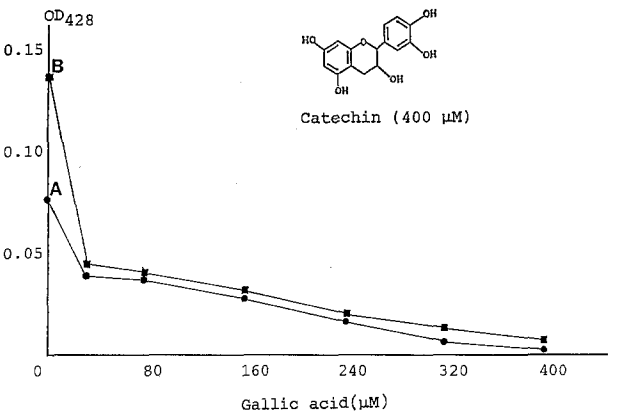

Figure 5. Antioxidant activity of gallic acid in the presence of catechin (400 µM) and the root exudate (10 µg protein). $A = 30$, $B = 90$ min.

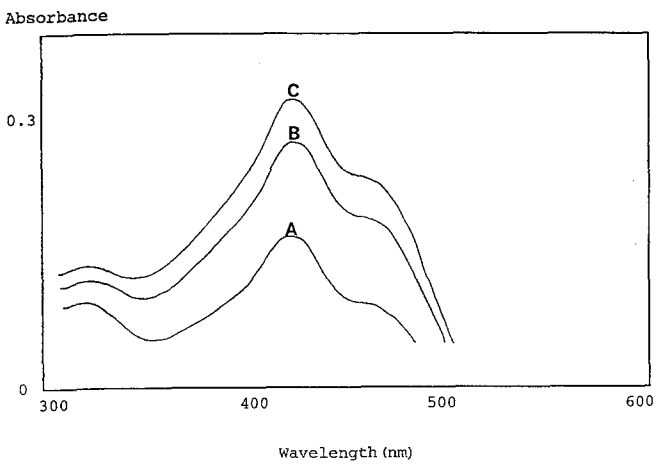

Figure 3. Exposure of catechin (final concentration = 0.3 mg/ml) to the root exudate of lettuce (1.5 µg protein) produced a product that absorbs at 428 nm. *A*, *B* and *C* are taken at intervals of 2 min. $A = 2$, $B = 4$, $C = 6$.

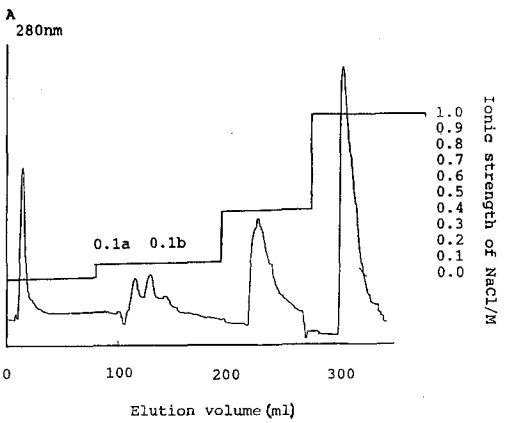

Figure 6. Stepwise gradient elution of the proteins in the root exudate of lettuce on DEAE-Sepharose. 1.0 × 25 cm (column SR 10/50). Sample: 1.0 ml lettuce root exudate (16.5 µg protein). Eluent: Buffer A was 20 mM Tris buffer, pH 7.3. Buffer B was Buffer A containing NaCl, using a stepwise gradient. Flow rate: 1.2 ml/min.

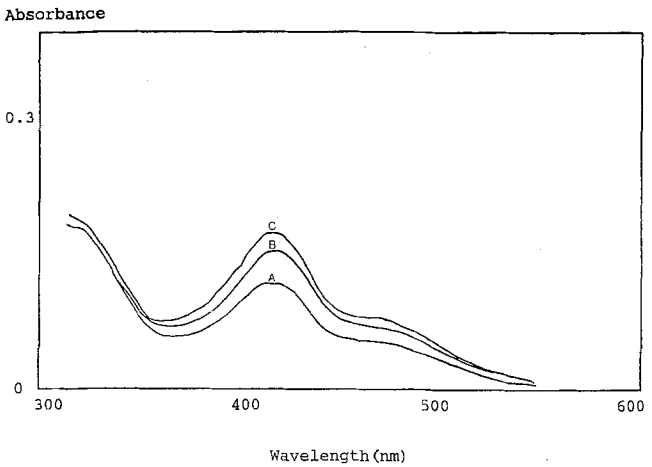

Figure 7. Exposure of catechin (final concentration = 0.5 mg/ml) to the protein fraction corresponding to peak 0.1b (50 ng protein) produced an oxidized product that absorbs maximally at 428 nm. Peak 0.1b was obtained by elution of the root exudate of lettuce seedlings with 0.1 M NaCl on DEAE Sepharose column. *A*, *B* and *C* are the absorption spectra taken at an interval of 10 min. $A = 10$, $B = 20$, $C = 30$ min.

tomato (*Lycopersicon esculentum*), alfalfa (*Medicago sativa*), rice (*Oryza sativa*) and corn (*Zea mays*) were used. All the seedlings that were screened released oxidase/s that were able to oxidize catechin.

The presence of oxidases in the tissues of plants [7] is well established, but this is the first time that it has been reported that oxidases are released from the roots of plants into the rhizosphere.

When some of the properties of these released oxidases and of other plant systemic oxidases that have been reported were compared, differences were found in the molecular weight and pH optimum [7]. However, 1-phenyl-3-(2-thiazolyl)-2-thiourea, an inhibitor that acts on the copper in the plant systemic oxidases [7], also inhibits this catechin oxidase.

Understanding of the role of this oxidase is still rudimentary, although it has been shown that it was able to reduce the growth inhibitory effects of some naturally occurring phenolics (eg. catechol and hydroquinone) through oxidation.

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1 Kubo, I., Pure appl. Chem. *61* (3) (1987) 373.
2 Hanke, F. J., and Kubo, I., LCGC *5* (3) (1987) 248.
3 Kamisaka, S., Plt Cell Physiol. *14* (1973) 747.
4 Kubo, I., Matsumoto, T., Hanke, F. J., Taniguchi, M., and Hayashi, Y., Experientia *41* (1985) 1462.
5 Bradford, M., Analyt. Biochem. *72* (1976) 248.
6 Laemmli, U. K., Nature *227* (1970) 680.
7 Mayer, A., and Harel, E., Phytochemistry *18* (1979) 193.
8 Merril, C. R. et al., Science *211* (1981) 1437.

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ACTH response induced by interleukin-1 is mediated by CRF secretion stimulated by hypothalamic PGE

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Summary. We investigated whether hypothalamic prostaglandin E_2 (PGE_2) and corticotropin releasing factor (CRF) are responsible for the development of the adrenocorticotropic hormone (ACTH) response induced by interleukin-1α (IL-1α). The present results show that ACTH responses induced by intravenous injection of IL-1α were suppressed by systemic pretreatment with indomethacin and that intrahypothalamic injection of PGE_2 stimulates the secretion of ACTH. Furthermore, systemic pretreatment with anti-CRF antibody significantly suppressed the ACTH response induced by intrahypothalamic injection of PGE_2. These data suggest that the ACTH response induced by IL-1 is mediated by CRF secretion stimulated by hypothalamic PGE_2.
Key words. Hypothalamus; interleukin-1; prostaglandin; corticotropin releasing factor; ACTH; acute phase response.

Interleukin-1 (IL-1), which is released by circulating and reticuloendothelial monocytes in response to a variety of pathogenic stimuli such as bacterial endotoxin, induces many immune and metabolic responses for host defence [1, 2]. IL-1 activates the arachidonic acid cascade system to synthesize and release prostaglandins (PGs), which act on the hypothalamus [3, 4] as possible final mediators to induce fever [5, 6]. Furthermore, recently IL-1 was found to stimulate the secretion of adrenocorticotropic hormone (ACTH) and glucocorticoid hormone when injected systemically [7]. The involvement of PGs has also been revealed [8–10] in the ACTH response induced by IL-1, since systemic pretreatment with the inhibitor of PG synthesis, indomethacin, significantly suppressed the