Cytokinins in Growing Potato Tubers*

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A vast amount of literature on the ubiquitous presence of endogenous cytokinins in various tissues of higher plants leaves little doubt that these substances play a possible role in regulation of developmental processes of the plants including potato. In fact, hormonal regulation of the potato growth due to the cytokinin has been the subject of extensive researches during life cycle of this plant, and indicated that the cytokinin has implicated, to a greater or lesser extent, in direction of stolon elongation^{16,17,18)} and breaking of dormancy⁵⁾ in concert with auxin and/or gibberellin. In the preceding papers^{13,14)}, we have presented an evidence for occurrence of a significant amount of cytokinins in the immature potato tubers which were just before starting vigorously their thickening growth, and suggested that an accumulation of these cytokinins seems to produce a beneficial effect on the tuber development as a cell division-inducing factor, citing an opinion that cytokinin may serve as a stimulus for induction of potato callus growth in response to auxin^{11,12)}. Recently, Forsline and Langille⁴⁾ have confirmed the presence of four compounds with cytokinin activity in potato plant and appeared that level of the individual cytokinins changed in connection with the variation of environmental conditions as day-length and temperature which directly regulate the tuberization of potato. However a question concerning the precise chemical nature of the cytokinins occurring in the potato tubers has not received a satisfactory answer. Therefore, the present investigation arises out our attempt to gain further details on the isolation and chemical

characterization of the endogenous cytokinins in the potato tuber.

Materials and Methods

Materials

Potato tubers (*Solanum tuberosum* L. cv. Irish Cobbler) which had been harvested at a month before reaching fully maturation were used for experimental materials throughout the present investigation. This is the stage when a maximum of cytokinin activity in the potato tuber was found as reported previously¹⁴⁾.

Extraction of cytokinin

One kilogram of freshly-harvested potato tubers was homogenized with sufficient amount of ethanol to produce a final 70% ethanolic extract and filtered. The filtrate was acidified to pH 2.5 with 1 N HCl and passed through a Dowex 50W-X4 H⁺ ion exchange resin (50-100 mesh) column of 150 ml volume at a flow rate of 500 ml/hr. The column was washed first with 300 ml of 70% ethanol, then with 500 ml of water, and finally the active materials were eluted with 750 ml of 3 N NH₄OH. To avoid the risk of heat decomposition of cytokinin, the column was cooled with ice-cold water until the exchange process had been complete. The eluate was evaporated *in vacuo* to about 50 ml at 37°C and this concentrate was extracted at pH 8.0 with three successive equal volumes of n-butanol, which were then combined and taken to dryness *in vacuo*. The aqueous fraction was discarded. The resulting residue was redissolved in a small volume of 80% ethanol and applied as a streak to Toyo No. 50 paper which had been previously washed with 1 N formic acid followed with distilled water thoroughly. The following solvents were used for development of paper chromatography: (A)

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iso-propanol-ammonia-water (10:1:1, v/v/v), (B) water saturated sec-butanol. The chromatograms were developed in an ascending fashion for all experiments. Each chromatogram was thoroughly air dried and cut into 10 equal strips corresponding to R_f . Each strip was placed into bioassay medium described below. The migration of active materials in the extract was always compared with that of authentic cytokinins. *Partition chromatography of cytokinins on Sephadex column*

Sephadex LH-20 chromatography was performed in essentially the same manner as described by Armstrong *et al.*¹¹. Active fraction released from the paper chromatograms was passed through a Sephadex LH-20 column (2.6×40 cm) which was eluted with 35% ethanol at a flow rate of 40 ml/hr. Ten milliliter fractions were collected, evaporated to dryness at 40°C and then bioassayed for cytokinin activity. The authentic cytokinins were also run on the different chromatography to detect the marker spots. In additional experiments, Sephadex G-10 column (1.5×30 cm) was also employed for further fractionation of samples, and active materials loaded on the column were eluted with distilled water at a flow rate of 50 ml/hr.

Cytokinin bioassay

Soybean (*Glycine max* Merrill cv. Acme) callus bioassay was carried out as described by Miller⁸. The fractions to be tested were incorporated into the Miller's basal medium supplemented with 3 mg/l IAA alone. Three small clumps (each ca. 8 mg in fresh weight) of callus stock were planted in each 100 ml Erlenmeyer flask containing 20 ml of the test medium. The cultures were maintained at 28° C under continuous white fluorescent illumination for 4 weeks. At the completion of each culture the increase in fresh weight has been calculated per explant; each value is an average of 6 replicates.

Experimental Results

In previous papers^{13,14}, it has already been presented an evidence for occurrence of two cytokinins in the potato tubers, and

their partition behavior in organic solvents and ion exchange resin indicated that the potato cytokinins were conspicuously detected in n-butanol phase which was extracted from the eluate of cation exchange resin with charging the ethanolic extract of potato tuber tissue. Taking this evidence into consideration, further information as to the chemical characteristics of these cytokinins are required. According to the results obtained herein (Fig. 1, A), when n-butanol extracts of samples were subjected to paper chromatography using solvent A, besides a small active peak at R_f 0 to 0.2, by far the most prominent peak was located at R_f 0.4–0.8 which were referred to as factor I and II, respectively. When using this solvent system, free base and riboside of cytokinin would usually migrate to the R_f range from 0.6 to 0.8 but separate incompletely, while its ribotide would be located the R_f interval between 0 and 0.2. While

Fig. 1. A. Distribution of the cytokinin activity of the Dowex 50W eluate of tuber extract after paper chromatography in solvent A. Two peaks of cytokinin activity are termed factor I (R_f 0–0.2) and factor II (R_f 0.4–0.8). B. Distribution of cytokinin activity of factor II which was eluted with 80% ethanol and rechromatographed with solvent B. Horizontal solid lines correspond to callus yield of controls and broken lines to that of 0.3 μ g/l zeatin.

Fig. 2. Elution profile of endogenous cytokinins from potato tuber tissues as separated on Sephadex LH-20 column. The positions of zeatin (Z), zeatin riboside (Z-R), N^6 -(Δ^2 -isopentenyl) adenine (IPA) and N^6 -(Δ^2 -isopentenyl) adenosine (IPA-R) are indicated by horizontal bars, and solid and broken lines mean the same as Fig. 1.

the position of factor I on the chromatogram, judged by the R_f value stated above, was roughly included in the region to which ribotides migrated, the factor II was found between R_f 0.4 and 0.8. There seems to be, therefore, existed some unknown cytokinin active substances at R_f 0.4–0.6 and which is unseparable from free base and riboside of cytokinin. In order to re-examine this problem, factor II was extracted from the chromatograms with 80% ethanol and rechromatographed using solvent B. From the histogram illustrated in Fig. 1, B, factor II was resolved into two major peaks which were distributed at R_f 0.4–0.5 and 0.6–0.9. Although no attempt was made to further identify in the present work, the active component with a lower migration rate seems to virtually correspond to the region of known migration for cytokinin glucoside⁹. As to another components distributed at R_f 0.6–0.9, it is capable of anticipating that these components may conceivable include zeatin, zeatin riboside, N^6 -(Δ^2 -isopentenyl) adenine (IPA) and N^6 -(Δ^2 -isopentenyl) adenosine (IPA riboside), citing evidence of earlier findings^{11,10}. In order to achieve further resolution of these

components, these were released to 80% ethanol from the chromatogram strips and evaporated to dryness followed to be taken up in 3 ml of 35% ethanol, and applied to the top of a Sephadex LH-20 column, then percolated with 35% ethanol to separate free base and riboside of cytokinins. Consequently, three distinct peaks with cytokinin activity were distributed on the histogram showing in Fig. 2, which peaks were henceforth termed peak A, B and C in order of increasing elution volume. These peaks were coeluted with available authentic cytokinins, viz. peak A with zeatin riboside, peak B with zeatin and peak C with IPA riboside. Among them, the most pronounced activity was found in the peak A corresponding to zeatin riboside. An additional evidence supporting the above finding that peak A and B corresponded to zeatin riboside and zeatin respectively was also obtained from chromatographic analysis by Sephadex G-10 column. The fractions in question eluted from the Sephadex LH-20 column were subjected again to Sephadex G-10 column chromatography, as described in Materials and Methods. Consequently these were dis-

Fig. 3. Elution profile of cytokinin activity. Fractions obtained from 190 ml to 270 ml of Sephadex LH-20 column as showing Fig. 2 were combined and rechromatographed with Sephadex G-10 column. The bars and lines mean the same as Fig. 1 and 2.

tinctively resolved into two major active fractions and exactly coeluted with zeatin riboside and zeatin as shown in Fig. 3.

Some possibility as to a presence of cytokinin ribotide deserves serious consideration since it was stated above the fact that there was a small but significant proportion of cytokinin activity in the factor I which may contain cytokinin ribotide. The effect of phosphatase on the presumed ribotide was determined. The R_f interval between 0 and 0.2 of the chromatograms being developed with solvent A was eluted and divided into three equal portions. One aliquot was incubated with 0.1 mg/ml alkaline phosphatase in 0.1 M glycine buffer (pH 10.5), 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ for 5.5 hr at 28°C. The other portion was used as control which being incubated with boiled enzyme solution. Both treated

Fig. 4. Cytokinin activity on the paper chromatograms being subjected to alkaline phosphatase treatment. An aliquot of active material (factor I) eluted from the 0 to 0.2 R_f region of chromatograms using solvent A was treated with alkaline phosphatase and rechromatographed in solvent B.

samples were chromatographed with solvent B and assayed. Although the control still showed an activity at R_f 0.1–0.4, phosphatase treatment yielded a more mobile product with cytokinin activity, which migrated between R_f 0.6–0.7 (Fig. 4). In view of the fact that the resulting product from the treatment appears to be a riboside, it seems logical to surmise that there were some cytokinin ribotides in the potato tubers. However at the moment it is an open question whether this cytokinin is a ribotide of zeatin or that of IPA. Subsequent experiment with the intention of separating to several ribotide was carried out. The remaining aliquot being previously treated with alkaline phosphatase was submitted to the Sephadex G-10 column chromatography. From the result showing in Fig. 5, a high active fraction and a second active one were clearly distinguished, of which the former fraction is quite similar to the elution pattern of zeatin riboside and the latter one to IPA riboside. Accordingly, it was verified that there were two kinds of cytokinin ribotide, namely the one is zeatin

Fig. 5. Elution profile of cytokinin activity of the alkaline phosphatase treated factor I after fractionation on Sephadex G-10 column. The bars and lines mean the same as Fig. 1 and 2.

ribotide and the other is IPA ribotide.

Discussion

From inspection of the data represented here as to the chemical characterization of the endogenous cytokinins in the growing potato tubers, it has led us to believe that at least six cytokinins appeared to present in them. Five of them behaved like zeatin, zeatin riboside, zeatin ribotide, IPA riboside and IPA ribotide in all respects of elution characteristics of Sephadex LH-20 and G-10 column chromatography. Although no further studies on the nature of the remaining component has been pursued, it is possible to be zeatin glucoside citing from the chromatographic evidence given by Henson and Wheeler⁶. On the other hand, all above evidence supports the opinion that major active component of naturally-occurring cytokinins in the potato tuber is zeatin riboside or its closely related compounds and the quantity of this compound is, on an average, of the order of 10^{-1} μ g zeatin equivalent per Kg of fresh tuber tissues. It is so far practically difficult to perform rigorous chemical identification,

because of the presence of a quite trace amount of cytokinin in the tubers.

Although it has been generally accepted as a fact that the cytokinin ribotides are insoluble in n-butanol since an earlier investigation by Miller⁹, the present data suffice to show an ample evidence that these ribotides are not completely insoluble in n-butanol but at least in part soluble in it, in considering partial migration of the compounds to the butanol phase during the successive partition with n-butanol. In view of the evidence stated above, it must not be overlooked that the bulk of ribotides seems to remain in the aqueous residue, because of their higher polarity. The determination of cytokinin activity in the aqueous residue faced a difficulty due to insufficient elimination of cumbersome impurity for cytokinin bioassay, whereby it was discarded in this experiment. On the assumption that the ribotides of cytokinin are physiologically less active than their corresponding free base and riboside² and these seem to be a storage form of cytokinin¹⁵, the ribotides should have an important implication on the development of potato tuber, so additional work is necessary to answer this question.

Another interesting point arising from this investigation is the fact that a significant amount of IPA riboside was also found in the ethanolic extract of potato tubers in addition to the riboside of zeatin. Although many investigations have confirmed the presence of zeatin and its related compounds in many plants, only limited information is presently available on the simultaneous occurrence of both derivatives of zeatin and IPA³. It has already been shown that the riboside of IPA was detected in the enzymatic hydrolysate of t-RNA¹³, and another studies on this line have been speculated a possibility that IPA is one of the precursor of zeatin¹⁰. The evidence is not yet complete enough to say this assumption, but it is most probable that the ribosides of zeatin and IPA existed together in the potato tuber tissues.

In conclusion, although some steps of this chemical characterization is not yet experimentally confirmed, there is ample evidence of the occurrence of six cytokinins

in the potato tubers. Thus a possible physiological role of these substances during tuber development will deserve in future further attention.

Summary

Extract from the tissue of growing potato tubers (*Solanum tuberosum* L.) exhibited cytokinin activity in the soybean callus assay. Based on the chromatographic analysis and phosphatase treatment, at least six kinds of cytokinin appeared to detect; e.g. (1) free zeatin, (2) zeatin riboside, (3) zeatin ribotide, (4) zeatin glucoside, (5) IPA riboside and (6) IPA ribotide.

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〔和 文 摘 要〕

生育中の馬鈴薯塊茎のサイトカイニンについて

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ダイズカルスバイオアッセイ法を用いて、生育中の馬鈴薯塊茎内に存在するサイトカイニンがいかなる種類のものであるかを検討した。

ペーパークロマトグラフィ、セファデックスカラムクロマトグラフィおよびアルカリ性フォスファターゼ処理等により、馬鈴薯塊茎内には下記の6種のサイトカイニンが存在することが判明した。

(1) ゼアチン, (2) ゼアチンリボシド, (3) ゼアチンリボチド, (4) ゼアチングルコシド, (5) イソペンテニルアデニンリボシド, (6) イソペンテニルアデニンリボチド。

これらの6種のサイトカニンの中で最も多量に存在するものは、ゼアチンリボシドであった。