Isolation of an Endogenous Elicitor Induced by Hydrogen Peroxide from Potato

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On treatment with hydrogen peroxide, potato tubers released an endogenous elicitor for phytoalexin production. The homogenous active substance, purified by gel filtration and anion exchange column chromatographies, is an acidic polysaccharide with MW 9,200. This constitutes the first isolation of a self-induced endogenous elicitor.

It has been postulated that plant could release endogenous elicitor in order to produce phytoalexin.[1] Two endogenous elicitors have been hitherto isolated from hydrolysates of plant cell walls with acid[2] or enzymes.[3,4] These are oligogalacturonides originated from pectin.[5] The pectin-degrading enzymes secreted by pathogens are thought to be involved in releasing the endogenous elicitor.[5] On the other hand, endogenous elicitors formed without involvement of pathogens have not been purified to homogeneity. Hargreaves et al. showed that bean hypocotyls released crude elicitors by freeze-thawing.[6,7] Phillips et al. reported partial purification and some characters of endogenous elicitors from UV-irradiated cell suspension cultures of red bean.[8] Recently, we reported the role of hydrogen peroxide (H_2O_2) as a dynamic trigger for phytoalexin production in potato plant.[9,10] We describe herein the first isolation of an endogenous elicitor activated and increased in potato tubers treated with H_2O_2.

When aged potato tuber tissues (cultivar Rishiri) were treated with an aqueous 1M H_2O_2 solution at 23 °C for 6 h, the resulting water extracts showed reproducibly the strongest elicitor activity for the production of rishitin, the representative potato phytoalexin (Figure 1).

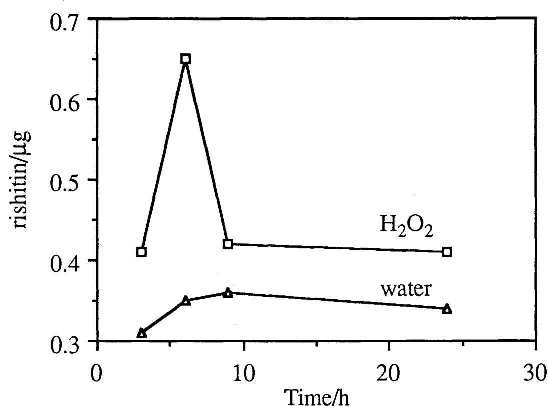

Figure 1. Comparison of the elicitor activities between the extracts obtained by incubation with H_2O_2 or water. The horizontal axis reveals the time of treatment with H_2O_2 or water to the holes of aged potato tuber slices. Each value represents the average of two experiments.

Potato tubers (Rishiri, 206 kg) were peeled and cut into slices (1.5 cm in thickness, 3~10 holes/slice), and 20,300 holes (1 cm in diameter, 1 cm in depth) were dug. They were aged in a dark at 23 °C for 16~18 h. The aqueous solution of 1M H_2O_2 was sprayed on the slices (8~10 ml/100 holes), which were allowed to stand for 6 h. The holes were then filled up with water. Aqueous solution was collected from the holes after 24 h and the same extraction was once repeated. The combined water extracts were washed with EtOAc, concentrated *in vacuo*, and lyophilized to give the crude extracts (37.2g). The crude extracts (3 g) were purified on a Sephadex G-25 column (4 cm x 120 cm, H_2O). The whole amount of the eluents and their hexose contents (with PhOH-H_2SO_4 method)[11] were monitored with OD_{210} and OD_{490}, respectively. Almost a half of the activity was recovered at the elution volume 525-825 ml. As a result, the active material (1.54 g) was obtained from 32.0 g of the crude extracts by repeating the experiments. Separation of the active component (50~80 mg) on a Sephacryl S-100 column (1.8 cm x 95 cm, 1/15M phosphate buffer, pH 6.5) gave good results only by using a buffer with high ionic strength. After desalting on a Sephadex G-25 column (H_2O), the active material (15~20 mg) corresponding to more than 80% of the elicitor activity was recovered at the elution volume 120-160 ml. Eventually, the active material (91.2 mg) was obtained from 545 mg of the Sephadex G-25 active material. The active component (20 ~25 mg) was absorbed on a DEAE Sephacel column (1 cm x 4.5 cm) equilibrated with 20mM phosphate buffer (pH 6.5). The column was washed with the same buffer, and eluted with stepwise gradient of 50-250mM NaCl solution in a buffer solution (50mM at each step). After desalting on a Sephadex G-25 column (H_2O), almost all activity was recovered at the fraction of 200mM NaCl. The DEAE active material was amounted to 14.9 mg starting from 69.8 mg of the S-100 active material. These data of the elicitor purification steps are summarized in Table 1.

The elicitor showed a single peak on a Sephadex G-75 column (2 cm x 101 cm, 1/15M phosphate buffer, pH 6.5). The elution peak of hexose content was in accordance with the elicitor activity, and could not be detected by absorption at 210 nm (Figure 2). The molecular weight was estimated to be ca. 9,200 in comparison with dextran standards. Electrophoresis of the elicitor was effected on a cellulose acetate membrane (6 cm x 10 cm, SEPARAX-SP, Fuji Photo Film Co., Ltd.) in an aqueous buffer solution including 10% Py, 0.3% AcOH, and 50mM EDTA (pH 6.5) at 0.8 mA/cm at 20 °C for 20 min.[12] Only a single spot [relative mobility, 0.7 as compared to a polygalacturonic acid from orange (1.0)] was detected by staining with a dye for acidic carbohydrates (0.5% alucian blue in 3% AcOH), followed by washing with 0.1% AcOH. No spots were observed when stained with a dye for protein (Ponceau solution, Jookoo Co., Ltd.). The results suggest that the elicitor is composed mostly of sugars. Colorimetric analyses of sugars in the elicitor revealed the presence of hexoses, uronic acids, pentoses, and deoxyhexoses (Table 2). These reveal that more than 86% of the elicitor molecule consists of sugars. The pure elicitor thus obtained caused accumulation of rishitin at a concentration of 50 µg/3 ml.

Oligogalacturonic acids have been already isolated as the

Table 1. Purification steps of an endogenous elicitor

step	weight/ mg	activity/ unit[b]	specific activity/ unit/mg
1. crude extracts	37,200 (32,000)[a]	2,480 (2,240)	0.07
2. Sephadex G-25	1,540 (545)[a]	1,027 (364)	0.67
3. Sephacryl S-100	91.2 (69.8)[a]	304 (230)	3.3
4. DEAE Sephacel	14.9	298	20

a) The amounts of samples applied to the next step are shown in parentheses.
b) One unit of sample caused accumulation of 10 μg of rishitin in the bioassay. The bioassay for the elicitor activity was performed as follows: to the aged potato tuber disks, the aqueous solution of the sample (3 ml) was inoculated into holes (1 cm in diameter, 1 cm in depth, 15 holes). After incubation in a dark at 23 °C for 24 h, the holes were filled with water. The water was collected after 24 h, followed once by the same way. The aqueous solutions were combined, extracted with EtOAc, and concentrated *in vacuo*. An aliquot of the hexane solution of the residue was spotted on a TLC plate (SiO_2) and developed with ether. Rishitin was detected by dipping the plate with conc. H_2SO_4 and immediately qantified as the red spot with TLC scanner by reflection at 500 nm. The activity represents the average amount of three experiments.

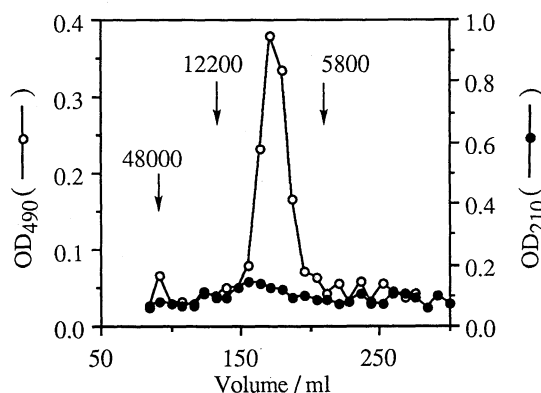

Figure 2. Molecular weight determination of the endogenous elicitor on a Sephadex G-75 column. The molecular weights of the dextran standards (48000, 12200, and 5800) are shown in the figure.

endogenous elicitors from the hydrolysates of plant cell walls (pectin) of bean.[2-5] Our results show the first isolation of an endogenous elicitor released by plant itself. The endogenous elicitor isolated from potato tubers on treatment with H_2O_2 has the following characteristic features. The molecular weight is relatively large as compared to oligogalacturonic acids and other sugar-containing exogenous elicitors.[13,14] It should be emphasized that the elicitor molecule contains both neutral and acidic sugars. Further chemical and biological characterization of the H_2O_2-induced endogenous elicitor is now in progress in our laboratory.

Table 2. Contents of reduced sugars by colorimetric analyses

sugars	contents[a]	methods[11]
hexoses	24% (glucose)	PhOH-H_2SO_4
uronic acids	27% (galacturonic acid)	carbazole-H_2SO_4
pentoses	23% (arabinose)	cysteine-H_2SO_4
deoxyhexoses	12% (rhamnose)	cysteine-H_2SO_4

a) A standard sugar is indicated in each parenthesis.

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