Isolation of a Specific Potato Tuber-Inducing Substance from Potato Leaves

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Potato tuberization is induced by an unidentified “tuberization stimulus” which is produced in the leaves. Recently, we confirmed the occurrence of two acidic substances in the leaves which appear to be the stimulus (Koda and Okazawa 1988). We report here the isolation of one of the substances from potato leaves. The molecular weight of the substance is 388. The substance is active in inducing tuberization in vitro at a concentration of 0.01 mg·liter\textsuperscript{-1} (ca. 3 \times 10\textsuperscript{-8} M).

Key words: Potato tuberization — \textit{Solanum tuberosum} — Tuber-inducing substance — Tuberization stimulus.

Potato tuberization is a physiological process to form a storage organ. The first sign of tuberization is the cessation of stolon elongation followed by a swelling at the sub-apical region. The swelling at this early stage is mainly brought about by radial cell expansion (Booth 1963, Cutter 1978, Koda and Okazawa 1983). Subsequently, vigorous thickening growth due to cell division and expansion occurs and starch accumulates in the cells. The tuberization is completed when apical and lateral meristems have fallen in the swelling tuber and have formed eyes.

The tuberization is controlled by environmental factors, mainly the photoperiod. Short days stimulate the process, while long days are inhibitory. The response to the photoperiod interacts with many other factors, including genotype (Ewing 1981), temperature (Burt 1964), nitrogen level (Krauss 1978) and age of mother tubers (Montaldi and Claver 1963).

With grafting experiments, Gregory (1956) and Chapman (1958) demonstrated the occurrence of a tuberization stimulus which is formed in the leaves under short days and transmitted to underground parts to induce tuberization. Recently, we confirmed the occurrence of two acidic substances in the leaves which are very active in inducing the tuberization in vitro (Koda and Okazawa 1988). The level of the substances in the leaves increased under short days, suggesting that these substances are the major factors of the tuberization stimulus. The present study was carried out to isolate the substances from potato leaves.

Materials and Methods

Bioassay for tuber-inducing substance—Bioassay for the tuber-inducing substances was performed by single-node stem segment culture in vitro as reported previously (Koda and Okazawa 1988). Single-node stem segments (ca. 2 cm long) prepared from etiolated potato shoots (\textit{Solanum tuberosum} L. cv. Irish Cobbler) were sterilized with 1\% sodium hypochlorite solution for 1 h and then washed thoroughly with sterile water. After removing both 5 mm ends, these segments were planted horizontally in a 100 ml flask containing 10 ml White’s basal medium supplemented with the sample to be tested. The medium was adjusted to pH 5.6 and solidified with 0.6\% Bacto-agar before autoclaving. The cultures were maintained at 25°C in the dark for 3 weeks, and then the rate of tuberization was calculated as (the number of tuberized laterals)/(the number of all emerged laterals).

Plant material for extraction and isolation of tuber-inducing substance—Seed tubers were planted in an experimental field early in May and raised in the usual manner. The plants began to form tubers late in June. Leaf blades (100 kg fr wt) were harvested from the plants late in July and immersed in 400 liters of 70\% ethanol for more than one month. After filtration, the ethanolic extract was used for the experiment.
Results

The ethanolic extract obtained from 10 kg fr wt leaves was concentrated in vacuo below 40°C to three liters, and the resulting aqueous concentrate was extracted successively 3 times with equal volumes of n-hexane and ethyl acetate. Strong tuber-inducing activity was found in the ethyl acetate fraction and the residual aqueous fraction (Fig. 1). The ethyl acetate fraction was divided into acidic and neutral/basic fractions by extraction with 1 M sodium bicarbonate. Most of the activity was found in the acidic ethyl acetate fraction. The active substance in this acidic fraction seemed to be unstable because the activity decreased greatly during the course of the isolation. Therefore, only the aqueous fraction was used to isolate the active substance.

The aqueous fraction (dry wt 128 g) obtained from 10 kg fr wt leaves was applied to a charcoal column (18.5 × 40 cm). The column was washed with 25 liters of water and then eluted with 25 liters each of 30% methanol, 100% methanol and 50% methanol in ethyl acetate. The eluates obtained with 100% methanol and 50% methanol in ethyl acetate exhibited strong tuber-inducing activity. These eluates were combined (dry wt 16.7 g), redissolved in 500 ml water and then passed through a Dowex 1 × 4 column (5.8 × 24 cm, 50-100 mesh, CH₃COO⁻ form). The column was washed with 5 liters of water and eluted with 5 liters of 2 M acetic acid. A strong tuber-inducing activity was found in the eluate. The washing water also showed slight activity.

The eluate (dry wt 5.0 g) was divided into five portions and each portion was fractionated on a Sephadex LH-20 column (4.4 × 30 cm) with 10% methanol. Eighty 16-ml fractions were collected. The activity was found in fractions 25–38. The active fractions were combined (dry wt 3.2 g) and applied to another Sephadex LH-20 column. The elution was carried out with 30% methanol in chloroform. Fifty fractions were collected. Fractions 20–25 exhibited the activity. The active fractions were combined (dry wt 1.1 g) and divided into 15 portions and each portion was separated on a Lobar LiChroprep RP-8 column (B type) with 50% methanol, giving 614 mg of active fraction.

Further purification was carried out by HPLC. Each one-fifteenth portion of the active fraction was separated using a preparative µ-Bondapak C₁₈ (19 × 150 mm) column with 10% acetonitrile at a flow rate of 9.9 ml·min⁻¹. According to the UV absorption at 210 nm, the eluate was divided into eight fractions. The activity was found in the sixth fraction. The active fraction (50 mg) was divided into 15 portions and each portion was then separated by an Aminex HPX-87 column (7.8 × 300 mm) with 0.01 M H₂SO₄ at a flow rate of 1 ml·min⁻¹. The eluate was divided into seven fractions. The fourth fraction exhibited the activity. To remove H₂SO₄, the active fraction was passed through a charcoal column (7 × 24 mm). The column was washed thoroughly with water and eluted with 50% methanol.

![Fig. 1 Tuber-inducing activities in ethyl acetate (○) and aqueous (●) fractions obtained from potato leaf blades.](image)

![Fig. 2 HPLC separation of a tuber-inducing substance on two Resolve C₁₈ columns connected in series with 30% methanol containing 0.13% acetic acid and tuber-inducing activity of separated fractions. The active fraction (15 mg) obtained from 10 kg fr wt leaf blades was purified by repeated HPLC. The eluate was divided into five fractions, and one-twentieth of each fraction was mixed with 50 ml of assay medium and assayed for tuber-inducing activity.](image)
methanol in ethyl acetate. The active fraction (15 mg) was divided into 15 portions and each portion was fractionated on two columns of Resolve C_{18} (3.9 × 150 mm, connected in series) with 30% methanol containing 0.1% acetic acid at a flow rate of 0.45 ml·min⁻¹. The eluate was divided into five fractions according to the UV absorption at 210 nm and the third fraction (6.7 mg) exhibited the strongest activity (Fig. 2).

This active fraction was divided into six portions which were then purified by Novapak C_{18} column (Radialpak cartridge, 8 × 100 mm). The column was eluted with 10% acetonitrile containing 0.1% acetic acid at a flow rate of 1 ml·min⁻¹. The elution profile monitored at 210 nm and the activity are shown in Fig. 3. The activity was found in the fourth and fifth fractions, which were combined (2.8 mg) and divided into three portions. Each portion was separated again using the same column with 3% tetrahydrofuran containing 0.1% acetic acid at a flow rate of 1 ml·min⁻¹. Fig. 4 shows the elution profile and the activity of the separated fractions. The first fraction showed the strongest activity. The active substance in the first fraction was also recovered from the second fraction by repeated HPLC. The first fraction (450 μg) was purified using the same column with 15% methanol containing 0.1% acetic acid. The elution profile monitored at 210 nm showed three peaks and the third peak (270 μg) exhibited the activity (Fig. 5). The substance present at the third peak seemed to be pure because it showed a single peak in analytical HPLC on columns of Aminex HPX-87, Resolve C_{18} and Novapak C_{18} (Fig. 6). From 100 kg fr wt potato leaf blades, we finally obtained 2.7 mg of the active substance.

The tuber-inducing activity of the isolated substance is shown in Fig. 7. The substance was active in inducing the tuberization in vitro at concentrations above 0.01 mg·liter⁻¹. The field desorption (FD) mass spectrum revealed that the active substance has a molecular weight of 388 (Fig. 8). The acetylated substance obtained by treatment with pyridine-acetic anhydride has a molecular weight of 556.

**Discussion**

After Gregory's suggestion (1956) about the occurrence of a specific tuberization stimulus in potato leaves, many studies were done to identify the stimulus (see reviews by Melis and Van Staden 1984, and Ewing 1985), most concentrating on the effects of known plant hormones on the tuberization. Only a few attempts have been made to isolate the stimulus directly from the leaves. Madec
The active fraction (450 μg, obtained by HPLC shown in Fig. 4) was purified by HPLC. The eluate was divided into three fractions, and one-twentieth of each fraction was used for assay.

(1963) reported that sap expressed from potato plants which had been grown under short days was capable of inducing tuberization when injected into a plant grown under long days. However, Simonds (1965) could not confirm Madec's result. This discrepancy seems to be due to

Fig. 5 HPLC separation of a tuber-inducing substance on Novapak C_{18} column with 15% methanol containing 0.1% acetic acid and tuber-inducing activity of the separated fraction. The active fraction (450 μg, obtained by HPLC shown in Fig. 4) was purified by HPLC. The eluate was divided into three fractions, and one-twentieth of each fraction was used for assay.

Fig. 6 HPLC profiles of the isolated substance on Aminex HPX-87 column with 0.01 M H₂SO₄ (A), on Novapak C_{18} Column with 7.5% acetonitrile containing 0.1% acetic acid (B), and on Resolve C_{18} column with 30% methanol containing 0.1% acetic acid (C).

Fig. 7 Tuber-inducing activity of the isolated substance. Numbers indicate the concentration of the substance in the assay medium (mg·liter⁻¹).
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Fig. 8 Field desorption mass spectrum of the isolated substance.

absence of accurate assay methods for the activity. In the present study we succeeded in isolating a substance which is active in inducing potato tuberization, using a single-node stem segment culture in vitro as an assay method.

The tuber-inducing substance isolated here seems to be a growth inhibitor, because tuberization begins just after cessation of stolon elongation and the tuberization stimulus, which moves acropetally, inhibits the shoot growth (Kumar and Wareing 1973). The substance may first cause a shift in growth of the stolon from elongation to radial expansion.

In a previous paper (Koda and Okazawa 1988), we suggested that one of the tuber-inducing substance is a glycoside. The peak at m/z 163 in the field desorption mass spectrum (Fig. 8) supports that this substance is a glycoside. The active substance detected in the acidic ethyl acetate fraction appears to be an aglycon of the isolated substance. The field desorption mass spectrum indicated that the isolated substance is different from known plant hormones. The threshold concentration of the substance for the activity was about $3 \times 10^{-8} \text{M}$. Determination of the chemical structure of this substance is currently in progress.

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References


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