Phytochemistry, Vol. 24, No 8, pp 1639–1643, 1985
Printed in Great Britain

0031–9422/85 $3.00 + 0.00

β-GALACTOSIDASES OF *LILIUM* POLLEN

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(*Revised received* 26 *November* 1984)

Key Word Index—*Lilium auratum*; Liliaceae; pollen; β-galactosidases, isoenzymes.

Abstract—Lily (*Lilium auratum*) pollen contains very high levels of β-galactosidase. There are three forms: β-galactosidase I and II differ in M_r, while β-galactosidase III is firmly bound in the pollen wall. The two cytoplasmic forms were separated and partially purified using a combination of chromatography on DEAE-cellulose, Sephadex G-200 and Sepharose 6B. Forms I and II appear to be glycoprotein in nature as shown by binding to Con A-Sepharose. The three enzymes were optimally active near pH 4, and all were inhibited by galactose and galactonolactone. The wall-bound enzyme, β-galactosidase III effectively hydrolysed nitrophenyl β-galactosidase but not lactose, and could not be released from the wall polysaccharide matrix by high salt concentrations or detergents. The total β-galactosidase activity of lily pollen remained constant during *in vitro* germination. A possible role for this enzyme may be in degradation of stylar arabinogalactans providing a carbon source for pollen tube nutrition.

INTRODUCTION

The present study involved extraction, partial purification and characterization of three forms of β-galactosidase in lily pollen, as a step to understanding the role of this enzyme in the regulation of pollen tube wall extension and in the breakdown of stylar arabinogalactans to release low M_r carbohydrates needed for pollen tube nutrition. Since lily pistils have hollow styles, pollen tubes grow superficially, that is, on the surface of the specialized epithelial cells which both cover the stigma and line the stylar canal. An exudate, secreted by these cells, surrounds the pollen tubes and this forms the only potential source of nutrition [1]. Chemical analysis of this exudate has revealed its arabinogalactan nature [2].

By means of labelling experiments, it has been shown that this complex carbohydrate is taken up by the growing pollen tubes. Although occurrence of the arabinogalactans has been demonstrated in stylar tissues of other plant species [3–5] little experimental evidence has been given for the presence of an enzyme system in pollen which can digest the stylar exudate material.

β-Galactosidase has been shown to effectively hydrolyse galactosidic linkages in various arabinogalactans and glycoproteins [6, 7]. Also, it has frequently been proposed that wall-bound glycosidases are correlated with cell elongation [8, 9]. Thus the present study was undertaken to determine the localization and properties of lily pollen β-galactosidase.

RESULTS AND DISCUSSION

Occurrence of different forms of β-galactosidase

The extracts of the mature lily pollen showed very high galactosidase activity as observed by hydrolysis of *o*-nitrophenyl-β-D-galactoside (*o*NPP). During isolation of the enzyme, preliminary experiments were performed to study the stability of galactosidase activity in the crude extracts.

The maximum stability of galactosidase in crude extract incubated at 37° was at pH 6.0, however at values below or above this pH there was a rapid loss of enzyme activity. Addition of proteinase inhibitors phenylmethylsulphonyl fluoride (PMSF) and leupeptin (0.1 mM) almost completely stopped the loss of activity observed at acidic pH range but not that at the alkaline range. The sulphydryl-protecting reagents, dithiothreitol (DTT) and mercaptoethanol, did not have any effect on stability of enzymatic activity in crude extracts. Considering the effects of pH on enzyme stability, the extraction of the pollen enzyme for partial purification purposes was done by using buffer at pH 6 supplemented with leupeptin. After chromatography on a Sepharose 6B column two different molecular mass forms of β-galactosidase were obtained (Fig. 1). The presence of 0.2% BSA in extract and elution buffers had no effect on stability or elution profiles of the enzyme.

Molecular forms of β-galactosidase

Relative molecular masses, M_r, of the two forms of β-galactosidase I and II were estimated from a plot of the log M_r of marker proteins versus their peak elution volume from a Sepharose 6B column (3.3 × 40 cm). The smaller, more abundant form β-galactosidase I had an M_r of 450 000 whereas the less abundant form II passed through the void volume of the Sepharose 6B column. The M_r exclusion limit of Sepharose is 4×10^6, so it is possible that the second form has a higher M_r than this value. However, since both forms are glycoproteins (see below), it can also be expected that the higher M_r form has a greater percentage of carbohydrate, so that due to the asymmetric shape of the molecule, it is excluded from the gel filtration column.

Partial purification of β-galactosidases

The partial purification procedure successfully resolved the two forms from each other and from certain

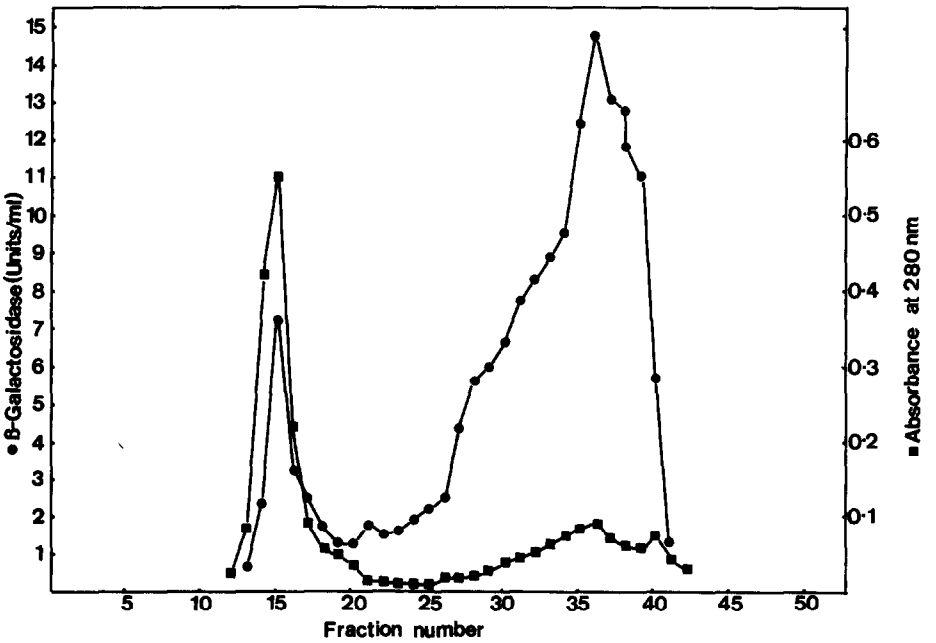

Fig. 1. Elution profile of lily pollen β-galactosidases on Sepharose 6B column (3.3 × 40 cms). The enzyme activity in the column eluate was determined by *o*NPG hydrolysis.

enzymes which would interfere in the kinetic studies. The first step was ion exchange chromatography using DEAE–cellulose. β-Galactosidase activity was not retained on the column. Using Sephadex G-200, the activity passed through the exclusion volume of the elution profile, and two separate M_r forms of the enzyme are resolved using a Sepharose 6B column (Fig. 1). Both of these forms bind firmly to the lectin concanavalin-A and are glycoproteins in nature. This was demonstrated using a Con-A Sepharose 4B column, when both forms eluted from the column with α-methyl mannoside. Both forms were stable for at least 3 months when stored frozen at 0° either in the presence of 1 mg/ml BSA or in its absence. Isoelectric focussing on polyacrylamide gels showed that both galactosidases have isoelectric points between 5.0 and 5.5, lying in the same range as reported for many microbial and animal cell β-galactosidases [10].

Occurrence of wall-bound β-galactosidase III

The washed pollen wall pellet obtained after extraction of the soluble fraction showed high β-galactosidase activity as determined by hydrolysis of *o*NPG. Before an extensive investigation of the pollen wall-bound β-galactosidase and its binding properties was made, the purity of the preparation was assessed. The wall pellets were devoid of hexokinase and malate dehydrogenase activities which ruled out the possibility of cytoplasmic contamination as a source of wall bound β-galactosidase. Extensive treatment of the cell wall pellets with the detergents Triton X-100, octyl-β-glucoside and Nonidet P-40 did not release any β-galactosidase activity from the walls indicating that contamination with membrane components was essentially absent.

To check the nature of binding of β-galactosidase to the pollen wall, aliquots of the pellet were treated with various ionic concentrations of borate buffer and other agents. None of the treatments proved effective in removing the bound β-galactosidase from the pollen walls. Similar results were obtained when pollen walls were prepared by the non-aqueous method described by Kivilaan *et al.* [11] and Murray and Bandurski [9]. A similar occurrence of invertase in cell walls of cotyledonary tissue of radish seedlings has been reported earlier [12, 13]. Also in a study on carrot roots it was possible to release wall bound invertase by treating isolated cell walls with hydrolytic enzymes [14]. When we attempted to carry out such an experiment with lily pollen walls, we observed that cellulase (Onozuka), cellulysin (Calbiochem) and pectinase (Sigma) contained β-galactosidase as impurity. As a result no further attempts to use enzymatic degradation of cell walls as a means to isolate wall-bound pollen β-galactosidase were carried out.

Tween 60, known to remove cell wall tannin–protein complexes did not release the enzyme. EDTA, dithiothreitol and 2-mercaptoethanol were also ineffective, suggesting that the enzyme is not bound to the pollen wall through divalent ions or disulphide linkages. Thus the possibility is that the enzyme is covalently bound to the pollen wall or may be physically entrapped within the wall polysaccharides.

The total β-galactosidase in the mature lily pollen was 0.5 μmol product/min/mg protein and there was no increase in this activity during *in vitro* germination up to 15 hr. No inductive effect of the presence of lactose in the germination medium on lily pollen β-galactosidase was obtained, nor was extracellular hydrolysis of lactose in the growth medium observed.

General properties of pollen β-galactosidases

β-Galactosidase I has a pH optimum of 4.0 and shows a broad range of activity between pH 2.8 and 6.0 while β-galactosidase II shows a pH optimum of 4.30 and is active

between pH 3.4 and 6.0. Neither form was active at a pH of 7.2 or higher. The optimum pH value for the wall-bound fraction is 4.0. The optimum pH values for these lily pollen enzymes lie in the same range as in other plant β-galactosidases [16–18].

Brief exposure to temperatures of 45° did not cause any inactivation, but, at higher temperatures both enzymes I and II rapidly lost their catalytic activity. However, the extent of denaturation was less in the case of the wall-bound β-galactosidase activity. The higher thermostability of wall-bound enzymes has also been demonstrated for invertases [19, 20].

Inhibition studies

The activities of all three forms were not significantly affected by EDTA (1–5 mM final concentration), Mg^{2+} and Mn^{2+} (5–10 mM). Zn^{2+} at 1 mM final concentration had no significant effect but higher concentrations (5 and 10 mM) showed significant stimulation of catalytic activity (40 and 55% respectively for all the enzyme forms). Both forms I and II were almost completely inhibited by 0.05 mM Hg^{+} but the wall-bound enzyme still retained about 25% of the original activity. All the three forms of the enzyme were not inhibited by 1–2 mM iodoacetamide and *N*-ethylmaleimide. *p*-Chloromercuribenzoate (*p*CMB) (0.01–0.1 mM) almost completely inhibited the activity of both cytoplasmic forms. With 0.01 mM *p*CMB the wall-bound β-galactosidase retained 87% of activity, while with 0.1 mM inhibitor, only 22% of the catalytic activity was still detectable. Cysteine (2 mM) had no effect on the activity of the soluble enzymes, but had a slight activating effect (20–30%) in the case of the pollen-wall enzyme. Neither of the three enzymes were affected by *o*-phenanthroline.

The β-galactosidases were inhibited by both galactose and D-galactonolactone. The latter reagent was converted to its most inhibitory form according to Levvy and Conchie [21–23]. The concentrations of D-galactonolactone for 50% inhibition were 0.7, 1.0 and 1.5 mM for β-galactosidases I, II and III respectively. L-Galactonolactone was not an effective inhibitor at concentrations as high as 10 mM. The concentrations of galactose for 50% inhibition were 25, 20 and 35 mM for β-galactosidases I, II and III. Glucose, fructose, arabinose and sucrose at 20 mM concentrations each did not inhibit the catalytic activities of the enzymes.

Substrate specificity

Both forms I and II of cytoplasmic β-galactosidase showed hydrolytic activity towards nitrophenyl-galactosides and lactose. Reaction rates for the enzymes were measured for concentrations of 0.1–10 mM for *o*- and *p*-nitrophenyl β-galactosides. Lactose was used at concentrations of 0.5–20 mM. The K_m values of the three fractions of the enzyme for three substrates are given in Table 1.

o-Nitrophenyl-β-galactoside (*o*NPG) proved to be the best substrate for the pollen β-galactosidases. β-Galactosidases I and II also hydrolysed cytochemical substrates such as 6-bromo-2-naphthyl-β-galactoside and 5-bromo-4-chloro-3-indoxyl-β-galactoside. In addition to low M_r substrates, both β-galactosidases I and II caused release of galactose residues from galactose rich polysaccharides, i.e. larch arabinogalactan and soluble polysaccharide fraction of lily stylar canals. The wall-bound enzyme activity showed unusual substrate specificity. Although it very effectively hydrolyses phenolic galactosides (nitrophenyl and naphthyl galactosides), it is strongly inhibited by D-galactonolactone, but cannot hydrolyse the β-galactosidic linkage of lactose. The immobile nature of the wall-bound enzyme cannot account for its inability to hydrolyse lactose since when Linko *et al.* [24] artificially entrapped purified *Aspergillus niger* β-galactosidase (EC 3.2.1.23) within cellulose fibres, there was no change in the lactose-hydrolysing ability of the enzyme. Earlier a β-galactosidase preparation from *Datura* cells was reported to hydrolyse *o*NPG but not lactose [25]. It was proposed that the *o*NPG hydrolysis is itself not a sufficient indicator of the ability of plant cells to hydrolyse lactose. Our present results strongly support this conclusion.

Table 1. Substrate specificities of different forms of β-galactosidase

Substrate	K_m values for β-galactosidase fraction (mM)		
	I	II	III
p-Nitrophenyl-β-D-galactoside	0.67	0.606	1.20
o-Nitrophenyl-β-D-galactoside	0.96	1.67	2.25
Lactose	7.15	14.90	No hydrolysis

The wall-bound β-galactosidases have been reported to be associated with the process of wall loosening during cell elongation [8, 9] and also have been proposed to release or process fungal cell wall components that elicit the production of phytoalexins [26, 27]. No suggestion for the possible role of intracellular β-galactosidases has been provided in any of the previous studies. As regards the functional significance of the intracellular β-galactosidases of lily pollen, the most logical role seems to be in the breakdown of stylar arabinogalactans which are taken up by the growing pollen tubes as polysaccharide fragments [1]. No increase in the activity of the β-galactosidase during pollen germination has been found, implying that synthesis of this enzyme is completed by pollen maturation. More investigations on the specifity of the wall-bound enzyme are needed before any physiological role can be attributed to it.

EXPERIMENTAL

Pollen collection. Pollen was harvested from field-grown *Lilium auratum* and stored at 4°. For *in vitro* germination, weighed samples of pollen were placed in a moist chamber for 1 hr before inoculating it in Petri-dishes containing Dickinson's [28] pentaerythritol medium maintained at 25 ± 2°. The germinated pollen was harvested by low speed centrifugation (400 *g*, 2 min) and then ruptured in K-Pi buffer (50 mM) as described later.

Enzyme preparation. The weighed amount of pollen was washed for 30 sec with chilled acetone (−20°) to remove most of the pollen coat, a pigmented lipid residue on the pollen surface. The pollen was then transferred to chilled (0–2°) 50 mM K-Pi buffer, pH 6.5 containing 1 mM DTT and 0.1 mM leupeptin, a

proteinase inhibitor, and ruptured in a chilled glass mortar. The homogenate was centrifuged for 10 min at 400 *g* and supernatant collected and cell debris was extracted × 3 by extraction buffer and centrifuged again.

The combined supernatant was shaken at 4° with insoluble polyvinylpyrrolidone to remove pigments and phenolics and then centrifuged. The cell wall pellet was further washed × 3 with extraction buffer and then stored at −70° for later analysis of insoluble β-galactosidase. From the supernatant the protein was precipitated with 100% saturated $(NH_4)_2SO_4$. The ppt was taken up in a minimum of cold 25 mM K citrate–Pi buffer containing 0.10 mM leupeptin and dialysed for 20 hr with two changes of the same buffer. The enzyme preparation was then placed on a DEAE 52 cellulose column (2.5 × 16 cm) pre-equilibrated with 50 mM K citrate–phosphate buffer, pH 6.5. For elution of the enzyme, a linear gradient of 0–0.5 M NaCl in the same buffer was applied. β-Galactosidase activity was not retained on the column, but 3–4 times purification was achieved.

The active fractions were concd by ultrafiltration and then applied on Sephadex G-200 column pre-equilibrated with 50 mM K citrate–phosphate buffer, pH 6. The proteins were eluted with the same buffer and the β-galactosidase activity eluted in the void vol. of the column.

The concd void vol. fraction was then applied to a Sepharose 6B (Pharmacia) column (3.3 × 40 cm) equilibrated with 50 mM K citrate–phosphate buffer. Using the same buffer, the β-galactosidase activity was eluted in two distinct fractions referred to as β-gal I and β-gal II (void vol. fraction)

Both of the active fractions were then separately subjected to lectin affinity chromatography on a Con A-Sepharose 4B column The column was equilibrated with 100 mM K citrate–phosphate buffer, pH 6.4 containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.25 M NaCl. The enzyme preparations were brought to similar conditions with respect to pH, divalent ions and ionic strength. The column was eluted thoroughly with this buffer until the *A* at 280 nm returned to zero. Elution of bound proteins was done with a linear gradient of 0–0.5 M of α-D-methylmannoside in the same buffer and subsequently by 0.2 M Na borate buffer at pH 6.7.

Protein content in the fractions eluted from all the columns was monitored by recording the *A* at 280 nm and by the assay of ref. [29].

β-Galactosidase activity assays. (i) *With* o- *and* p-*nitrophenyl-β-galactosides.* The incubation mixture consisted of 60 μl, 200 mM acetate buffer, pH 4.8; 200 μl of 30 mM *o*- or *p*-nitrophenyl β-D-galactoside and 40 μl enzyme. The incubations were performed for 15 min at 35°. (ii) *With lactose.* D-Glucose, the hydrolysis product was determined by glucose oxidase–peroxidase chromogen assay [30]. (iii) *With naphthyl and indoxyl β-galactosides.* The galactose released by the hydrolysis of these substrates was determined [31] using D-galactose dehydrogenase (Boehringer–Mannheim) After termination of β-galactosidase reaction by brief boiling, 50 μl of galactose-containing soln was transferred to the reaction mixture consisting of 75 μl 100 mM Tris buffer, pH 8.6, 25 μl 16.5 mM NAD^+ soln; 5 μl D-galactose dehydrogenase (25 U/ml) This was incubated at 37° for 20 min and the *A* at 340 nm was measured An appropriate control was run which had no galactose. (iv) *With galactans.* The galactanase activity was assayed by measuring the release of reducing sugars from larch arabinogalactan and high M_r polysaccharide fraction from lily stylar canal exudate This polysaccharide was prepared from unpollinated lily pistil by using the method described earlier [32] The incubation mixture comprised 200 μl of 200 mM acetate buffer, pH 4.8, 100 μl of 1% polysaccharide and 200 μl of the enzyme preparation The incubation was then performed at 37° and 50 μl aliquots were removed every 2 hr. The reaction was terminated by heating at 100° and soln was analysed for reducing sugars by dinitrosalicylate method as used earlier in ref. [19].

Assay of wall-bound β-galactosidase. Prior to assay, the aliquots of washed wall pellet were suspended in H_2O and assayed for hexokinase and malate dehydrogenase activities to check the purity of the fraction. The β-galactosidase assay mixture consisted of 100 μl of pollen wall suspension in 200 mM K citrate–phosphate buffer, pH 4.8, 100 μl of 50 mM *o*-nitrophenyl-β-D-galactoside (pH 4.8). The incubation was at 35° for 20 min. After incubation the soluble reaction mixture was separated by centrifugation and to an aliquot (100 μl), 900 μl of 300 mM Na_2CO_3 was added. The *A* was then measured at 420 nm. Similarly, the products of other substrates were determined in cell wall free supernatant by using the same methods described for soluble fractions.

Isoelectric focusing. Isoelectric focusing was performed using polyacrylamide gels having pH 3.5–9.5 range ampholytes (Biorad). Both samples of crude extract and partially purified preparations were used. The pH gradient was determined by using marker proteins (Pharmacia) of known isoelectric points. The gels were stained for β-galactosidase using 6-bromo-2-naphthyl-β-D-galactoside as a substrate [33].

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