β-1,3-Glucanase activity in the stigma of healthy petunia flowers

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Abstract

β-1,3-Glucanase activity was detected in extracts of different tissues of healthy mature petunia flowers except the filament. The stigma was studied further as it had the highest enzyme activity and there is a paucity of information on the occurrence of this enzyme in this tissue. Specific activity of the enzyme was found to increase within the stigmatic tissue from early development until just before anthesis. Following non-denaturing polyacrylamide gel electrophoresis at pH 8.8, extracts of dehiscent stigma seem to contain three acidic isoforms of β-1,3-glucanase. Crude extracts of stigma was passed through a pachyman affinity column. A fraction of affinity-purified active β-1,3-glucanase enzyme was found to have no antifungal activity against *Trichoderma viride*, *Phloma clematidina* and *Cladosporium fulvum*.

Additional key words: PAGE, pathogenesis-related proteins, *Petunia hybrida, Solanaceae.*

Introduction

β-1,3-glucanase is one of the most well known pathogenesis-related (PR) proteins that have been found in seeds or leaves (Suo and Leung 2001, Leubrer-Metzger 2003, Wu and Bradford 2003, Saikia *et al*. 2005). An apparent role for β-1,3-glucanase is in defense against phytopathogens such as fungi (Egea *et al*. 1999, Xing *et al*. 2003, Funnell *et al*. 2004, Kim *et al*. 2004). Recent studies have also provided evidence implicating a role for β-1,3-glucanase in several developmental processes (Delp and Palva 1999, Buchner *et al*. 2002, Leubrer-Metzger 2003) including pollen maturation (Bucciaglia *et al.* 2003). The occurrence of the enzyme activity in the anther is apparently associated with a critical stage in pollen development which involves the dissolution of the callose-containing wall (mainly β-1,3-glucan) surrounding the tetrads. Without this biochemical event, free microspores would not be released into the anther locule. Although β-1,3-glucanase has been studied at the molecular level in floral tissues besides anthers (Delp and Palva 1999, Yamguchi *et al*. 2002, Wu and Bradford 2003), the occurrence of the enzyme in other flower parts has not been studied. Futhermore, it is largely unclear as to what, if any, functional/developmetnal role the enzyme might play in these tissues.

Here, we focused on differences in β-1,3-glucanase activity among the different parts of healthy petunia flowers. Further, we examined the enzyme activity in the light of its specific role in the stigma using microarray analysis (Tung *et al*. 2005).

Materials and methods

Plants: Seeds of *Petunia hybrida* line 'Mitchell' were kindly provided by Dr. R. Gardner (University of Auckland, New Zealand) and were germinated on potting mix. When seedlings had four mature leaves, they were transplanted into individual pots and maintained in a growth room at 22 °C, 16-h photoperiod, irradiance of 100 μmol m^{-2} s^{-1} provided by fluorescent tubes, and relative humidity of 70 %. Flowering began 2 - 3 months after seed had been sown and at this point collection of floral tissues began. Petunia plants over seven months of age were not included in this study, as the floral morphology of these plants were not consistent. Healthy

Received 7 December 2005, *accepted* 25 August 2006.
Abbreviations: PAGE - polyacrylamide gel electrophoresis; PR - pathogenesis related.
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petunia flowers normally display the following developmental stages: stage 1 - very young petunia flower, tip of petals just beginning to extend past the sepals (12 - 14 mm); stage 2 - corolla extending to approximately half of the full length (30 - 35 mm); stage 3 - corolla fully extended, petals just beginning to open; stage 4 - petals fully open at an angle of 90° to the floral stem axis and anther predehiscent (predehiscent flowers); stage 5 - petals fully open at an angle of 90° to the floral axis and anther dehiscent (dehiscent flowers). Flower parts were collected at the appropriate developmental stages for enzyme studies.

Enzyme extraction and assay: Plant material was homogenised in sodium phosphate buffer (pH 6.8) with a mortar and pestle. The homogenate was centrifuged for 15 min at 23 000 *g* at 4 °C (*Eppendorf centrifuge 5403*, New Jersey, USA) and the supernatant was used as enzyme extract.

Enzyme extracts were incubated with 0.1 % (m/v) laminarin at 37 °C for 6 h and the control contained enzyme extract only. After incubation, laminarin was added to the control before 0.02 cm^3 was removed and mixed with 5 cm^3 of HBH reagent (para-hydroxybenzoic acid) for reducing sugar assay using the procedure of Lever (1973). The mixtures were placed in a boiling water bath for 5 min and then immediately placed in cold water (4 °C). The absorbance was read at 420 nm in a *Novaspec II* (*Pharmacia Biotech*, New Jersey, USA) spectrophotometer. A standard curve was constructed using a range of glucose concentrations. Protein concentrations of crude enzyme extracts were determined using Coomassive blue dye according to Bradford (1976) and bovine serum albumin (fraction V) was used to establish a protein standard curve.

Isozyme gel electrophoresis: Polyacrylamide gel electrophoresis (PAGE) at pH 8.8 for acidic β-1,3-glucanase isozyme determination was performed according to the *BioRad* (Hercules, USA) *mini-Protean II* dual slab cell instruction manual, with the exception that SDS (sodium dodecyl sulphate) was not added to the gel. Tracking dye was bromophenol blue (0.003 %, m/v) in 20 % (m/v) sucrose. Gel electrophoresis running conditions were 200 V for approximately 35 - 45 min. Staining for β-1,3-glucanase activity on the gel was based essentially on the method of Pan *et al*. (1989). Briefly, after electrophoresis, native PAGE gels (each containing two replicates of the appropriate enzyme extracts) were cut in half lengthwise, washed with distilled water three times and incubated in 0.05 M sodium acetate (pH 5.0) buffer for five times. One half of each gel (containing one set of the replicates of different extracts) was incubated at 37 °C for 3 h in a solution containing 10 cm^3 of the sodium acetate buffer and 0.133 g laminarin (the enzyme substrate) dissolved in 10 cm^3 distilled water. The remaining half was incubated under identical conditions, except that laminarin was omitted from the incubation solution. Both gels were rinsed in distilled water, fixed in a solution of methanol, acetic acid and distilled water (5:2:5, v/v) for 5 min and washed with distilled water again. The gel was then placed in a solution of 2,3,5-triphenyltetrazolium chloride (0.15 g) in 100 cm^3 of 1 M NaOH and placed in a boiling water bath until red bands appeared (usually within 5 to 10 min). Stained gels were put immediately into 7.5 % (v/v) acetic acid to reduce background staining.

Affinity column chromatography: An affinity column packed with pachyman (*Calbiochem-Novabiochem Corporation*, La Jolla, USA), was prepared and used for isolation of β-1,3-glucanase from petunia stigma extracts essentially as described by Krebs and Grumet (1993). Fractions of 1.5 cm^3 each were collected from the column. First the column was washed with 0.02 M sodium acetate buffer containing 0.5 M NaCl until absorbance of fractions (at least 10 consecutive ones) containing no or little protein, before it was washed with 25 cm^3 of 0.5 % (m/v) reduced laminarin solution. Fractions corresponding to peak activity were pooled, placed in a dialysis tube (mol. mass cut-off of 10 000) and concentrated using solid PEG (mol. mass of 20 000) and stored at -20 °C. Samples from affinity fractions were run on SDS-PAGE according to the method of Laemmli (1970). Protein concentrations of fractions collected from the pachyman affinity column were determined using *Sigma Kit No. BCA-1* (bicinchoninic acid protein determination) according to the instructions of the supplier.

Antifungal tests: Three different pathogenic fungal species were tested, *Trichoderma viride* (S7), *Cladosporium fulvum* (C24), and *Phoma clematidina* (C125), all from the University of Canterbury Fungal Collection. Numbers in parenthesis refer to isolate numbers.

Antifungal effects of three solutions were tested by the method of Mauch *et al*. (1988). Fungal plugs were placed on one-fifth strength potato dextrose agar (*Oxoid*, Hampshire, UK) plates and incubated for 24 h at 26 °C to allow for initial vegetative growth. At this time, sterile filter paper discs (4 mm diameter) were laid on the agar surface, and 0.02 cm^3 of the solutions to be tested were applied to the discs. The plates were further incubated at 26 °C for 2 - 4 d after the onset of treatment. All test solutions were filtered through a 0.22 μm membrane filter prior to application. The solutions tested were: *1*) approximately 5.12 mg cm^{-3} of affinity-purified β-1,3-glucanase which had first been concentrated against PEG as described above, *2*) half-strength affinity-purified enzyme; *3*) boiled enzyme control (undiluted enzyme was boiled for 5 min), and *4*) sterile water control.

Data analysis: One way analysis of variance (*ANOVA*) and *t*-tests were performed using *Microsoft Excel* data analysis tools. Standard error was also calculated using the descriptive statistics function of *Microsoft Excel*. Multiple analysis of means was performed using the Newman-Kuels test (Zar 1984). Significance was calculated to a minimum of 95 % confidence level.

Results and discussion

Localization of β-1,3-glucanase activity: Extractable β-1,3-glucanase was detected in different parts of mature dehiscent petunia flowers except the filament (Fig. 1). Extracts of stigma and style had the highest enzyme activity, followed by those of anther and ovary, while those of petal and sepal had the least enzyme activity. These results are different from a previous report (Leung 1992) on the localization of chitinase activity within a healthy petunia flower. A majority of the chitinase activity was found in the stigma, much less in the petal and not detectable in other reproductive parts of the flower. Thus β-1,3-glucanase and chitinase activities do not necessarily co-localize in all parts of a healthy petunia flower.

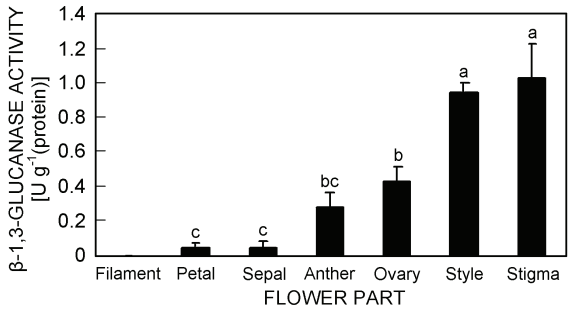

Fig. 1. Specific β-1,3-glucanase activity in extracts of petunia flower tissues at the dehiscence stage. Units of activity represent μg glucose released per hour. Data are from two experiments with values representing the mean of four replicates ± SE. The experiment was repeated three times with similar results. Values with the same letter are not significantly different.

This is the first report showing β-1,3-glucanase activity in petal and sepal of healthy flowers. It is not known how this enzyme within these tissues is of use to the plant. The occurrence of the enzyme activity in the anther could be associated with pollen maturation (Delp and Palva 1999, Leubrer-Metzger 2003) but that in the ovary and stigma is presently unknown. Interestingly, it has recently been hypothesized that the proteins in the stigma might be beneficial for pollen germination/pollen tube growth on stigma (Verhoeven *et al*. 2005). The possibility that β-1,3-glucanase could be one of the stigma proteins that plays such a functional role is intriguing but remains to be investigated further.

Developmental control of stigma β-1,3-glucanase activity: To complement the previous study on the occurrence of chitinase in the stigma of healthy petunia flowers (Leung 1992) and in light of the potential importance of β-1,3-glucanase in the stigma (Tung *et al*. 2005, Verhoeven *et al*. 2005), the rest of the present study focussed on β-1,3-glucanase activity in the petunia stigma. A time course study showed that activity of β-1,3-glucanase in the stigma increased as the flower grew and developed to the stage where the anthers were predehiscent. Then the enzyme activity dropped off slightly after the anthers were dehiscent (Fig. 2). This time course of changes in the enzyme activity is similar to that of chitinase in the stigma (Leung 1992), suggesting that both enzymes might be under the same developmental control. A multiple comparison of means using the Newman-Keuls test showed that β-1,3-glucanase activity within the stigma of predehiscent flowers was not significantly higher than that of the two preceding and one following stages. Stigmata of all developmental stages other than 4 were not significantly different from each other.

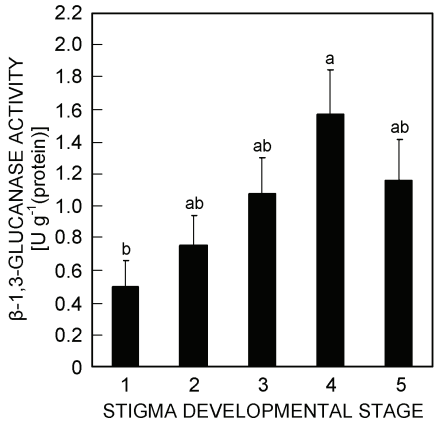

Fig. 2. Activity of β-1,3-glucanase in extracts of stigma from different developmental stages: 1 - very young petunia flower-tip of petals just beginning to extend past the sepals, 2 - corolla extending, approximately half of the full length (30 - 35 mm), 3 - corolla fully extended, petals just beginning to open, 4 - petals fully open at an angle of 90 ° to the floral stem axis, anthers predehiscent, 5 - petals fully open at an angle of 90 ° to the floral stem axis, anthers dehiscent. Other details as in Fig. 1.

Isozyme analysis: Analysis of the β-1,3-glucanase isozymes present in the extracts of stage 5 stigmatic tissue was performed according to the procedure described by Pan *et al.* (1989). After several preliminary experiments, it was found that 15 % acrylamide gel and at least 15 μg of crude protein extract of stigmata loaded in

each lane and 3-h enzyme assay would give the optimal resolution of isozyme bands (Fig. 3). On a gel at pH 8.8, two bands of isozmyes were clearly detectable. There seemed to be another band which was very diffuse at a time of enzyme assay that enabled the resolution of the other two isoforms (Fig. 3). None of the control gels (incubated without laminarin) displayed bands. Using an

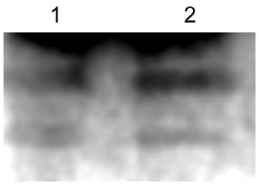

Fig. 3. Non-denaturing PAGE analysis of β-1,3-glucanase activity in crude stigma extracts. The gel was at pH 8.8. *Lane 1* - 30 μg protein; *lane 2* - 15 μg protein. Gel a was incubated with laminarin (substrate) for 3 h before staining with 2,3,5-triphenyltetrazolium chloride in 1 M NaOH.

acidic gel system (pH 3.8), only one isozyme was detected although the resolution seemed to be poor (data not shown). This is the first report on the occurrence of multiple isozymes of β-1,3-glucanase in stigma although leaves and seeds are known to have several isoforms of the enzyme (Suo and Leung 2001, Leubrer-Metzger 2003). Further work is required to elucidate what function each isozyme might have in the stigma.

Purification of β-1,3-glucanase using affinity chromatography: Concentrated extracts from 1000 stage 5 petunia stigmata were passed through a column containing pachyman, an insoluble β-1,3-glucan, for affinity absorption of β-1,3-glucanase protein which was eluted subsequently from the column by the addition of laminarin (Fig. 4). There were high levels of activity present in the fractions prior to the addition of reduced laminarin, suggesting that over 90 % of the β-1,3-glucanase activity in the crude petunia stigma extracts was not tightly bound onto the pachyman column. In the work on purification of β-1,3-glucanase from crude celery root extract using the same affinity column method (Krebs and Grumet 1993), the yield of enzyme activity was higher and close to 20 %. Following addition of reduced laminarin, a large peak of β-1,3-glucanase activity corresponding to the peak in protein level was detected. A second volume of reduced laminarin was also added in an attempt to elute the more tightly bound enzyme from the column, resulting in the last peak of enzyme activity (fraction 67) and protein content.

Fractions corresponding to peak protein and enzyme activity were pooled and concentrated (fractions 46 - 54 and 64 - 68). The protein concentration in these pooled affinity-purified fractions was about 0.0125 mg cm^{-3} which was just sufficient for checking purity on SDS-PAGE gel and antifungal tests (a primary objective of the present study) but not high enough for other more detailed characterizations such as isozyme analysis. A single major band was observed for both concentrated extracts (Fig. 5) indicating that a highly purified fraction of stigma β-1,3-glucanase has been obtained. The same result was obtained in the previous report on a single affinity purification step from crude celery root extract (Krebs and Grumet 1993). Our experiment was attempted three times and considerable effort was spent each time to collect 1000 stigmata from flowers of the same develop

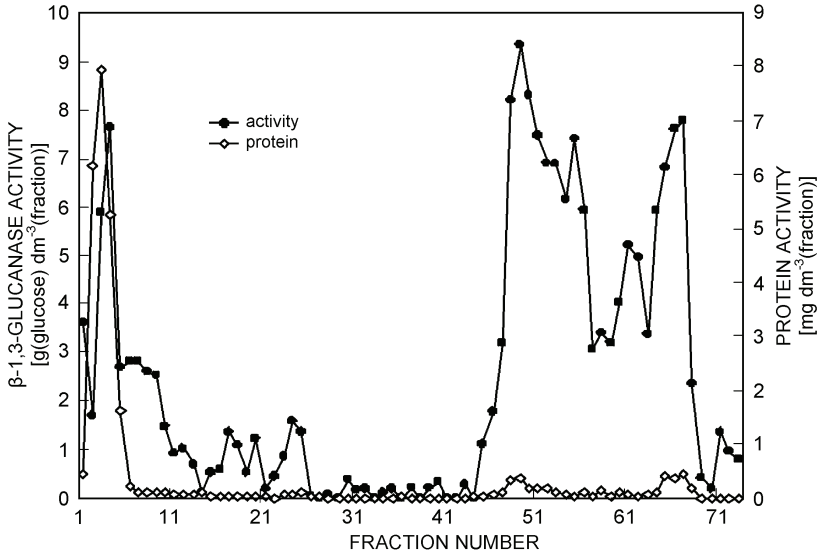

Fig. 4. Affinity chromatography of crude extract of 1000 dehiscent petunia stigmata. Elution profiles of total protein and β-1,3-glucanase activity from a *Pachyman* affinity column are shown. Protein and enzyme activity levels are indicated for fractions that followed the void volume. Wash 1: fractions 1 - 22 (33 cm^3 of 20 mM Na-acetate, 0.5 M NaCl, pH 5.2); wash 2: fractions 23 - 44 (33 cm^3 20 mM Na-acetate, pH 5.2); laminarin eluate 1: fraction 45 - 64 (30 cm^3 of 0.5 % reduced laminarin); laminarin eluate 2: fractions 65 - 73 (13.5 cm^3 of 0.5 % reduced laminarin).

mental stage. Future studies will need to examine more closely the operational parameters of this affinity column method and other enzyme protein purification approaches to optimize enzyme yield and purification.

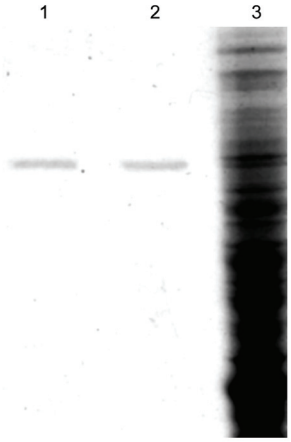

Fig. 5. SDS-PAGE gel showing purified petunia stigma β-1,3-glucanase protein (approximately 35 kD) from a *Pachyman* affinity column. *Lane 1* - concentrated fractions 46 - 54 (10 μg); *lane 2* - concentrated fractions 64 - 68 (10 μg); *lane 3* - crude stigmatic extract (5 μg). Bands in *lanes 1* and *2* were computer enhanced as in the original photo they did not show up sufficiently on a print.

Antifungal activity test: The affinity-purified β-1,3-glucanase after having been concentrated against powder PEG 20 000 from 0.0125 mg cm^{-3} to approximately 12.5 mg cm^{-3} was tested against three fungal species, *Phoma clematidina, Cladosporium fulvum,* and *Trichoderma viride* for antifungal activity. No direct antifungal activity was observed against any of the fungi tested. Microscopic observations of the fungi surrounding the disks revealed no discernible difference between them and hyphae surrounding the control disks (data not shown). These results suggest that at least some of the β-1,3-glucanase activity in petunia stigma does not have antifungal activity. In contrast, the pachyman affinity column-purified β-1,3-glucanase activity of celery root had hydrolytic activity against isolated cell walls of the celery wilt pathogen, *Fusarium oxysporum* (Krebs and Grumet 1993). Furthermore, Mauch *et al*. (1988) found that 0.01 - 0.03 mg cm^{-3} (concentrations much lower than those used in the present study) of β-1,3-glucanase purified from pea pods inhibited growth of *T. viride.*

In conclusion, the physiological significance of β-1,3-glucanase in the stigma of healthy petunia flowers remains to be investigated further. However, a least a purified fraction of the enzyme seems to be devoid of antifungal activity.

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