Note

Purification of Phosphoinositol Kinase from Suspension-cultured Cells of Rice (Oryza sativa L.)

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Phosphoinositol kinase was purified from suspension-cultured rice (Oryza sativa L.) cells. The apparent molecular mass of the rice enzyme was estimated to be 58 kDa by SDS–PAGE and 234 kDa by Toyopearl HW-55S gel filtration, indicating that it is a tetrameric enzyme. The enzyme absolutely required Mg\(^{2+}\) for the activity, but Mn\(^{2+}\) and Ca\(^{2+}\) did not affect it. In addition, this kinase phosphorylated inositol monophosphate to phytate. Judging from these data, rice phosphoinositol kinase was concluded to be a different enzyme distinguished from the other plant phosphoinositol kinases.

Inositol phosphates are accumulated as a phosphate storage substance, phytin, in seeds and other storage tissues, and they are a component of inositol phospholipids.\(^1\) It has been proposed, that inositol 1,4,5-trisphosphate has an important role in a plant signal transduction system, since it stimulates the release of Ca\(^{2+}\) from the internal store in plant cells.\(^2\) In addition, phospholipase C, which catalyzes cleavage of PIP\(_2\) to inositol 1,4,5-trisphosphate, has been purified from rice cells.\(^3\) Two phosphorylation pathways from myo-inositol to phytate have been proposed: one of them proceeds via the stepwise phosphorylation of inositol phosphates, and phosphoinositol kinase catalyzes stepwise phosphorylation of inositol phosphates but not phosphorylate myo-inositol.\(^4\) The other proceeds via polyinositolphosphate intermediates. Inositol 1,4,5-trisphosphate is released from PIP\(_2\) by phospholipase C and phosphorylated to phytate.\(^5,6\) In rice cells, it has been observed that a series of inositol phosphates are accumulated during the growth cycle.\(^3\) myo-Inositol kinase and phospholipase C producing inositol 1,4,5-trisphosphate from PIP\(_2\) have been identified in the cells.\(^7,8\) In this communication, we report the purification and some properties of a phosphoinositol kinase from rice suspension-cultured cells.

For standard assay of phosphoinositol kinase, a reaction mixture consisting of 200 mM Tris–HCl buffer (pH 7.2), 5 mM MgCl\(_2\), 10 mM 2-mercaptoethanol, 1 mM inositol trisphosphate, 1 mM [γ-\(^{32}\)P]-ATP (1850 kBq/mol), and enzyme in a final volume of 1 ml was incubated for 2 h at 30°C. The reaction was stopped in boiling water for 2 min, and then the mixture was centrifuged at 15,000 × g for 10 min; phytate hydrolysate (1 μmol phosphorus) was added to the supernatant as a carrier. \(^{32}\)P-Labeled inositol phosphates obtained from the phosphorylation of the kinase reaction were put on an AG-1X2 column (0.9 x 4 cm; Bio-Rad). The column was washed with water, and inositol phosphates were eluted with 60 ml of 0.2 N HCl and 100 ml of 1.0 N HCl sequentially. The eluate with 1.0N HCl containing total reaction products (inositol tetrakisphosphate ~ phytate) were concentrated and dried by a rotary evaporator, and dissolved in 1.0 ml of water. The radioactivity was measured by a liquid scintillation counter (Aloka LSC-900) using a toluene–Triton X-100 scintillator system. The kinase activity was not detected in the step before Con A-Sepharose column chromatography, since there was strong phosphatase activity in the enzyme sample.

Suspension-cultured cells derived from embryos of rice seeds (Oryza sativa L. cv. Nipponkai) were grown in MS medium\(^9\) for 4 days at 28°C as described in the previous report,\(^9\) since the maximum expression of phosphoinositol kinase activity in the rice cells occurred in the middle of log phase, around at 3–4 days of the culture period (data not shown). Rice cells (785 g fresh weight) harvested were homogenized with two volumes of 20 mM Tris–HCl buffer (pH 7.2) containing 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 20,000 × g for 20 min, and then the supernatant was fractionated with ammonium sulfate. The fraction from 40% to 60% saturation was collected and dialyzed against buffer A (20 mM Tris–HCl [pH 7.2], 5 mM 2-mercaptoethanol, 1 mM MnCl\(_2\), 1 mM CaCl\(_2\), and 0.5 mM NaCl) and put on Con A-Sepharose (2.5 x 20 cm; Pharmacia Fine Chemicals) equilibrated with buffer A. The pass-through fraction from the column was precipitated in 60% saturation of ammonium sulfate. The pellet was dissolved in buffer B (20 mM Tris–HCl [pH 7.6], 2 mM 2-mercaptoethanol and 40% glycerol) and dialyzed against buffer B. The dialyze was put on DEAE-Toyopearl (2.5 x 65 cm; Tosoh) equilibrated with buffer B. After the column was washed with 1 liter of buffer B, proteins were eluted with 2.5 liters of a linear concentration gradient of 0 to 0.3 M NaCl in buffer B. The active fractions were pooled and concentrated by Amicon PM-30. The concentrated enzyme was put on Toyopearl HW-55S gel filtration column (2.2 x 115 cm; Tosoh) equilibrated with buffer B. The proteins were eluted with buffer B at a flow rate of 0.5 ml/h. The active fractions were pooled and concentrated by Amicon PM-30. The concentrated enzyme was dialyzed against buffer B and then put on a 5′AMP-Sepharose affinity column (1.5 x 8 cm; private produces) equilibrated with buffer B. After washing the column with 40 ml of buffer B, proteins were eluted with 1 mM ATP in buffer B. The active fractions (fraction No. 48–55) were pooled and concentrated. All purification steps were done at 0 to 4°C.

A summary of the purification of rice phosphoinositol kinase is given in Table I. The specific activity of the purified enzyme was 1.8 μmol/min/mg protein. The separation profile of phosphoinositol kinase on a 5′AMP-Sepharose affinity column is shown in Fig. 1. The distribution of phosphoinositol kinase activity coincided with the protein peak observed. Native and SDS–PAGE analyses indicate that the enzyme preparation obtained by the 5′AMP-Sepharose column chromatography is a homogeneous protein (Fig. 2). Apparent molecular size estimated by a Toyopearl HW-55S gel filtration was 234 kDa (Fig. 3), and the molecular mass of the subunit was estimated by SDS–PAGE to be 58 kDa (Fig. 2B). These data appear to indicate that the rice phosphoi-
nositol kinase exists as a tetrameric protein. Chakraborti
and Biswas have reported that phosphoinositol kinase purified
from cotyledons of mung beans germinated for 24 h had two
different forms (phosphoinositol kinase-A and B). 10 Phosphoino-
sitol kinase-A was composed of two subunits (82 kDa and 62 kDa)
and phosphoinositol kinase-B had three subunits (82 kDa, 39 kDa,
and 34 kDa). Rice phosphoinositol kinase absolutely required
Mg$^{2+}$ for the enzyme activity, but Mn$^{2+}$ and Ca$^{2+}$ did not affect
it (data not shown). The mung bean phosphoinositol kinase also
required Mg$^{2+}$, and Mn$^{2+}$ was twice as effective as Mg$^{2+}$ for
the enzyme activity. 10 Judging from these data, we concluded that
rice phosphoinositol kinase is a different enzyme distinguished
from mung bean phosphoinositol kinases.

However the structures of the enzyme reaction products have
not yet been decided; rice phosphoinositol kinase catalyzes the
phosphorylation of each inositol phosphate (inositol monophos-
phate-inositol pentakisphosphate) to their corresponding higher
phosphorylated ones (Table II). In the hypothetical pathway via
polynosphoinositol intermediates, Raboy has mentioned a possi-
bility that plant phosphoinositol kinases are analogous to in-
ositol 1,4,5-trisphosphate and inositol 1,2,4,5-tetrakisphosphate
kinases isolated from animal cells. 11 We identified stereoisomers
of inositol tetrakisphosphates, which were inositol 1,3,4,5-tetra-
ksiphosphate, inositol 1,3,4,5-tetrakisphosphate, and inositol
pentakisphosphates, two of which were inositol 1,2,3,4,5-pent-
aksiphosphate and inositol 1,2,4,5,6-pentakisphosphate, that

**Table I. Purification of Rice Phosphoinositol Kinase**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total activity (units$^a$)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg protein)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>—</td>
<td>13512</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>—</td>
<td>4791</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A-Sepharose</td>
<td>2072</td>
<td>1449</td>
<td>1.4</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>1664</td>
<td>107</td>
<td>15.5</td>
<td>11.1</td>
<td>80.3</td>
</tr>
<tr>
<td>Toyopearl HW-55S</td>
<td>1636</td>
<td>52.9</td>
<td>30.9</td>
<td>22.1</td>
<td>80</td>
</tr>
<tr>
<td>5'AMP-Sepharose</td>
<td>369</td>
<td>0.2 1844</td>
<td>1317</td>
<td>17.8</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ One unit has been defined as 1 nmol $^{32}$P converted to inositol phosphates per minute.

**Fig. 1.** Separation Profile of Rice Phosphoinositol Kinase by 5'AMP-
Sepharose Affinity Chromatography.

- ○ - , activity; — , protein.

**Fig. 2.** Native and SDS-PAGE of Purified Rice Phosphoinositol Kinase.

(A) Native-PAGE: The gel was stained with Amido black.

(B) SDS-PAGE: The gel was stained with CBB.

Phosphorylase b (97.4 kDa), glutamate dehydrogenase (55.4 kDa), lactate dehydroge-
nase (36.5 kDa), and trypsin inhibitor (20.1 kDa) were used as molecular mass
standards on SDS gels.

**Fig. 3.** Estimation of the Molecular Size of Phosphoinositol Kinase.

1. ferritin (450 kDa); 2. catalase (240 kDa); 3. albumin from bovine serum (68 kDa);
4. albumin from hen egg (45 kDa); 5. cytochrome c (12.5 kDa).

**Table II. Substrate Specificity of Rice Phosphoinositol Kinase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (μmol/min/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>Inositol monophosphate</td>
<td>0.37</td>
</tr>
<tr>
<td>Inositol bisphosphate</td>
<td>0.49</td>
</tr>
<tr>
<td>Inositol trisphosphate</td>
<td>1.84</td>
</tr>
<tr>
<td>Inositol tetrakisphosphate</td>
<td>1.92</td>
</tr>
<tr>
<td>Inositol pentakisphosphate</td>
<td>3.08</td>
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Substrates were prepared from phytate by hydrolysis according to the
methods of Hayakawa et al. 13 The enzyme activity was measured by
variable methods described in the text. Inositol phosphates were eluted
from an AG-1X2 column as follows: inositol monophosphate was eluted
with 0.1 N HCl, inositol bisphosphate was eluted with 0.15 N HCl, inositol
trisphosphate was eluted with 0.2 N HCl, inositol tetrakisphosphate was
eluted with 0.3 N HCl, and inositol pentakisphosphate was eluted with
0.5 N HCl. After substrates were eluted, the reaction products were eluted
with 1 N HCl.
occurred in rice cultured cells (unpublished data). It has been proposed that not only inositol 1,4,5-trisphosphate but also inositol 1,3,4,5-tetakisphosphate is a candidate for a second messenger molecule in animal cells. To clarify the possibility that plant phosphoinositide kinases may be involved in signal transduction system via polyphosphoinositide, we are now examining the physiological roles of inositol tetrakisphosphate and inositol pentakisphosphate produced by rice phosphoinositol kinase.

References