Starch Granules and Phoshorylases from Pollens

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X-Ray diffraction was applied to the starch granules prepared from pollens of *Typha latifolia*, *Pinus thunbergii* and *Zea mays*. From the comparison of diffraction patterns, starch granules from pollens were judged to be A-type, which is observed in starch granules from cereals.

Phosphorylases were purified from pollens of *Zea* and *Pinus*. *Zea* phosphorylase gave a single protein band on polyacrylamide gel electrophoresis, the position of which coincided well with that of the enzyme activity.

Comparative activities of phosphorylases for soluble starch, amylopectin and starch granules from pollens were determined in both directions of Pi release and G-1-P formation. In both directions, *Zea* phosphorylase had almost similar activities for soluble starch, amylose A and starch granules of *Zea* pollen except for 4-fold activity for amylose A in the direction of G-1-P formation. *Pinus* phosphorylase had very low activities for starch granules from *Pinus* pollen in both directions.

It was suggested that the properties of phosphorylases obtained from the pollens involving *Typha* pollen reflect a physiological feature of each pollen.

Key words : Pollen starch, Pollen phosphorylase, *Zea* pollen, *Pinus* pollen.

Introduction

Starch content of pollen is highly variable, comprising as much as 22.4% of the dry weight of *Zea mays* L. down to 2.6% in *Pinus thunbergii* Parl.[1] The content extremely changes during the periods of maturity, germination and tube elongation. The synthesis of starch is catalyzed by starch synthase (EC 2. 4. 1. 21) and its degradation is dependent mainly on amylases (EC 3. 2. 1. 1, 3. 2. 1. 2) and phosphorylase (EC 2. 4. 1. 1).

We observed that phosphorylase activities were highly detected in pollens of plants tested, but amylase activities were usually low levels. *Typha* pollen contained two phosphorylases, which differed from each other in the activity for starch granules from the same pollen.[2] In the direction of Pi release, the one maintained a high activity comparable to those for other substrates, while the other showed a very low activity. This fact suggests that two phosphorylases take different roles in the synthesis and degradation of starch in pollens.

In this paper, we deal with the analysis of X-ray diffraction of starch granules from pollens and the comparative activities of phosphorylases purified from pollens of *Zea* and *Pinus*

for soluble starch, amylose and starch granules from pollens.

Materials and Methods

1. Pollen

Mature pollen grains of *Typha latifolia* were collected from the plants in a paddy field at Nisshin-cho, Aichi prefecture in June 1986, dried overnight at room temperature and stored below −20℃ until use. Mature pollen grains of *Zea mays* were collected from the plants cultivated at Meijo University, Nagoya in August 1987, dried in a desiccator for 2 days and stored below −20℃ until use. Mature pollen grains of *Pinus thunbergii* were collected from the plants at Ohtaka Ryokuchi Park, Nagoya in May 1987, dried overnight at room temperature and stored below −20℃ until use.

2. Chemicals

Amylose A (M. W. approximately 2,900), soluble starch and amylopectin were obtained from Nacalai Tesque Inc. Phosphoglucomutase and glucose 6-phosphate dehydrogenase were from Boehringer Mannheim GmbH. Epoxy-activated Sepharose 6B and Sephadex G-200 were from Pharmacia Fine Chemicals. DE-52 was from Whatman Ltd. DEAE Bio-Gel A and Bio-Gel A-0.5m were from Bio-Rad Laboratories. TSK-GEL TOYOPEARL HW-55S and DEAE-TOYOPEARL 650S were from Toyo Soda Manufacturing Co. $NADP^+$was from Kohjin Co., Ltd. Other reagents used were of analytical grade.

3. Preparation of amylose-Sepharose 6B

Amylose-Sepharose 6B was prepared with the same methods as described previously.[2]

4. Preparation of starch granules

Starch granules from pollens of *Typha* and *Pinus* were prepared with the same methods as described previously.[2, 3] Starch granules from *Zea* pollen were done according to the preparation methods of starch granules from *Typha* pollen.[2] Starch granules from *Zea* seeds were prepared as follows: the seeds were treated in a Waring blender for 5 min and the homogenate was filtered through cheese cloth. Then the mixture was centrifuged at 1,250×g for 10 min and the precipitate was treated according to the preparation methods of starch granules from *Typha* pollen.[2]

5. X-Ray diffraction analysis

The X-ray diffraction was carried out at room temperature with a type of Geigerflex diffractometer (Rigaku Denki). The measurement conditions were as follows: target, Cu; filter, Ni; voltage, 30kV; current, 15 mA; receiving slit, 0.2mm; time constant range, 1-4 sec; counting range, 1×10^2, 1×10^3; scanning speed, 2° / min. About 80 mg of starch was loaded on a glass holder without introducing a preferred orientation of starch.

6. Enzyme assay

Phosphorylase activity was measured in the both directions of Pi release and glucose

1-phosphate (G-1-P) formation with the same methods as described previously.[2]

7. Protein measurement

Protein was measured with a modification[4] of Lowry's method with bovine serum albumin as the standard.

8. Purification of phosphorylase

Thirty grams of *Zea* pollen were suspended in 300 ml of 50 mM imidazole-HCl buffer (pH 7.0) containing 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 10% sucrose. Twenty-ml portions were disintegrated for 2 min in a Teflon-glass homogenizer at about 2,000 rpm and the combined mixture was centrifuged at 10,000×g for 10 min. Solid ammonium sulfate was added to the supernatant (205 ml) to 30% saturation with constant stirring, left for 1 hr and centrifuged at 12,000×g for 10 min. To the supernatant, solid ammonium sulfate was added to 90% saturation with constant stirring and then the mixture was left for 1 hr. The precipitate collected by centrifugation at 12,000×g for 10 min was dissolved in 10 mM imidazole-HCl buffer (pH 7.0) containing 1 mM DTT, 1 mM EDTA and 10% sucrose (Buffer A) and the solution was dialyzed against the same buffer. The dialysate (114 ml) was put on a column (3.5×33 cm) of DEAE Bio-Gel A equilibrated with Buffer A. After the column was washed with Buffer A, the enzyme was eluted with a linear gradient of 0-0.5 M NaCl (total volume 1.2 liter) in Buffer A. The active fractions (65 ml) were collected, concentrated to about 5 ml with a collodion bag and put on a column (2×110 cm) of Sephadex G-200 equilibrated with Buffer A and then the enzyme was eluted with the same buffer. The active fractions (35 ml) were put on a column (2×4.5 cm) of amylose-Sepharose 6B equilibrated with Buffer A. After the column was washed with Buffer A and Buffer A containing 0.5 M NaCl, the enzyme was eluted with Buffer A containing 0.1% amylose A. The active fractions (10 ml) were collected, concentrated to about 5ml with a collodion bag and put on a column (2×95 cm) of Bio-Gel A-0.5m equilibrated with Buffer A. The enzyme was eluted with the same buffer.

Thirty grams of *Pinus* pollen were suspended in 300 ml of 50 mM Tris-acetate buffer (pH 7.0) containing 1 mM DTT and 10% glycerol. Ten-ml portions were disintegrated for 10 min in a Teflon-glass homogenizer at about 2,000 rpm and the combined mixture was centrifuged at 12,000×g for 10 min. Solid ammonium sulfate was added to the supernatant (220 ml) to 30% saturation with constant stirring, left for 1 hr and centrifuged at 12,000×g for 10 min. Solid ammonium sulfate was added to the supernatant to 70% saturation with constant stirring and then the mixture was left for 1 hr. The precipitate collected by centrifugation at 12,000×g for 10 min was dissolved in 10 mM Tris-acetate buffer (pH 7.0) containing 1 mM DTT and 10% glycerol (Buffer B), and then the solution was dialyzed against the same buffer. The dialysate (49 ml) was put on a column (1.5×27 cm) of DEAE Bio-Gel A equilibrated with Buffer B. After the column was washed with Buffer B, the enzyme was eluted with a linear gradient of 0-0.5 M NaCl (total volume 600 ml) in Buffer B. The active fractions (58 ml) were collected,

concentrated to about 5 ml with a collodion bag and put on an HPLC column (3.2×45 cm) of TSK-GEL TOYOPEARL HW-55S equilibrated with Buffer B. The enzyme was eluted with the same buffer at a flow rate of 1 ml / min. The active fractions (27 ml) were collected and put on an HPLC column (2.2×20 cm) of DEAE-TOYOPEARL 650S equilibrated with Buffer B. The enzyme was eluted with a linear gradient of 0-0.5 M NaCl (total volume 500 ml) in Buffer B at a flow rate of 1 ml / min. The active fractions (43 ml) were collected and dialyzed against Buffer B.

9. Electrophoresis

Polyacrylamide gel electrophoresis was done by the method of Davis.[5] Electrophoresis in 5% polyacrylamide disc gel was done for 3 hr at 2.5 mA per column in Tris-glycine buffer (pH 8.3). After electrophoresis, protein was stained with Coomassie brilliant blue G-250. The activity staining of phosphorylase was done as follows : a gel was soaked in an assay solution for Pi release at 37℃ for 30 min and then transferred into 2% potassium iodide containing 0.2% iodine. SDS-polyacrylamide gel electrophoresis was done by the method of Weber and Osborn[6] as the conditions described previously.[2]

Results

1. Starch granules from *Zea* pollen

For the preparation of starch granules from pollen, the combination of centrifugation at a low gravity (19 × g) and a high gravity (1,250 × g) was very effective. The yield of starch granules from *Zea* pollen was 11.5% of the fresh pollen weight used. The value was higher than that (6.7%) of *Typha* pollen. The shape was an ellipsoid similar to that from *Typha* pollen[2] and their lines of apsides were about 1 μm under the observation by a scanning electron microscope (data not shown).

2. X-Ray diffraction

The X-ray diffraction analysis was done on amylose A, amylopection, soluble starch, starch granules from *Zea* seeds and starch granules from pollens of *Typha*, *Zea* and *Pinus*. As shown in Fig. 1, amylose A and soluble starch had some sharp X-ray diffraction peaks, while starch granules from each pollen showed only small undulation patterns. The diffraction pattern of starch granules from *Zea* seeds, which is known to be A-type, was similar to those of starch granules from pollens. Amylopectin gave no definite peak.

3. Purification of phosphorylases

The summary of the purification of phosphorylase from *Zea* pollen is shown in Table 1. The enzyme was purified 893-fold with a yield of 5.1%. The specific activity was 175 units / mg. As shown in Fig. 2, the enzyme gave a single protein band on polyacrylamide gel electrophoresis, the position of which coincided well with that of the enzyme activity. The enzyme also gave a single band with a molecular weight of 103,000 on SDS-polyacrylamide gel elec-

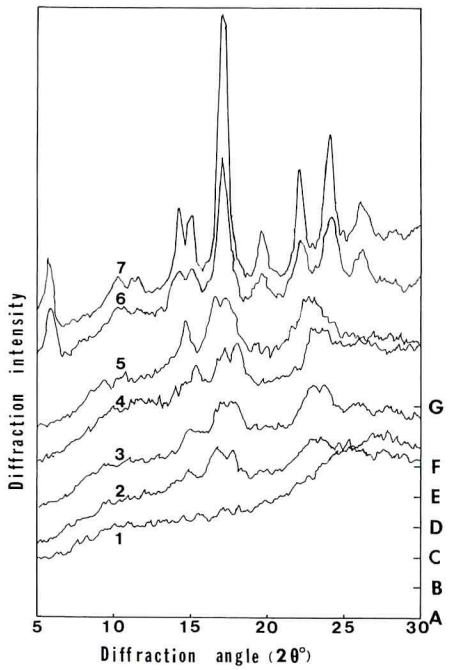

Fig. 1. X-Ray diffraction profiles of starches.
Experimental conditions are described in **Materials and Methods.** Starch specimens : 1, amylopectin , 2, starch granules from *Pinus* pollen , 3, starch granules from *Typha* pollen ; 4, starch granules from *Zea* pollen ; 5, starch granules from *Zea* seeds ; 6, soluble starch , 7, amylose A.
A, B, C, D, E, F or G shows zero position of diffraction intensity for each starch 1, 2, 3, 4, 5, 6 or 7 respectively

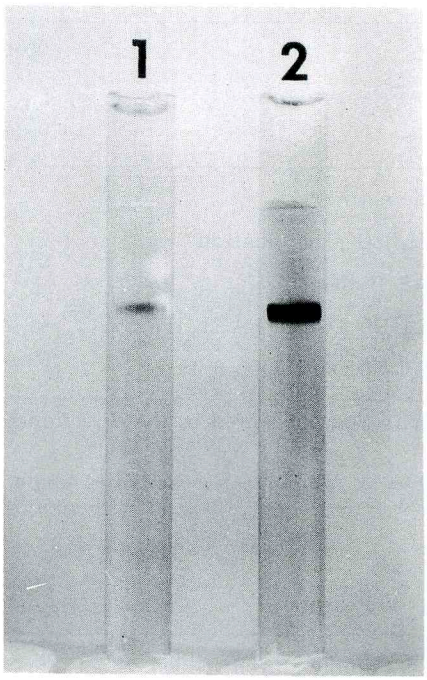

Fig. 2. Polyacrylamide gel electrophoresis of purified phosphorylase from *Zea* pollen.
Seven μg each of enzyme was used for protein staining and activity visualization. Experimental conditions are described in **Materials and Methods.**
1, protein staining ; 2, activity staining.

Table 1. Purification of phosphorylase from *Zea* pollen

Step	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)
Crude extract	205	720	3,670	0.196	100
$(NH_4)_2SO_4$ fractionation	114	715	1,970	0.363	99.3
DEAE Bio-Gel A	65.0	547	563	0.972	76.0
Sephadex G-200	35.0	285	157	1.82	39.6
Amylose-Sepharose 6 B	10.0	85.9	0.360	239	11.9
Bio-Gel A-0.5m	26.5	36.7	0.210	175	5.1

Activity was measured in the direction of Pi release.

Table 2. Purification of phosphorylase from *Pinus* pollen

Step	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)
Crude extract	220	185	985	0.188	100
$(NH_4)_2SO_4$ fractionation	49	115	461	0.249	62.2
DEAE Bio-Gel A	58	100	112	0.893	54.1
TSK-GEL TOYOPEARL HW-55S	27	65.3	39.0	1.67	35.3
DEAE-TOYOPEARL 650S	43	49.2	22.2	2.22	26.6

Activity was measured in the direction of Pi release.

Table 3. Comparative activities of phosphorylase from *Zea* pollen for different substrates

Substrate	Relative activity (%)	
	Pi release	G-1-P formation
Soluble starch	100	100
Amylose A	73.2	427
Native starch granules from *Zea* pollen	57.1	108

Activity was measured at pH 6.0 in the direction of Pi release and at pH 6.4 in the direction of G-1-P formation. Each activity was expressed as a percentage of activity for soluble starch.

Table 4. Comparative activities of phosphorylase from *Pinus* pollen for different substrates

Substrate	Relative activity (%)	
	Pi release	G-1-P formation
Soluble starch	100	100
Amylose A	505	290
Native starch granules from *Pinus* pollen	21.8	3.51

Activity was measured at pH 6.0 in the direction of Pi release and at pH 6.8 in the direction of G-1-P formation. Each activity was expressed as a percentage of activity for soluble starch.

trophoresis (data not shown).

The summary of the purification of phosphorylase from *Pinus* pollen is shown in Table 2. The enzyme was purified 12-fold with a yield of 26.6% but it was not electrophoretically pure. The specific activity was 2.22 units / mg.

4. Comparative activities for different substrates

As shown in Table 3, in the direction of Pi release, phosphorylase from *Zea* pollen showed a half of activity for starch granules from the same pollen in comparison with that for soluble starch. In the opposite direction, the enzyme activity for amylose A was four times higher than that for soluble starch.

As shown in Table 4, in both directions, phosphorylase from *Pinus* pollen had high activities for amylose A but showed very low activities for starch granules from the same pollen.

Discussion

Hizukuri classified native starch granules from plants into three types, A, B and C by X-ray diffraction patterns.[7] The A and B types are independent but the C-type is probably a mixture of A and B types by various proportions.[8] The starch granules from pollens seem to belong to A-type which is observed in starch granules from cereals and pulses such as rice, corn and green pea. Interestingly, the diffraction pattern of starch granules from *Zea* pollen was similar to that of starch granules (15 μm in diameter)[9] from the same seeds (Fig. 1). This result suggests that .these two starch granules have a similar structure in spite of a large difference in the size.

The affinity chromatography on an amylose-Sepharose 6B column was effective on purification of phosphorylases from pollens. All the phosphorylases were adsorbed to the column but showed different behaviors on elution. Phosphorylases from *Zea* pollen were selectively eluted with 0.5% amylose A after the column was washed with 1 M NaCl, having a strong resemblance to P-1 from *Typha* pollen.[2] Phosphorylase from *Pinus* pollen were easily eluted with a low concentration of NaCl, having a resemblance to P-2 from *Typha* pollen.[2]

The relative activities of phosphorylases for soluble starch, amylose A and starch granules from pollens were investigated in the same manner as that of phosphorylases from *Typha* pollen.[2] In the direction of Pi release, phosphorylase from *Zea* pollen had similar activities for soluble starch and amylose A, resembling to P-1 from *Typha* pollen, except for having a slightly low activity for starch granules from the same pollen. Phosphorylase from *Pinus* pollen showed a significantly lower activity for starch granules from the same pollen than that for soluble starch, resembling to P-2 from *Typha* pollen.

In the direction of G-1-P formation, phosphorylase from *Zea* pollen had similar activities for soluble starch and starch granules from the same pollen, resembling to P-1 and P-2 from *Typha* pollen, except for having 4-fold higher activity for amylose A. It is noteworthy that phosphorylase from *Pinus* pollen exhibited an extremely low activity for starch granules from the same pollen.

These results suggest that P-1 from *Typha* pollen and phosphorylase from *Zea* pollen can participate in both directions of synthesis and degradation of starch granules in the pollens,

while P-2 mainly works in degradation, and also phosphorylase from *Pinus* pollen is hard to act on starch granules from the same pollen in both directions. Steup *et al.*[10] reported two phosphorylases, chloroplast phosphorylase and nonchloroplast phosphorylase, from spinach leaves which were differed in degradation activity for starch granules.

The results obtained here are likely to reflect the difference of physiological features of three kinds of pollens. *Typha* pollen can synchronize the synthesis and degradation of starch during germination and pollen tube elongation. *Zea* pollen containing above 20% of starch performs one sided digestion of starch because of guaranteeing the speedy germination and tube elongation. On the other hand, *Pinus* pollen synthesizes starch in accordance with the germination and tube elongation and lies in dormancy for a year. Thus, the pollen probably needs not a rapid degradation of starch.

This work offered an available evidence on the participation of phosphorylase regarding the synthesis and degradation of starch in pollen.

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花粉のデンプン粒とホスホリラーゼ

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ガマ，マツおよびトウモロコシ花粉から調製したデンプン粒に対し，X線回折による分析を行った。回折像の比較から花粉のデンプン粒は穀類のデンプン粒に見られるA型に属することがわかった。

ホスホリラーゼをトウモロコシおよびマツ花粉から調製した。トウモロコシ花粉のホスホリラーゼは，ポリアクリルアミドゲル電気泳動によって，活性と一致する単一のタンパク質バンドを与えた。

ホスホリラーゼの活性を可溶性デンプン，アミロースAおよび花粉デンプンを基質として，合成方向と分解方向で比較した。トウモロコシ花粉のホスホリラーゼは可溶性デンプン，アミロースおよびトウモロコシ花粉デンプンに対して，両方向でほぼ同程度の活性を示したが，例外的に分解方向でアミロースに対して4倍高い活性を示した。マツ花粉のホスホリラーゼは両方向でマツ花粉デンプンに対し，極めて低い活性を示した。

ガマ花粉を含め，これらの花粉から得られたホスホリラーゼの性質は花粉の生理的性質を反映していることが示唆された。