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PHOSPHATASES FROM POLLEN OF *BRASSICA CAMPESTRIS* AND *LILIUM REGALE*

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Key Word Index—*Brassica campestris*; Cruciferae; rape seed; *Lilium regale*; Liliaceae; pollen wall; phosphatases.

Abstract—Soluble and wall-bound acid phosphatases isolated from rape seed pollen showed similar properties except for the pH optimum curve which was elevated for the cell wall enzyme. About 50% of the phosphatase activity of washed pollen wall preparations could be solubilized with Triton X-100, compared with only *ca* 20% for the corresponding preparation from lily pollen. A comparison of the wall-bound acid phosphatase of rape seed and lily pollen showed a marked difference in specificity towards fructose-6-phosphate and glucose-6-phosphate. A Mg^{2+}-dependent alkaline pyrophosphatase was obtained from rape seed pollen but this activity could not be detected in cell wall preparations.

INTRODUCTION

Acid phosphatase (EC 3.1.3.2) is known from cytochemical studies to be present in pollen grains particularly in the intine [1, 2]. This enzyme is active as an antigen [3] and so could be involved in pollen–stigma recognition. Acid phosphatase might also play a role in growth of the germinating pollen tube.

Due to the importance of acid phosphatase in pollen physiology, the cytochemical approach has been extended to the partial purification of soluble and pollen-wall enzymes. The two types of pollen used enabled comparison between species with distinctly different pollination physiology. *Brassica campestris* is a dicotyledon with tricellular pollen and a sporophytic system of self-incompatibility, while *Lilium regale* is a monocotyledon with bicellular pollen. *Lilium longiflorum* is known to exhibit gametophytic self-incompatibility [4].

In addition to the isolation and study of acid phosphatases, a Mg^{2+}-dependent alkaline pyrophosphatase (EC 3.3.1.1) was found to be present in *B. campestris* pollen as a soluble enzyme, but activity could not be detected in pollen wall preparations.

RESULTS AND DISCUSSION

Wall-bound acid phosphatase activity

Pollen wall preparations were obtained using methods based on those described for wall preparations of invertase from lily pollen [5]. As shown in Table 1, the washed pollen wall preparation was found to retain activity when incubated in sodium chloride solution (0.5 or 1.0 M) or Tween 20 (1% or 5%) but some activity was removed with 1% Triton X-100. This loss of activity was not simply due to denaturation of enzyme as the activity lost from the wall preparation could be recovered in the extractant solution. It appears that some of the wall-bound enzyme may be associated with the cell membrane, as Triton X-100 is known to be effective in disruption of membranes [6], although the release of enzyme adsorbed to cell wall fragments during homogenization is a further possibility. The solubilization of pollen wall phosphatase with Triton X-100 was more pronounced with *Brassica* pollen than with *Lilium*. This result is of interest because of the known differences in the cytology of pollen of these genera and the difference in their self-incompatibility systems [4]. In *Brassica*, sodium chloride appeared to stimulate activity. This stimulation could result from decreased denaturation compared with controls incubated in distilled water, as incubation was for 1 hr at 20°.

Table 1. Effect of various extractants on acid phosphatase activity of pollen wall preparations

Extractant*	Enzyme activity as a percentage of control†	
	Brassica preparation	*Lilium* preparation
Sodium chloride (0.5 M)	118.0	89.0
Sodium chloride (0.1 M)	120.0	95.8
Tween 20 (1%)	92.5	98.1
Tween 20 (5%)	90.4	108 0
Triton X-100 (1%)	55.3	80.5

*Extractant incubated with wall material for 1 hr at 20° whilst shaking (150 cycles/min) before centrifuging to collect wall material and assaying for residual activity.

†Results average of duplicates. Control samples incubated with distilled water. Absolute activities for the control were 2 0 pkat for *Brassica* and 1.1 pkat for *Lilium*.

The pollen wall acid phosphatase of *Brassica* had an optimum pH plateau from *ca* 4.7 to 5.7. The K_m was 0.5 mM using *p*-nitrophenylphosphate as substrate. A number of substances inhibited the wall preparation, ammonium molybdate (0.1 mM) caused 83% inhibition,

sodium fluoride (10 mM) 80% inhibition and inorganic phosphate at 10, 20 and 50 mM caused 10%, 33% and 53% inhibition, respectively. Zinc chloride, ferrous sulphate, cupric sulphate, calcium chloride and magnesium chloride all at 1 mM had no effect on the activity. Ethylene diamine tetraacetic acid (EDTA) (10 mM) and mercuric chloride (5 μM) were also without effect. The specificities of the pollen wall phosphatase for rape seed and lily preparations are shown in Table 2. Both showed substantial pyrophosphatase and ATPase activities but differed in the level of glucose-6-phosphatase and fructose-6-phosphatase activities, these being higher in lily pollen. The specificity of the wall phosphatase was further studied using mixed substrates with each substrate at a concentration of 10 mM. These experiments showed that activities were not additive either for the lily preparation or for rape seed. This is evidence for a single acid phosphatase in each of the wall preparations. The difference in specificity between lily and rape seed preparations (using fructose-6-phosphate or glucose-6-phosphate as substrate) could indicate either a structural difference in the active centre region or steric effects imposed by the matrix in which the enzyme is embedded.

Soluble acid phosphatase activity

Purification of the soluble acid phosphatase activity of rape seed involved using Sepharose 6B and DEAE-Sephadex to produce two partially purified fractions. Fraction 1 was eluted rapidly from DEAE-Sephadex using 20 mM Tris–HCl buffer, pH 6.8, and fraction 2 required sodium chloride (0.5 M) elution. Fraction 2 was used as the enzyme source for studies of the effect of pH, substrate concentration and effectors on activity.

The soluble phosphatase from rape seed pollen had a pH optimum of 4.6 with a sharper maximum than for the wall phosphatase. Differences in the shape of the pH curve are known to be associated with moving from the soluble to the particulate state [7]. The soluble phosphatase had a K_m of 0.8 mM using *p*-nitrophenylphosphate as substrate. This value is comparable with those from other tissues, e.g. 0.3 mM for acid phosphatase from scutella of germinating maize [8] and 0.6 mM for acid phosphatase from cultured tobacco cells [9]. Neutral phosphatase activity from *Spirodela* had a K_m of 0.7 mM for *p*-nitrophenylphosphate [10]. Inhibitors had similar effects to those observed with the wall enzyme. Ammonium molybdate (0.1 mM) caused 78% inhibition, sodium fluoride (10 mM) 73% and inorganic phosphate (10 mM) 12%. Zinc chloride, ferrous sulphate, copper sulphate, calcium chloride and magnesium chloride all at 1 mM and EDTA (10 mM) were without effect, as also described for the wall preparation.

The specificities of fractions 1 and 2 are compared in Table 2. Fraction 2 has more acid pyrophosphatase activity than fraction 1. This is interesting in view of the finding (see below) that alkaline pyrophosphatase activity was found only in this fraction and not in fraction 1 or in wall preparations. Otherwise, the specificity of the soluble acid phosphatase of rape seed pollen is similar to soluble acid phosphatase from other sources [9, 11–13] in having substantial pyrophosphatase and ATPase activities relative to *p*-nitrophenylphosphatase activity.

Comparison of wall acid phosphatase with soluble acid phosphatase of *Brassica* pollen, indicates very similar properties except for the elevated pH optimum curve in the case of the wall enzyme. The soluble pollen acid phosphatase has properties similar to soluble acid phosphatases from other sources.

Mg^{2+}-stimulated alkaline pyrophosphatase activity

This activity could not be detected in wall preparations from *Brassica* pollen but was present in the fraction 2 soluble enzyme as shown in Table 3. No activity could be detected in the fraction 1 soluble enzyme. The activity of the enzyme reaches a maximum at *ca* pH 8.5. Mg^{2+}-dependent alkaline pyrophosphatase has been studied in leaves [14, 15] and has previously been reported as a soluble enzyme in pollen of *Typha latifolia* and other species [16]. This enzyme is known to decrease during leaf senescence, consistent with the view that highest alkaline pyrophosphatase activity is associated with cells active in biosynthesis [17]. In pollen, the role of this pyrophosphatase may be to allow the biosynthesis of UDP-glucose to proceed by removal of pyrophosphate. This may be

Table 2. Specificities of wall phosphatase preparations from *Brassica* and *Lilium* and DEAE–Sephadex peaks of soluble acid phosphatase from *Brassica* (fractions 1 and 2)

	Activity with *p*-nitrophenylphosphate (%)			
	Wall preparations		Soluble acid phosphatase from *Brassica*	
Substrate	*Brassica*	*Lilium*	Fraction 1	Fraction 2
p-Nitrophenylphosphate	100*(98)	100(31)	100(121)	100(80)
Glucose-6-phosphate	14	51	28	20
Fructose-6-phosphate	12	44	7	2
Fructose diphosphate	16	40	31	33
ATP	60	76	61	67
Inorganic pyrophosphate	45	67	56	115
Phytate	0	2	0	0

* Incubations were for 20 min at 37° and each included a substrate control used to correct values for non-enzyme phosphate production. Values shown in parentheses are the absolute activities in pkat

Table 3 Effect of pH and presence of Mg^{2+} on the pyrophosphatase activity of fraction 2 soluble phosphatase preparation from *Brassica* pollen

pH	% Pyrophosphatase activity relative to pH 8.5 (+Mg^{2+}) activity	
	+Mg^{2+}	−Mg^{2+}
7.5	67	78
8.0	88	61
8.5	100*	45
9.0	96	23
9.5	49	4
10.0	45	7
10.5	29	8

*Absolute activity 40 pkat.

important during the conversion of starch to sucrose which is known to occur during pollen germination [16].

EXPERIMENTAL

Pollen collection. Pollen of *Brassica campestris* was collected from anthers harvested from plants grown in growth chambers. Pollen of *Lilium regale* was obtained from flowers purchased at a local market. Collected pollen was shown by microscopy to be free of anther debris. Lily pollen was washed rapidly (×3) with Me_2CO to remove pigments.

Preparation of soluble enzyme. Pollen (2 g) was treated with liquid N_2 and 20 ml MES buffer, pH 6.5 (50 mM). The mixture was ground with a mortar and pestle with further additions of liquid N_2 and, after thawing, was centrifuged at 39 000 *g* for 20 min. The pellet obtained was used for preparation of the wall enzyme (described below). The supernatant was applied to a column of Sepharose 6B (3.3 × 55 cm) and eluted with 50 mM Tris–HCl, pH 7. Fractions (5 ml) were collected and assayed, the activity being confined to a single eluted peak. The active fractions were pooled and passed through a DEAE-Sephadex column (1.5 × 40 cm). This was eluted initially with 20 mM Tris–HCl, pH 6.8 (1 ml fractions) resulting in elution of a single peak of activity here referred to as fraction 1. Further elution (5 ml fractions) with 0.5 M NaCl in 20 mM Tris–HCl, pH 6.8, produced a second peak of activity referred to as fraction 2.

Preparation of wall enzyme The pellet obtained from centrifuging the pollen extract (see above) was taken up in 50 mM MES buffer, pH 6.5, and centrifuged at 3000 *g* for 15 min. The pellet obtained was washed (×4) before taking up in 5 ml MES buffer and incubating with 45 ml Triton X-100 (1%) for 1 hr at 20°, whilst shaking at 150 cycles/min. The mixture was centrifuged for 10 min at 39 000 *g* and the pellet washed (twice) with MES buffer. The final pellet was taken up in buffer and stored frozen.

Assay of acid phosphatase. p-Nitrophenylphosphate (2.5 μmol) was used, together with NaOAc buffer, pH 4.8 (100 μmol) and suitably diluted enzyme or wall preparation in a total vol. of 0.25 ml. The reaction was stopped using 0.75 ml KOH (M) and the *A* at 405 nm determined. Assays, using wall preparation, were carried out in centrifuge tubes and the wall material removed by brief centrifugation before the addition of KOH. This avoided spurious colour development due to alkali leaching yellow pigment from the pollen wall. When substrates other than *p*-nitrophenylphosphate were used, phosphate was determined by the method of ref. [18]. The K_m was determined with the aid of the Lineweaver–Burk plot.

Assay of Mg^{2+}-stimulated alkaline pyrophosphatase activity. Mg^{2+} (10 μmol), PPi (1 μmol), Tris buffer (50 μmol, pH 9) and suitably diluted enzyme in a total vol. of 0.5 ml was incubated at 37°. The reaction was stopped using the mixed reagent for P analysis described in ref. [18].

Protein assay. As described in ref [19].

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