Degradation of Phytic Acid and Changes of Phosphatase Activities during Cultivation of *Typha* Pollen

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Typha pollen contained phytic acid in which about 50% of the total phosphorus in the extract was comprised. Phytic acid remained about 35% of that of mature pollen in the first 1 h-cultivation but almost disappeared by 3 h-cultivation when the pollen tube elongated sufficiently. During cultivation inositol-trisphosphate, which is a final hydrolytic product by a phytic acid-specific phytase, was not accumulated. The extracts of *Typha* pollen showed some activity peaks of phosphatases for *p*-nitrophenylphosphate, phytic acid and inositol 1, 2, 3-trisphosphate, when fractionated on a DEAE-cellulose column. In comparison with the activities in the extract of ungerminated pollen, those in the extract after 1 h-cultivation increased remarkably for three substrates and the activity for inositol-trisphospahte was more elevated after 3 h-cultivation.

Key words : Inositol 1, 2, 3-trisphosphate, Phosphatase, Phytase, Phytic acid, *Typha* pollen.

Phytic acid, *myo*-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate, is a normal component in plants and especially abundant in mature seeds and tubers. Cosgrove suggested five physiological roles of phytic acid as a phosphorus reserve store, an energy store, a competitor of ATP, an immobilizer of multivalent cations and a regulator of the level of inorganic phosphate.[1]

Jackson *et. al.* have reported that plant species with a style length of 5 mm or more contain phytic acid in the range of 0.05-2.1% by weight and suggested a role of phytic acid as a precursor for cell wall synthesis necessary for rapid tube elongation.[2] Lin *et al.* reported that the breakdown of phytic acid proceeds at a constant rate by 3 h during germination in lily pollen.[3] Inositol, which occurs by the complete dephosphorylation of phytic acid after germination, is utilized for phospholipid biosynthesis and pectin synthesis.[4] On the other hand, inositol trisphosphate derivatives, inositol 1, 4, 5-trisphosphate, 1, 3, 4-trisphosphate have been known as a second messenger for calcium release.[5]

We have found a new type of phytase in *Typha* pollen,[6] differing from 3-phytase and 6-phytase which have previously been reported.[7] It was specific for phytic acid and showed a product-specificity, yielding an inositol-trisphosphate as a final hydrolysis product.

In this paper, the degradation of phytic acid and changes of phosphatase activities for *p*-nitrophenylphosphate, phytic acid and inositol 1, 2, 3-trisphosphate as substrates were examined to clarify the utilization of inositol or its derivatives in germination of *Typha* pollen.

Materials and Methods

1. Pollen and cultivation

Mature pollen grains were collected from a plant (*Typha latifolia* L.) in a paddy field at Nisshin-cho, Aichi prefecture in June 1989. They were dried for 2 days at room temperature and stored below −20℃ until use. Cultivation of the pollen was done on a 1.5% agar medium containing 3% sucrose at 30℃.

2. Analysis of phosphate compounds in extracts from the pollen

Eight grams of the pollen or the culture from 8 g of the pollen were added into 80 ml of boiling 1 mM EDTA and heated for 10 min. After cooled, 10 ml-portions of the mixture were disintegrated for 10 min in a Teflon-glass homogenizer and the combined homogenates were centrifuged at 20,000× g for 10 min. The supernatant was put on a column (0.8×16 cm) of Dowex 1×2 and washed with 20 ml of water. Then the materials were eluted with a linear gradient of 0 to 0.6 M HCl (total volume of 700 ml). A control run on a Dowex column was done using the partial hydrolysate of phytic acid (300 mg) obtained by autoclaving at pH 4.0 for 40 min as the same method described above.

One ml of each fraction containing phosphate compounds was mixed with 0.2 ml of 60% perchloric acid and organic phosphates were dephosphorylated by heating at 180℃ for 4 h. The Pi content in both fractions before and after the dephosphorylation was measured by the method of Furchgott and Gudareff.[8]

3. Determination of inositol content

Inositol content was examined by the method of Lornitzo[9] using the dephosphorylated fractions for Pi measurement described above.

4. Preparation of inositol 1, 2, 3-trisphosphate

The phytase was purified from *Typha* pollen as described previously by the authors.[6] Two hundred milliliter of the reaction mixture containing 25 mM Tris-HCl buffer (pH 8.0), 2.5 mM sodium phytate, 2.5 mM $CaCl_2$ and 4.25 units of purified phytase was incubated at 37℃ for 20 h. Then the mixture was added with a small amount of Amberlite IR 120B (H^+) and lowered pH to about 3.0. After the mixture was centrifuged at 20,000× g for 10 min, the supernatant was put on a column (1×26 cm) of Dowex 1×2 and washed with 20 ml of water. Then the materials were eluted with a linear gradient of 0 to 0.6 M HCl (total volume of 1600 ml). Organic phosphate in the fractions were dephosphorylated by the method described above. The fractions containing inositol-trisphosphate were collected, neutralized with 1 M LiOH and evaporated to dryness under a reduced pressure. The residue was washed twice with methanol,

once with acetone and dried in a desiccator. The obtained material (170 mg) was identified as the *myo*-inositol 1, 2, 3-trisphosphate with NMR analysis by Dr. L. Persson of Perstorp Carbotec Co., Sweden.

5. Fractionation of phosphatases

Three grams of the pollen or the culture from 3 g of the pollen were suspended in 30 ml of 10 mM Tris-HCl, pH 7.5, containing 10% glyceorl and 1 mM 2-mercaptoethanol. Ten-ml portions of the suspension were disintegrated for 10 min in a Teflon-glass homogenizer and the combined homogenates were centrifuged at 20,000× g for 10 min. The supernatant was dialyzed against the above buffer and put on a column (1.5×25 cm) of DEAE-cellulose equilibrated with the same buffer. After the column was washed with 40 ml of the same buffer, the enzymes were eluted with a linear gradient of 0 to 0.6 M NaCl in the same buffer (total volume of 200 ml).

6. Assay of phosphatases

Phosphatase activities were determined using *p*-nitrophenylphosphate, sodium phytate and inositol 1, 2, 3-trisphosphate as substrates. The reaction mixture contained 0.1 M buffer (Tris-acetate, pH 6.5 or Tris-HCl, pH 8.0), 2 mM substrate (0.5 mM for inositol-trisphosphate) and appropriately diluted enzyme in a total volume of 1 ml. The assay mixture for sodium phytate at pH 8.0 contained 2 mM $CaCl_2$ in addition to the above. The reactions were done at 37℃ for 30 min and stopped by the addition of 0.5 ml of 0.3 M perchloric acid. The Pi liberated was measured by the method of Furchgott and Gudareff.[8] One unit of enzyme was defined as the amount that released 1 μmole of Pi per min under the above conditions.

Results and Discussion

The existence of phytic acid was confirmed in the extract from *Typha* pollen as shown in Fig. 1-B. The content of Pi derived from phytic acid was estimated to be approximately a half of the content of total Pi, which was shown by the sum of Pi in hydrolysate of each fraction, in the extract. Phytic acid content decreased to 35% of that of mature pollen in the first 1 h-cultivation when the length of pollen tube was almost comparable to a diameter of native pollen bodies and almost degraded by 3 h-cultivation when the length of pollen tube elongated sufficiently, that is, more than several times that of native pollen bodies. The result is similar to the breakdown of phytic acid during germination in lily pollen[3] and suggests the utilization of inositol, sugar moiety of phytic acid, as a precursor material for tube cell wall polysaccharide synthesis.

During the cultivation, inositol-pentakisphosphate and inositol-tetrakisphosphate were not accumulated (Fig. 1-C, D). The identification of the peak of inositol-trisphosphate was somewhat difficult, but its remarkable existence or accumulation was denied by comparing the elution positions of Pi and inositol with that of standard inositol-trisphosphate.

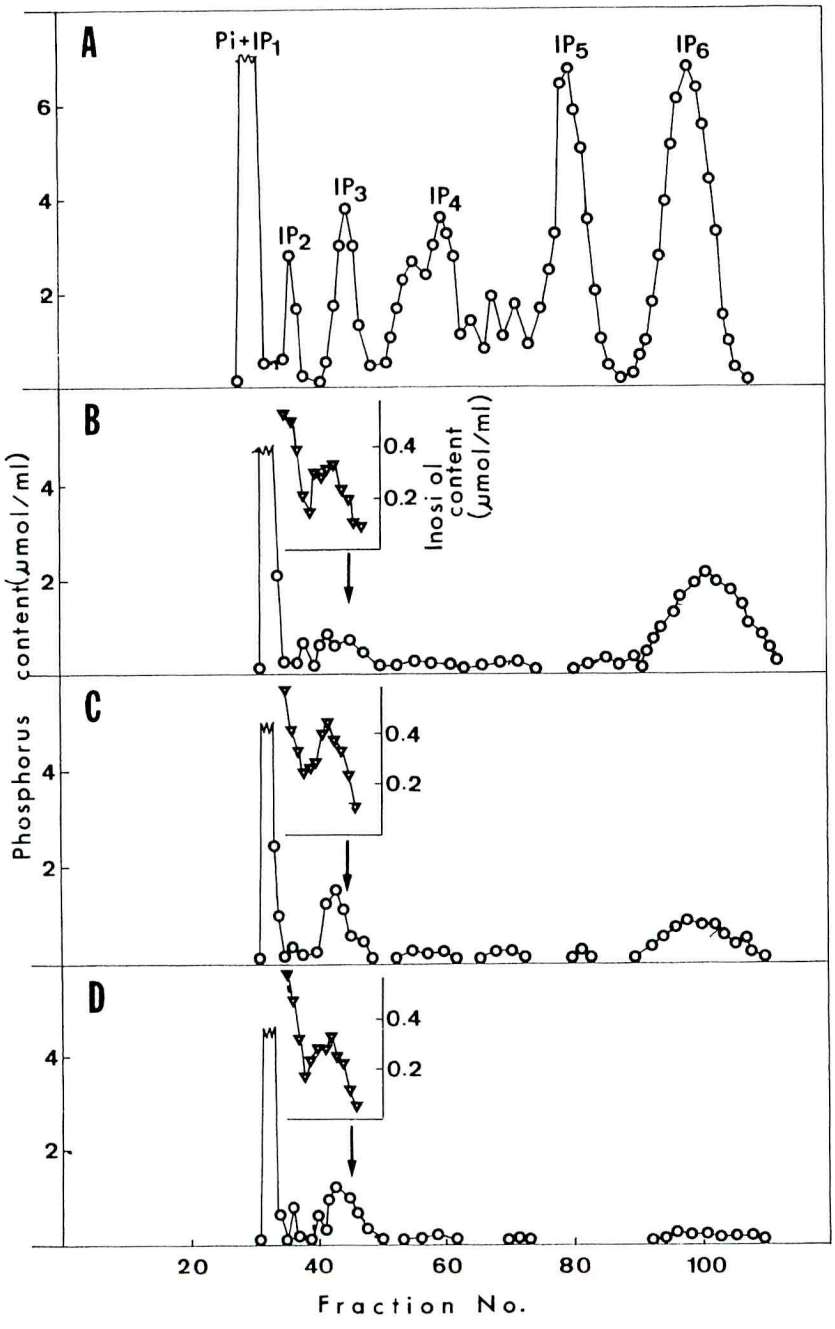

Fig. 1. Elution patterns of the extracts from *Typha* pollen on a column of Dowex 1×2.
(A) Partial hydrolysate of phytic acid ; (B) Extract from the pollen before cultivation ; (C) Extract from the pollen after 1 h-cultivation ; (D) Extract from the pollen after 3 h-cultivation. Phosphorus content was indicated as amount of Pi in each fraction after the complete dephosphorylation treatment. IP_1, IP_2, IP_3, IP_4, IP_5 and IP_6 are inositol-monophosphate, inositol-bisphosphate, inositol-trisphosphate, inositol-tetrakisphosphate, inositol-pentakisphosphate and inositol-hexakisphosphate respectively. The arrows in Fig. 1-B-D show the elution position of inositol-trisphosphate in Fig. 1-A.

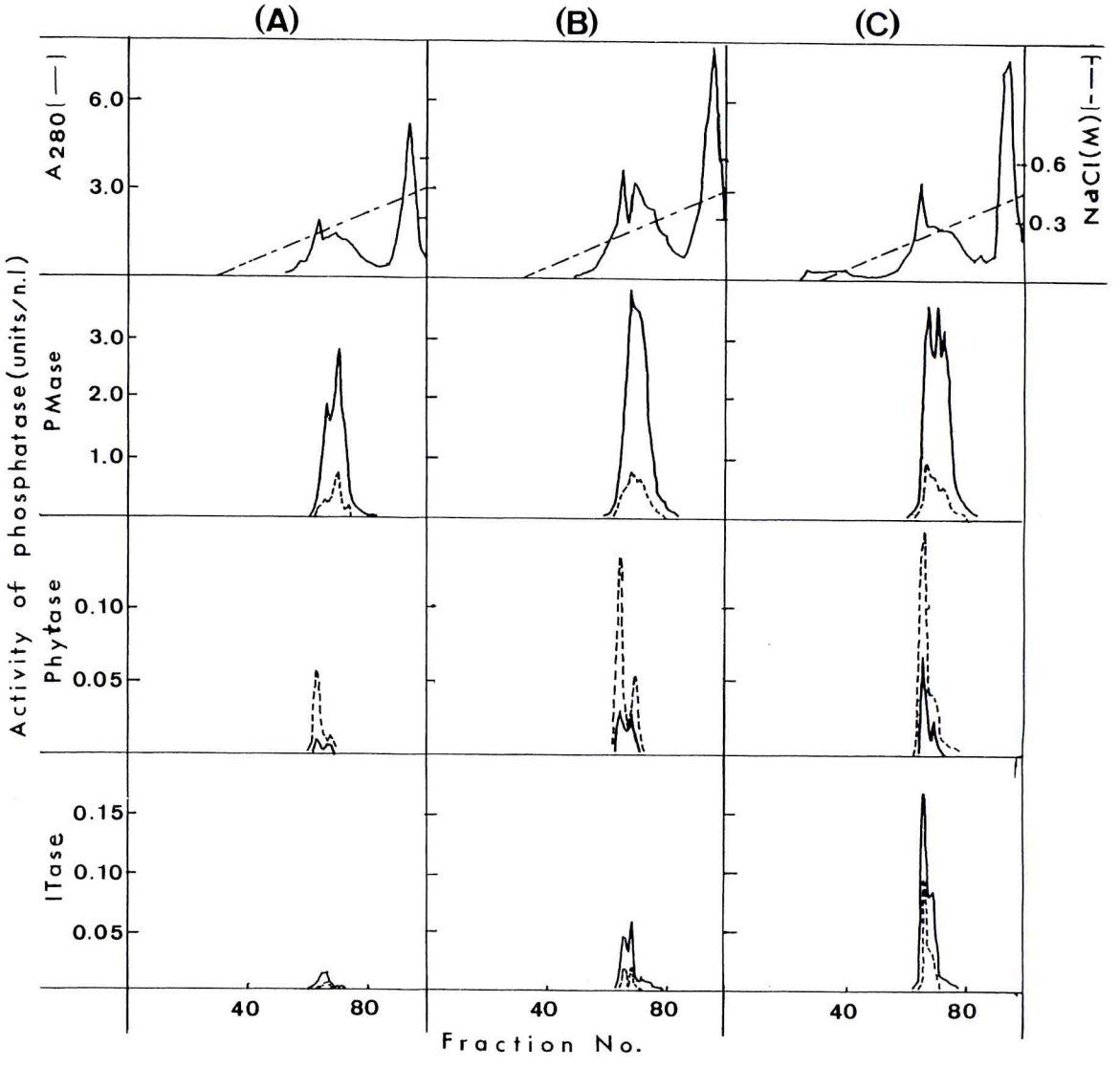

Fig. 2. Elution patterns of phosphatases of the extracts from *Typha* pollen on a DEAE-cellulose. (A) Extract from the pollen before cultivation ; (B) Extract from the pollen after 1 h-cultivation ; (C) Extract from the pollen after 3 h-cultivation. A_{280} shows absorbances at 280 nm. PMase, Phytase and ITase show the activities for *p*-nitrophenylphosphate, phytic acid and inositol 1, 2, 3-trisphosphate respectively. ———, Activity in Tris-acetate buffer (pH 6.5) ; ------, Activity in Tris-HCl buffer (pH 8.0) ;

As shown in Fig. 2, some activity peaks of phosphatases for each substrate were detected in both extracts from ungerminated and germinated pollens. The activities for *p*-nitrophenylphosphate and inositol 1, 2, 3-trisphosphate were stronger at pH 6.5 but that for phytic acid was higher at pH 8.0, suggesting the dependency on the phytic acid-specific phytase.[6] After 1 h-cultivation there was an increasing change of each activity for three substrates and the activity

for inositol-trisphosphate was more markedly elevated between the cultivation of 1 h and 3 h.

The results obtained here make us imagine that the mode of degradation of phytic acid and its derivatives are fairly complicated. We suppose that phytic acid is attacked giving rise to inositol-trisphosphate mainly by phytic acid-specific phytase and partially by nonspecific phosphatases, and inositol-trisphosphate or lesser inositol phosphates is also degraded by some phosphatases including a newly synthesized phosphatase having a stronger activity for inositol-trisphosphate. During this process, inositol-trisphosphate is not accumulated, therefore it seems that the product-specificity of the phytic acid-specific phytase, yielding an inositol-trisphosphates as a final hydrolysis product,[6] will not have specialized meaning, such as a second messenger,[5] in pollen germination.

There are many reports on the appearance of newly synthesized enzymes during germination of pollen. We confirmed the increase of phosphatase activities in *Typha* pollen and are now examining the isolation and properties of these phosphatases.

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ガマ花粉培養中のフィチン酸の分解とホスファターゼの活性変動

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ガマ花粉はフィチン酸を含んでおり，それに由来するリン酸は抽出物中の全リン酸の約50%を占めていた．フィチン酸は1時間の培養で約35%残存していたが，花粉管が十分に伸長した3時間培養後には完全に消失した．フィチン酸特異的フィターゼによるフィチン酸の最終分解産物であるイノシトール1，2，3-三リン酸は，培養中に蓄積しなかった．

ガマ花粉抽出物は *p*-ニトロフェニルリン酸，フィチン酸およびイノシトール1，2，3-三リン酸を基質としたとき，DEAE-セルロースカラムクロマトグラフィによって分画されるいくつかのホスファターゼ活性ピークを示した．成熟花粉と比較すると，3種の基質に対するホスファターゼ活性は1時間培養後著しく増加したが，イノシトール-三リン酸に対する活性は3時間培養でさらに高められた．