Intracellular Localization of Lunularic Acid and Prelunularic Acid in Suspension Cultured Cells of Marchantia polymorpha

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ABSTRACT

Intracellular localization of lunularic acid and prelunularic acid in suspension cultured cells of Marchantia polymorpha L. was studied. The sum of both compounds was determined as lunularic acid group (LNAs) because of the instability of prelunularic acid to convert into lunularic acid.

Mechanical disruption of the cells followed by differential centrifugation showed that LNAs was associated with the supernatant of 100,000g centrifugation. Protoplasts isolated from the cells were osmotically ruptured and the distribution of LNAs among the organelles was examined by discontinuous density gradient centrifugation of the protoplast contents. Successful isolation of intact chloroplasts, mitochondria and peroxisomes free from cytoplasm indicated that LNAs was not accumulated in these organelles. Flotation techniques resulted in an efficient isolation of pure vacuoles and revealed that LNAs was distributed almost equally in the vacuoles and cytoplasm.

Lunularic acid (Fig. 1) was first isolated from a liverwort, Lunularia cruciata L. Dum, and proposed as an endogenous growth-regulatory substance in liverworts on the basis of its inhibitory activity on growth and the alteration of its content in response to photoperiod (27). Pryce (19) proposed that lunularic acid might be a biological equivalent of ABA in liverworts because of their complementary occurrence in the plant kingdom and structural similarity between ABA and lunularic acid. On the other hand, Gorham and Coughlan (5) reported that lunularic acid has an inhibitory effect on photosynthetic functions in isolated chloroplasts and mentioned the possibility that this may be responsible for the growth-regulatory properties of lunularic acid. However, since then, the mode of lunularic acid action has not been studied and, accordingly, the role of this compound as an endogenous growth regulator in liverworts is still obscure.

Recently, we found prelunularic acid (Fig. 1) in the cultured cells of Marchantia polymorpha and also proved the ubiquitous occurrence of this compound in liverworts (16, 17). Prelunularic acid is easily converted into lunularic acid and is supposed to be a direct precursor of lunularic acid. Furthermore, we showed that almost all of lunularic acid in both intact liverworts and cultured cells determined previously had been derived from prelunularic acid during extraction (2). These facts necessarily lead to the reconsideration of the hitherto suggested physiological role of lunularic acid, and to the examination of whether prelunularic acid has the same activity as lunularic acid. However, the direct assay of the effect of prelunularic acid on growth is troublesome because it readily converts into lunularic acid. Another approach to the solution of this problem is to determine the accumulation site of these two compounds in the plant cells.

In this paper, we describe the intracellular localization of lunularic acid and prelunularic acid in the cultured cells of M. polymorpha, and discuss the nature of these compounds.

MATERIALS AND METHODS

Plant Material. The cell suspension culture of Marchantia polymorpha L. was grown in MSK-2 medium supplemented with 2% glucose and 1 mg/L of 2,4-D (9, 18).

Differential Centrifugation. The cells (20.4 mg/ml dry weight) were harvested by centrifugation on the 11th day after inoculation on fresh medium. After washing with a solution containing 0.3 M sucrose, 25 mM Tris (pH 8.0), 1 mM MgCl2, and 1 mM EDTA by centrifugation, the cells were resuspended in the same solution. This cell suspension was repeatedly sonicated for 30 s with an ultrasonic vibrator at 80 W (20 kHz, UR-200F, Tomy Seiko Co., Ltd., Tokyo, Japan) resulting in complete cellular disruption. The homogenate was subjected to a three-step differential centrifugation: 1,500g for 5 min, 10,000g for 60 min, and 100,000g (Hitachi 65P) for 60 min. LNAs in the pellet of each step and in the supernatant of final centrifugation was extracted and determined. In a separate experiment, the cells were also disrupted using a Teflon homogenizer instead of the ultrasonication, and fractionated in the same way as above.

Isolation and Rupture of Protoplasts. The cells were harvested by filtration and suspended in a maceration solution to give a final cell density of 5 mg dry weight ml−1. The maceration solution consisted of the freshly dissolved 2.5% (w/v) Driselase (Kyowa Hakko Kogyo Co., Ltd. Tokyo, Japan), 0.7 M mannitol, 5 mM Mes (pH 5.5), and 1 mM CaCl2, and was used after centrifugation at 1,500g for 10 min. This cell suspension was incubated under a gentle shaking in a L-type test tube at 30°C for 2.5 h. After removal of cell clumps by filtration through a nylon mesh (40 μm diameter), the protoplasts were collected by centrifugation at 1,500g for 5 min. The pellet was washed twice with and resuspended in an isolation solution containing 0.5 M mannitol, 25 mM Tris (pH 8.0), and 1 mM MgCl2 at a concentration of about 2 × 107 protoplasts ml−1. The protoplasts were ruptured by diluting the protoplast suspension with twice the volume of a solution containing 25 mM Tris (pH 8.0), 10 mM EDTA, 1 mM MgCl2, and 0.15% (w/v) BSA to give a final concentration of 0.17 M mannitol and by gentle pipetting with a Pasteur pipette about 10 times. The crude mixture obtained was subsequently used for isolation of organelles, while an aliquot was saved for analysis of LNAs1 and markers.

Density Gradient Centrifugation. Isolation of organelles was

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1 Abbreviations: LNAs, lunularic acid group; G3P dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase; PEP carboxylase, phospho-enolpyruvate carboxylase.
The amount of LNAs in the fractions obtained by the flotation method was determined in a simpler way. An aliquot of each fraction was mixed with acetic acid to a final concentration of 25% (v/v) and evaporated to dryness on a centrifuging evaporator at 40°C (Model EC-10, Tomy Seiko Co., Ltd.) resulting in complete conversion of prelunularic acid into lunularic acid. The residue was dissolved in 60% acetonitrile containing 0.1% acetic acid and passed through a Sep-pak C18 cartridge before the LNAs content was measured by HPLC.

**Enzyme Assay.** Activities of α-mannosidase and acid phosphatase were measured by following the amount of p-nitrophenol released from p-nitrophenyl-α-D-mannopyranoside and p-nitrophenyl-phosphate, respectively, according to the method of Boller and Kende (3). Fumarase was assayed by the method of Racker (20). Cyt c oxidase was determined as previously described (7). NADP G3P dehydrogenase, PEP carboxylase, and 3-P glycerate kinase were determined by following the change in absorbance of a pyridine nucleotide at 340 nm (10, 26). Catalase activity was assayed as described previously (24).

**Other Assays.** Chl content was assayed according to the previous paper (18). Protein, precipitated with 5% TCA, was measured by the method of Lowry et al. (13).

### RESULTS

**Differential Centrifugation of Cell Homogenate.** Examination under light microscopy indicated that the cells were completely disrupted by the ultrasonication employed. The pellets of centrifugations at 10,000 g and 100,000 g were rich in chloroplast segments. The amount of LNAs determined in each fraction are shown in Table I. More than 98% of LNAs in the cells was recovered in the supernatant after the final centrifugation at 100,000 g. When the cells were broken more gently by using a Teflon glass homogenizer, the similar results were obtained except that 11.5% of LNAs attributable to unbroken cells was found in the pellet of 1,500 g centrifugation.

**Isolation of Organelles by Density Gradient Centrifugation.** The pellets of centrifugation at 10,000 g and 100,000 g were rich in chloroplast segments. The amount of LNAs determined in each fraction were shown in Table I. More than 98% of LNAs in the cells was recovered in the supernatant after the final centrifugation at 100,000 g. When the cells were broken more gently by using a Teflon glass homogenizer, the similar results were obtained except that 11.5% of LNAs attributable to unbroken cells was found in the pellet of 1,500 g centrifugation.

**Distribution of LNAs.** The sum of lunularic acid and prelunularic acid was determined as the amount of LNAs. These two compounds in an aliquot (0.5 ml) of each fraction were extracted with boiling methanol containing 1% (v/v) acetic acid (6 ml) and then roughly purified by a Sep-pak C18 cartridge (Waters Associates). Quantitative determination of LNAs in the extract was carried out according to a previous paper (2). Briefly, prelunularic acid was converted into lunularic acid by alkaline treatment (1 N NaOH, 10 min). After acidification, lunularic acid was extracted with ethyl acetate, and subsequently transferred into 60% acetonitrile containing 0.1% (v/v) acetic acid. The amount of lunularic acid was determined by HPLC fitted with a Partisil ODS column (i.d. 4 mm × 25 cm) by comparing the peak height monitored at 285 nm with that of standard solution. The elution solvent was 50% (v/v) acetonitrile in water containing 20 mm ammonium acetate adjusted to pH 3.6 with acetic acid.

**Table I. Distribution of LNAs in the Fractions Obtained by Differential Centrifugation of Cell Homogenate of M. polymorpha**

The cell homogenate was subjected to three-step differential centrifugation. The amount of LNAs in each fraction was determined and expressed by the values per g dry weight of cells used.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>LNAs µmol g⁻¹ dry wt</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,500 g pellet</td>
<td>0.08</td>
<td>0.4</td>
</tr>
<tr>
<td>10,000 g pellet</td>
<td>0.12</td>
<td>0.5</td>
</tr>
<tr>
<td>100,000 g pellet</td>
<td>0.14</td>
<td>0.6</td>
</tr>
<tr>
<td>100,000 g supernatant</td>
<td>21.86</td>
<td>98.5</td>
</tr>
<tr>
<td>Total</td>
<td>22.20</td>
<td></td>
</tr>
<tr>
<td>Original cells</td>
<td>21.43</td>
<td></td>
</tr>
</tbody>
</table>
activity of mannosidase in this fraction was concentrated 44-fold comparing with that in the crude mixture. The assay of cytoplasmic marker enzymes, PEP carboxylase and 3-P glycerate kinase, indicated that this vacuolar fraction was not contaminated with a significant amount of cytoplasm. Almost 25% of LNAs was recovered in this fraction proving that a large amount of LNAs was located in the vacuoles. On the other hand, the 14% Metrizamide layer including the pellet (cytoplasmic fraction) contained almost all activities of cytoplasmic marker enzymes. This fraction also included 37.6% of mannosidase and 66.4% of LNAs.

On the basis of these results, the proportion of LNAs originally present in the vacuole to the total amount in protoplast was calculated. In the calculation, the following two assumptions have been made: (a) the activities of mannosidase and PEP carboxylase represent the amount of vacuole and cytoplasm, respectively; and (b) LNAs is localized only in vacuole and cytoplasm, which are designated \( L_v \) (vacuolar LNAs) and \( L_c \) (cytoplasmic LNAs), respectively. Then, the relative amount of LNAs present in vacuolar fraction (25.1%) is obtained from the vacuoles (49.2%) and the cytoplasm (0.8%). This leads to the following equations:

\[
L_v + L_c = 1 \\
0.492 \cdot L_v + 0.008 \cdot L_c = 0.251
\]

A value 0.50 is obtained for \( L_v \) by solving these equations, which indicates that 50% of total LNAs in protoplast is located in vacuole. Similar equations are also obtained for cytoplasmic fraction:

\[
L_v + L_c = 1 \\
0.376 \cdot L_v + 0.959 \cdot L_c = 0.664
\]

The solution of these equations gives a value 0.51 for \( L_v \). This result from cytoplasmic fraction also indicates that a half amount of LNAs is localized in vacuole. In two other separate experiments undertaken in the same way (data not shown), close values for \( L_v \) were obtained from both vacuolar and cytoplasmic fractions.

**DISCUSSION**

The differential centrifugation of homogenate of cultured cells of *M. polymorpha* proved that LNAs was present not in the bound form with cell walls or membranous components but in a soluble form in the matrix of organelles or cytoplasm.

The isolation of intact organelles was carried out using protoplasts, since it has the advantage of reducing the risk of organelle breakage. Sedimentation centrifugation showed that LNAs was accumulated in neither chloroplasts, mitochondria nor peroxisome, although these organelles, especially the chloroplasts, are plausible sites of synthesis and accumulation of these compounds. The results from this centrifugation also suggested that LNAs are present in the vacuole and/or cytoplasm. The separation of vacuole from cytoplasm was more effectively carried out by flotation centrifugation using Metrizamide gradient in fairly short time (10 min) under weak centrifugal force (400g). With these conditions, only the vacuole migrated upward. This appeared to be more effective in reducing the contamination of vacuoles, not only with other organelles but also with cytoplasm, than the sedimentation method. The vacuoles thus obtained contained a considerable amount of LNAs.

Our experiment did not exclude the possibility that LNAs, which are small molecules, may have leaked out of the vacuole. Leigh et al. (12) observed that incomplete lysis and leakage of the contents occurred when the vacuoles (in 1.2 m sorbitol) originated from beet root were suspended in a hypotonic medium (0.3 m sucrose). However, nicotin (21), a cyanogenic glucoside,
Table II. Distribution of LNAs and Markers in Fractions Obtained by Density Gradient Centrifugation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>LNA S</th>
<th>Mannosidase</th>
<th>Acid Phosphatase</th>
<th>PEP Carboxylase</th>
<th>3-P-G Kinase</th>
<th>Protein</th>
<th>Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol %</td>
<td>nmol min⁻¹%</td>
<td>nmol min⁻¹%</td>
<td>nmol min⁻¹%</td>
<td>%</td>
<td>µg %</td>
<td>µg %</td>
</tr>
<tr>
<td>0% Metrizamide</td>
<td>6</td>
<td>2.0</td>
<td>2.6</td>
<td>2.7</td>
<td>38</td>
<td>4.5</td>
<td>ND</td>
</tr>
<tr>
<td>0%/7% interface</td>
<td>73</td>
<td>25.1</td>
<td>47.5</td>
<td>49.2</td>
<td>353</td>
<td>41.5</td>
<td>1.2</td>
</tr>
<tr>
<td>7% Metrizamide</td>
<td>19</td>
<td>6.5</td>
<td>10.2</td>
<td>10.6</td>
<td>86</td>
<td>10.1</td>
<td>5.0</td>
</tr>
<tr>
<td>Total</td>
<td>193</td>
<td>66.4</td>
<td>36.3</td>
<td>37.6</td>
<td>374</td>
<td>34.9</td>
<td>145.4</td>
</tr>
<tr>
<td>Crude mixture</td>
<td>295</td>
<td>105.0</td>
<td>851</td>
<td>841</td>
<td>151.6</td>
<td>154.2</td>
<td>7722</td>
</tr>
</tbody>
</table>

* ND, not detected.

dhurrin (22), and shikimic acid (8) were reported to be retained in the vacuoles isolated osmotically as in our experiment.

Estimation of the ratio of vacuolar and cytoplasmic LNAs was performed by regarding activities of mannosidase and PEP carboxylase to represent the amount of vacuole and cytoplasm, respectively. Mannosidase has been reported to occur only in vacuoles in plant cells from various tissues including suspension cultured cells (3). PEP carboxylase is not a strict marker of cytoplasm as shown by its occurrence in chloroplasts in some cases (23). However, its exclusive or predominant location in the cytoplasm seems to be more general (14, 26). The results obtained by calculations of the vacuolar fraction showed that LNAs was almost equally distributed in vacuole and cytoplasm. This conclusion was corroborated by consistency with the results derived from the cytoplasmic fraction.

We did not determine the localization of labile prelunularic acid independently of lunalric acid. However, the intracellular distribution of LNAs described above can be reasonably recognized as that of prelunularic acid, since at least 99.8% of LNAs has been proven to be prelunularic acid in our previous paper (2). The fact that a considerable amount of prelunularic acid was distributed in the cytoplasm suggested that this compound does not have any inhibitory effect comparable to lunalric acid on cellular processes. If it does, prelunularic acid present in cytoplasm at high concentration would be expected to express a recognizable degree of inhibition of cellular growth. But the growth of *Marchantia polymorpha* cells is actually comparable to that of cultured cells of other plants. This suggestion is also supported by our preliminary experiment in which exogenously applied prelunularic acid did not show any noticeable inhibitory activity on the growth of gamma of *Marchantia polymorpha* as lunalric acid did (data not shown).

On the other hand, the result that at least half of the prelunularic acid was localized in the vacuole reminds us of the phenomenon of metabolic excretion of secondary metabolites, especially that of phenolics. Localization of anthocyanin (25) and coumarin (15) in the vacuole have been shown by organelle fractionation. Tannin has been shown to be accumulated in the ER and small cytoplasmic vacuoles as well as in the vacuole by electron microscopic examination of cultured cells of white spruce (4). Such compounds deposited in vacuole are believed not to express growth-regulatory effects.

On the basis of the facts mentioned above, we reconsider the previously proposed growth-regulatory role of endogenous lunalric acid itself. Prelunularic acid is very unstable and can be easily converted into lunalric acid nonenzymically. We question how such a spontaneous conversion can control the concentration of lunalric acid as a growth regulator, if any, and also why the labile precursor is accumulated in such a large quantity.

In general, many phenolics, for example cinnamic acid derivatives such as coumarin, and depsids such as chlorogenic acid, are also clearly able to exert significant inhibitory effects on the growth process of plant tissues when applied exogenously. However, this does not necessarily imply that they act in the same way as endogenous growth regulator. Although the putative growth regulator role of phenolics has been postulated by a number of investigators (6), it is still uncertain whether they actually play any physiological role apart from an ecological one. The situation seems to be same for lunalric acid and prelunularic acid. It is worthwhile to mention that the amount of phenolic compounds in plant tissue generally increases when the plant is exposed to unfavorable conditions for growth. The amount of LNAs in the cultured cells of *Marchantia polymorpha* was also remarkably increased in a medium depleted of phosphate (1). In this sense, they can be regarded as a kind of stress metabolite. The accumulation of such stress compounds under unfavorable conditions is often accompanied by the decline of metabolic activities of the tissue. However, this does not necessarily mean that the stress metabolites formed, including lunalric acid and prelunularic acid, suppress the metabolic activities of the plant leading to inhibition of growth. More information is required to make clear the physiological role of endogenous phenolic compounds.

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