Contribution of Phosphatases in Phytic Acid Metabolism during Cultivation of *Typha* Pollen

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Phosphatases in the extract from the cultured cell of *Typha* pollen were separated into 22 fractions by means of protamine treatment, anion exchange chromatography, affinity chromatography and gel-filtration. They were roughly classified into five groups on the basis of the substrate specificity. All of phosphatases in each group showed poor activities for phytic acid and inositol 1, 2, 3-trisphosphate (ITP). Although a phosphatase (D1A3G2) among them acted clearly for both substrates, its activity was only 13% for ITP and 6% for phytic acid as compared with that for *p*-NPP. From the results obtained, it was concluded that phytic acid in *Typha* pollen during cultivation is attacked by phytic acid-specific phytase to give rise to ITP, then to inositol monophosphate by the action of D1A3G2, and inositol monophosphate is finally hydrolyzed into inositol and Pi in cooperation with some nonspecific phosphatases.

Key words : Phosphatase, Phytic acid, Inositol phosphate, Phytase, *Typha* pollen.

Phytic acid is important for mature seeds and tubers 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]. In pollen, it is suggested that inositol, a sugar moiety of phytic acid, is utilized for phospholipid biosynthesis, pectin synthesis[2] and cell wall synthesis[3]. This is surmised from the fact that phytic acid in lily pollen and *Typha* pollen almost disappears after 3 hr of germination[4,5]. The degradation process of phytic acid, however, seems to be complex. In *Petunia* pollen the activity degrading phytic acid develops after 2 hr of germination as a result of translation of a stable RNA already prepared in ungerminated pollen[6]. Lily pollen has three kinds of phytases which differ in pH optimum[4,7]. pH optimum 6.5-phytase is induced during germination[4], and pH optimum 8.0-phytase is localized at the membrane of a storage body[8] and effectively extracted with detergent[7]. *Typha* pollen contains a phytase which is highly specific for phytic acid and yields inositol trisphosphate as a final hydrolysis product[9]. The activity hydrolyzing inositol trisphosphate was very low in mature pollen but increased remarkably during cultivation[5].

We tried to clarify the degradation process of phytic acid to inositol and Pi in *Typha* pollen.

Since the first hydrolysis of phytic acid in *Typha* pollen is done predominantly with the phytic acid-specific phytase, the second process, that is the hydrolysis of inositol trisphosphate, must be examined. In this paper we describe the isolation and classification of phosphatases in the extracts from the cultured *Typha* pollen, and discuss the contribution of the classified phosphatases in phytic acid metabolism.

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 1991. 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. Chemicals

p-Nitrophenyl phosphoric acid disodium salt (*p*-NPP) and phytic acid sodium salt were purchased from Nacalai Tesque Co. DE-52 (cellulose anion exchanger) was obtained from Whatman Paper Ltd. Epoxy-activated Sepharose 6B and HiLoad 26/60 Sephacryl S-200 HR were from Pharmacia LKB Biotechnology. Ultra Thimble UH100/10 was from Schleicher & Schuell. Inositol 1, 2, 3-trisphosphate (ITP) was prepared as described in a previous paper[5].

3. Assays of phosphatase, ITase and phytase

Phosphatase activity was determined using *p*-NPP as a substrate. The reaction mixture in 1.0 ml consisted of 2 mM *p*-NPP, 0.1 M Tris-acetate buffer, pH 6.5 and appropriately diluted enzyme. The reaction was 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[10]. The assay of ITase was done as the above methods except of using 0.5 mM ITP as a substrate. The assay of phytase was done as the above methods except of using 2 mM sodium phytate as a substrate and 0.1M Tris-HCl, pH 8.0 containing 2 mM $CaCl_2$ as a buffer. One unit of enzyme was defined as the amount that cleaves phosphate ester corresponding to 1 μmole per min under the above conditions.

4. Protein determination

Protein was measured by a modification of Lowry's method[11] with bovine serum albumin as standard. On the chromatogram, protein content was shown with an absorbance at 280 nm.

5. Preparation of Sepharose gel associated with phytic acid-partial hydrolysate

Sodium phytate 1.2 g was dissolved in 100 ml of water, adjusted to pH 4.0 with 1 N HCl, filled up to 200 ml with water and autoclaved for 40 min at 120℃. The obtained phytic acid-partial hydrolysate was adjusted to pH 10.0 with 1 N NaOH. Ten grams of epoxy-activated Sepharose 6B were washed with water and 100 ml of 0.1 M carbonate buffer, pH 10.0 on a glass filter. The gel was suspended in the above phytic acid-partial hydrolysate solution and the mixture was kept at room temperature for 16 hr with gentle stirring. After the gel was washed in turn with water, 0.1 M carbonate buffer, pH 8.0 and 0.1 M acetate buffer, pH 4.0 on a glass filter, free

oxirane groups of the gel were blocked with 1 M monoethanolamine. The gel was again washed with water on a glass filter and used for the affinity chromatography of phosphatases.

6. Separation of phosphatases

Typha pollen of 20 g was cultivated at 30 ℃ for 4 hr. The cultured cell was suspended in 200 ml of 10 mM Tris-HCl, pH 7.5 containing 1 mM 2-mercaptoethanol and 0.5 M sucrose (buffer A) and 10-ml portions were disintegrated for 10 min in a teflon-glass homogenizer. The homogenate was centrifuged at 23,000×g for 10 min and the supernatant was added with 1/20 volume of 1% protamine sulfate. The mixture was stood for 30 min and again centrifuged at 23,000×g for 20 min. The collected supernatant was dialyzed against buffer A and the dialysate was put on a DE-52 anion exchange column (3×50 cm) equilibrated with buffer A. After the column was washed with 100 ml of buffer A, proteins were eluted with a linear gradient of 0 to 0.6 M NaCl in buffer A (total 600 ml). Three active fractions, D1 (No. 92-97), D2 (No. 98-103) and D3 (No. 104-111) were separately collected and dialyzed against 10 mM Tris-acetate, pH 6.5 containing 1 mM 2-mercaptoethanol and 0.5 M sucrose (buffer B). They were individually put on a column (1×30 cm) of Sepharose gel associated with phytic acid-partial hydrolysate and the column was washed with 80 ml of buffer B. Proteins were eluted with a linear gradient of 0 to 0.1 M NaCl in buffer B (total 200 ml) and continuously with 100 ml of 0.5 M NaCl in buffer B. Three active fractions from D1, six active fractions from D2 and six active fractions from D3 were obtained by the chromatographies. These active fractions except two fractions having low activity were concentrated respectively to about 1.5 ml using Ultra Thimble UH100/10 membrane under a reduced pressure and each of them was put on a HiLoad Sephacryl S-200 HR column (2.6×60 cm) and gel-filtrated with buffer A containing 0.1 M NaCl.

Results

1. Separation of phosphatases

Phosphatases in the extract from the 4 hr-cultured cell of *Typha* pollen were purified by means of protamine treatment, DE-52 anion exchange chromatography, affinity chromatography using gel associated with phytic acid-partial hydrolysate and gel-filtration. Fig. 1 shows the anion exchange chromatogram. Most amount of ITase activity was comprised in fraction D1. Purification diagram after protamine treatment is drawn in Fig. 2. Phosphatases were separated into 22 fractions. Fractions in which the peak activity of phosphatase was beyond 0.5 unit/ml on gel-filtration, were D1A2G, D1A3G1, D1A3G2, D2A3G1, D2A4G, D3A4G and D3A5G. The bulk of ITase activity was finally brought in D1A3G2 fraction. Purification steps of D1A3G2 are summarized in Table 1. The specific activity for *p*-NPP increased only 6-fold, while that for ITP was enhanced about 50-fold.

2. Substrate specificity of phosphatases

The substrate specificity of the separated phosphatases was measured using 15 kinds of organic phosphate compounds and inorganic pyrophosphate. On the basis of the results shown in Table 2, phosphatases were roughly classified as follows; (1) group which acts to the same extent for *p*-NPP, ATP, ADP, UTP and PPi, to about 50% of the activity toward *p*-NPP for GlyP, PyrP and NADP, and to a certain extent for AMP, G1P, G6P and F6P. D1A1G1, D1A2G, D2A1, D2A2G1,

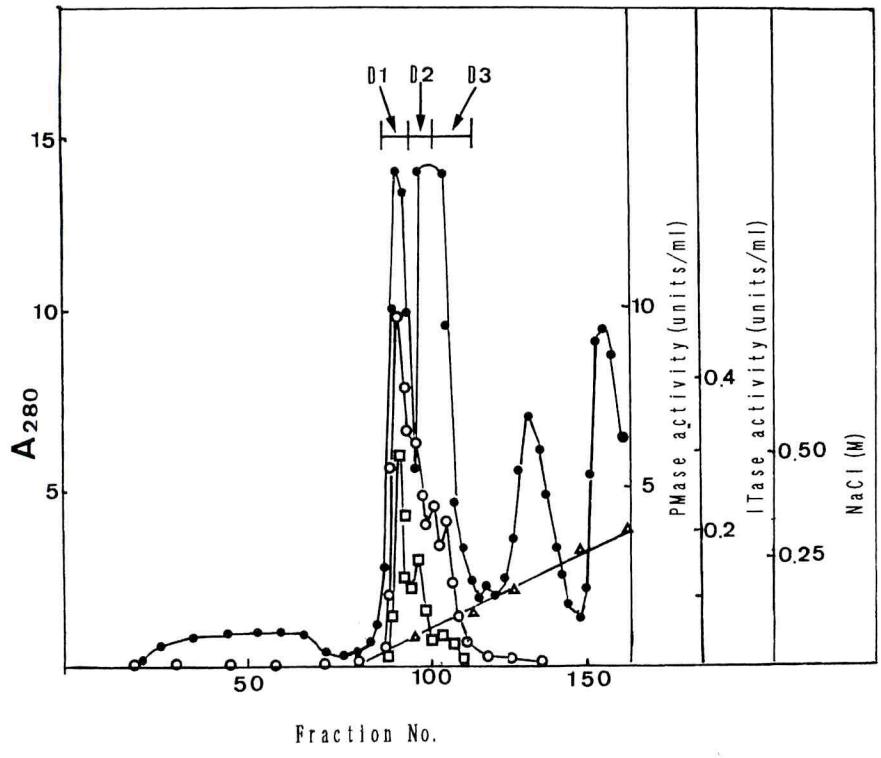

Fig. 1. Elution pattern of phosphatases in the extract from the 4 hr-cultured *Typha* pollen on a DE-52 anion exchange column. For the conditions of chromatography, see **Materials and Methods.** Fractions of 6 ml were collected. ○ — ○, phosphatase (PMase) activity ; □ — □, ITase activity ; ● — ●, absorbance at 280nm ; △ — △, NaCl concentration.

D2A3G1, D2A4G, D2A5G1, D3A1 and D3A2G1 belong to this group. (2) group which acts more preferentially for ATP but lesser for ADP and UTP than for *p*-NPP. D1A1G2, D1A3G1, D2A2G2, D2A3G2 and D3A2G2 belong to this group. (3) group which acts more preferentially for a specially fixed substrate than for *p*-NPP. D2A6G1 and D2A6G2 belong to this group. (4) D1A3G2 which acts more preferentially for ATP, ADP, UTP, PPi and PEP than for *p*-NPP. (5) group which shows the highest activity for *p*-NPP and less activity for all other substrates. D2A5G2, D3A3G, D3A4G, D3A5G and D3A6G belong to this group.

All of phosphatases in each group showed poor activities for phytic acid and ITP. Although D1A3G2 acted clearly for both substrates, its activity was only 13% for ITP and 6% for phytic acid as compared with that for *p*-NPP. The activities for *myo*-inositol monophosphates were measured of the main phosphatases. As shown in Table 3, most phosphatases except D1A3G2 acted better for *myo*-inositol monophosphates than for ITP and were apt to work more preferentially for *myo*-inositol 1-monophosphate than for *myo*-inositol 2-monophosphate.

3. Properties of D1A3G2

Optimum pH, time course of the reaction for ITP and metal requirements were measured of

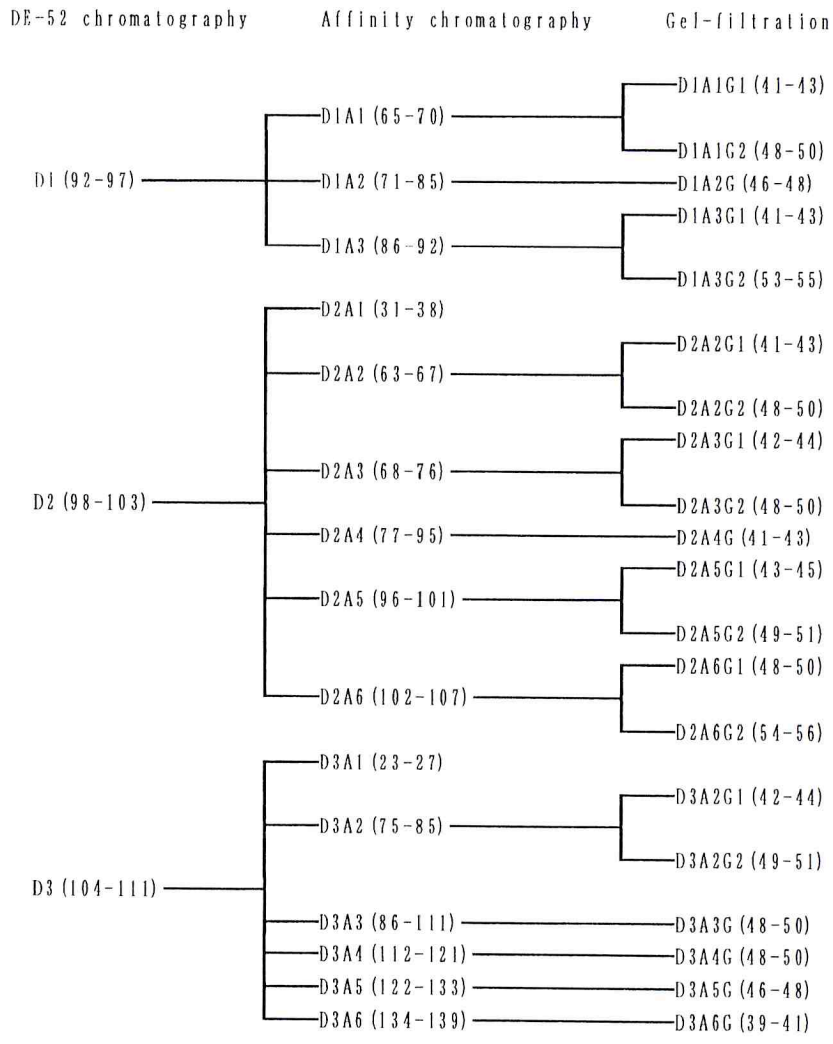

Fig. 2. Purification diagram of phosphatases in the extract from the 4 hr-cultured *Typha* pollen.

Table 1. Summary of purification of D1A3G2

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg protein)	Yield (%)
Crude extract	285	2605	1190 (16.0)	0.457(0.0061)	100
Protamine treatment	260	1552	1064 (14.6)	0.686(0.0094)	89.4
DE-52 chromatog.	32.0	199.2	241 (5.11)	1.210(0.0257)	20.3
Affinity chromatog.	18.5	38.1	37.0(3.22)	0.971(0.0845)	3.1
Gel-filtration	21.0	3.84	11.2(1.25)	2.917(0.326)	0.94

The values were obtained for *p*-NPP as a substrate and those in parentheses were did for ITP.

Table 2. Substrate specificity of phoshatases

Enzyme	*p*-NPP	ATP	ADP	UTP	PPi	PEP	AMP	G1P	G6P	F6P	G1yP	PyrP	NADP	NaphP	Phytate	ITP
D1A1G1	100	93	69	66	84	66	13	8	24	19	42	45	48	36	1	1
D1A1G2	100	126	66	40	24	16	0	1	1	2	3	6	7	4	0	0
D1A2G	100	125	82	79	109	76	18	9	30	23	52	62	65	31	0	1
D1A3G1	100	122	73	55	58	42	7	3	10	11	20	28	31	14	0	0
D1A3G2	100	176	151	157	156	123	3	45	14	9	13	12	23	53	6	13
D2A1	100	118	87	75	96	34	12	5	14	14	34	46	49	26	0	0
D2A2G1	100	99	86	82	104	77	17	10	33	23	56	58	65	29	0	1
D2A2G2	100	135	65	30	42	21	3	2	2	2	3	7	8	6	1	0
D2A3G1	100	120	85	67	93	70	16	8	27	24	46	56	53	36	0	2
D2A3G2	100	115	63	32	14	18	1	5	1	0	3	7	6	5	0	0
D2A4G	100	121	119	103	86	58	14	8	20	16	31	44	45	29	0	1
D2A5G1	100	111	137	99	85	90	25	26	31	31	44	49	56	46	1	1
D2A5G2	100	72	91	38	19	14	3	3	3	8	3	9	15	14	0	1
D2A6G1	100	84	64	28	40	11	44	14	8	5	1	153	12	8	0	0
D2A6G2	100	14	13	14	8	9	0	2	0	52	241	2	1	4	0	0
D3A1	100	97	107	85	110	90	53	44	71	56	66	60	77	94	1	7
D3A2G1	100	132	112	105	102	91	18	11	23	23	56	43	77	66	0	2
D3A2G2	100	125	125	54	19	13	0	1	0	2	2	6	4	6	0	0
D3A3G2	100	82	53	32	19	11	1	3	1	2	3	6	7	17	0	0
D3A4G	100	56	15	9	10	4	2	1	1	1	1	2	1	4	0	0
D3A5G	100	23	15	2	0	1	17	0	0	1	1	2	2	3	0	0
D3A6G	100	62	66	51	73	54	39	32	49	43	48	47	54	69	0	4

Abbreviation : *p*-NPP, *p*-nitrophenylphosphate ; ATP, adenosine 5'-triphosphate ; ADP, adenosine 5'-diphosphate ; UTP, uridine 5'-triphosphate ; PPi, inorganic pyrophosphate ; PEP, phospho (enol) pyruvate ; AMP, adenosine 5'-monophoshate ; G1P, glucose 1-phosphate ; G6P, glucose 6-phosphate ; F6P, fructose 6-phosphate ; G1yP, 2-glycerophosphate ; PyrP, pyrdoxal 5'-phosphate ; NADP, Nicotinamide adenine dinucleotide ; NaphP, α-naphtylphosphate ; ITP, Inositol 1, 2, 3-trisphosphate.

Table 3. Activity of the main phosphatases for inositol phosphates derivatives

Enzyme	*p*-NPP	ITP	*myo*-Inositol 1-monophosphate	*myo*-Inositol 2-monophosphate
D1A2G	100	1	13	5
D1A3G1	100	1	10	2
D1A3G2	100	14	9	6
D2A3G1	100	2	13	3
D2A4G	100	0	1	1
D3A4G	100	0	0	0
D3A5G	100	0	1	1
D3A6G	100	3	19	5

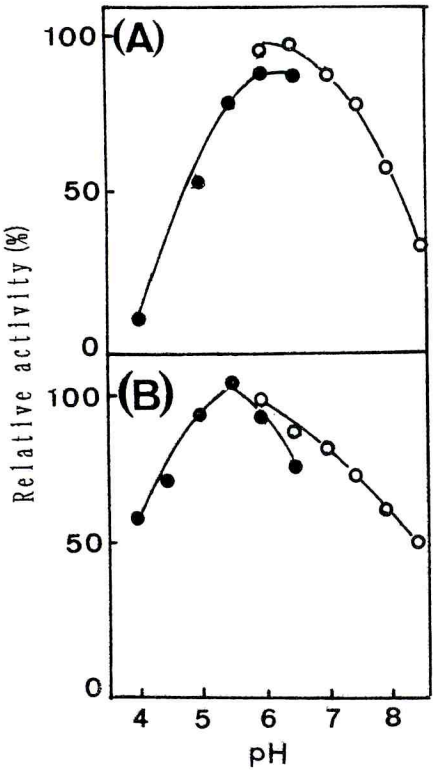

Fig. 3. Effect of pH on the activity of D1A3G2. Activity was measured in the standard assay methods except that the indicated buffers were used. (A), Activity for *p*-NPP ; (B), Activity for ITP ; ● — ●, Sodium acetate buffer ; ○ — ○, Tris-HCI buffer.

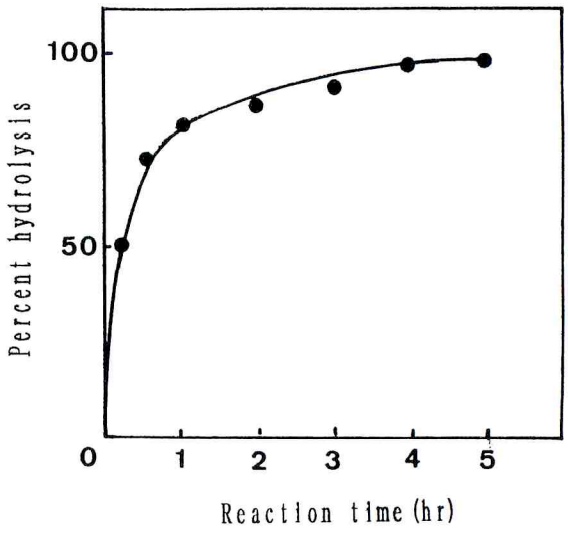

Fig. 4. Time course of the reaction for ITP by D1A3G2. Ten-ml of the reaction mixtures consisted of 0.1 M Tris-HCI buffer, pH 6.5, 0.15 mM ITP and D1A3G2 of 0.12 unit for ITP were incubated. An aliquot, 1.0 ml, was withdrawn at the time indicated and mixed with 0.5 ml of 0.3 N perchloric acid. Pi released was measured as described in **Materials and Methods.** Percent hydrolysis corresponds to the percentages of Pi released to the total phosphate esters in ITP.

D1A3G2. The effect of pH on the activity of D1A3G2 was investigated for *p*-NPP and ITP. As shown in Fig. 3, optimum pH was 6.5 for *p*-NPP and 5.5 for ITP.

The time course of ITP hydrolysis by D1A3G2 is shown in Fig. 4. Seventy % of Pi was released within the first 30 min of the reaction and then it took for 4.5 hr until the rest of Pi was completely liberated. The effect of divalent cations on the activity of D1A3G2 for ITP is shown in Table 4. No divalent cation enhanced the activity except that Mn^{2+} was a little effective.

Table 4. Effect of divalent cations on the activity of D1A3G2 for ITP

Cation added (1 mM)	Relative activity (%)
None	100
$CaCl_2$	98.5
$MgCl_2$	94.3
$MnCl_2$	125.4
$CoCl_2$	113.4
$CuSO_4$	31.3
$ZnCl_2$	6.0

Discussion

The dephosphorylation pathways of phytic acid have been reported by Irving[12]. He suggested that there are two main pathways by phytases, the one yields L-*myo*-inositol 1, 2, 3, 4, 5-pentakisphosphate as an initial reaction product and *myo*-inositol as a final reaction product, and the other yields at first L-*myo*-inositol-1, 2, 4, 5, 6-pentakisphosphate and at last *myo*-inositol 2-monophosphate. We found a new type of phytase from *Typha* pollen[9]. It yields inositol trisphosphate (ITP), which was identified as inositol 1, 2, 3-trisphosphate by NMR analysis[13], as a final reaction product. As ITP was not accumulated in *Typha* pollen during cultivation, the existence of enzymes hydrolyzing ITP was presumed. In fact it was found that the activity for ITP appeares newly during cultivation[5]. From the results obtained we proposed the mode of degradation of phytic acid in *Typha* pollen as follows; at the first stage phytic acid is attacked mainly by phytic acid-specific phytase and partially by nonspecific phosphatases to inositol trisphosphate or lesser inositol phosphates, and at the second stage their intermediates is degraded by some phosphatases[5].

We searched the enzymes which act at the second stage in this experiment. Phosphatases in the extract from the 4 hr-cultured *Typha* pollen were separated into 22 fractions by means of protamine treatment, the anion exchange chromatography, the affinity chromatography associated with phytic acid-partial hydrolysate and gel-filtration. As shown in Table 1, all phosphatases had much lesser preference for phytic acid than for other phosphate compounds. These results support our presumption that the first stage of hydrolysis of phytic acid almost depends on phytic acid-specific phytase[5]. The highest activity for ITP was gained by D1A3G2. D3A1 and D3A6G which had a weak activity for ITP are considered not to be important for ITP hydrolysis because of being

the low active fractions. D1A3G2 was an acid phosphatases without a requirement of divalent metal ions and acted more preferentially for ATP, ADP, UTP, PPi and phospho (enol)pyruvate known as high energy compounds than for *p*-NPP. Time course of the reaction for ITP by D1A3G2 was also examined. As shown in Fig. 3, phosphate ester of ITP was completely hydrolyzed after 5hr, however 70% release of Pi was observed within the first 30 min of the reaction. This may mean that inositol monophosphate is hard to be hydrolyzed by D1A3G2. Some phosphatases showed higher activities than D1A3G2 for inositol 1-monophosphate and similar activities for inositol 2-monophosphate. Adding with these facts, we modified the main degradation pathway of phytic acid in *Typha* pollen during germination as follows ; Phytic acid is attacked by phytic acid-specific phytase to give rise to inositol trisphosphate, then to inositol monophosphate by a sort of phosphatase, D1A3G2, which is synthesized during germination, and inositol monophosphate is hydrolyzed into inositol and Pi in cooperation with some nonspecific phosphatases.

Finally it should be noted that phytic acid metabolism in pollen is regulated in mRNA level for phytase production in *Petunia* and lily pollens and for ITP-hydrolyzing phosphatase production in *Typha* pollen, even though the reason is not clear.

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ガマ花粉培養中のフィチン酸代謝におけるホスファターゼの寄与

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ガマ花粉の培養細胞抽出液のホスファターゼをプロタミン処理，陰イオン交換クロマトグラフィ，アフィニティクロマトグラフィおよびゲルろ過によって22の画分に分けた．これらの基質特異性を調べ，おおまかに5つのグループに分類した．どのグループのホスファターゼもフィチン酸やイノシトール 1, 2, 3-トリリン酸にはきわめて低い活性を示した．これらの中で D1A3G2 は両基質に作用したが，それぞれの基質に対する活性は *p*-ニトロフェニルリン酸に対する活性と比較し僅か13%および6%であった．得られた結果から，ガマ花粉培養中のフィチン酸はまずフィチン酸特異的フィターゼの作用でイノシトールトリリン酸となり，ついで D1A3G2 の作用でイノシトールモノリン酸にまで分解され，さらにいくつかの非特異的ホスファターゼの共同作用によってイノシトールと無機リン酸を生じるものと結論した．