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Maria F. Chen

Preliminary determination of the number of β-1,3-glucanase isoforms and their cumulative activity in the leaves of the strawberry plant (*Fragaria x ananassa* Dutch. Cultiva Chandler)

Maria F. Chen
Thesis Advisor: Dr. Ding S. Shih, Department of Biological Sciences
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I. Objectives:

There are two main objectives in this project. The first is to establish an assay and ideal assay conditions for characterizing β-1,3-glucanase activity from crude extracts isolated from the strawberry plant. The second objective is to determine the number of isoforms of β-1,3-glucanase. Having this preliminary information will be useful in later plans to isolate and characterize purified β-1,3-glucanases from the strawberry plant.

II. Introduction

During the past few decades, the commercial acreage of strawberry plants, an important crop to Louisiana, has decreased. Factors contributing to the decrease are diseases, such as the anthracnose crown rot caused by the fungus *Colletotrichum fragariae*, that lead to considerable loss of crop (Smith and Black, 1990). One solution to this problem has been to develop strawberry cultivars resistant to diseases through traditional plant breeding techniques. Another possible and more expedient solution is to use transgenic techniques to incorporate genes that confer disease resistance to cultivars that have good horticultural characteristics but are susceptible to disease. This latter approach requires further study of the defense mechanisms found in the strawberry plant.

Plants activate a variety of defense mechanisms against pathogen attack. One of these mechanisms is the accumulation of a class of proteins called "pathogenesis related proteins" or PR proteins. Accumulation of such proteins often corresponds to disease resistance (Broglie and Broglie, 1993). Of interest are β-1,3-glucanases and chitinases, 2 sub-classes of PR proteins. It is thought that β-1,3 glucanases and chitinases defend plants by hydrolyzing β-1,3-glucan and chitin, which are the main carbohydrate components of most fungal cell walls. Specifically, chitinases catalyze the hydrolysis of β-1,4- (2 acetamido-2-deoxy)-D-glucosidic linkages in chitin while β-1,3- glucanases catalyze the hydrolysis of β-D-glucosidic linkages in β-1,3-glucans (Cabello, et al 1994). β-1,3-glucanases and chitinases have been show to synergistically inhibit the growth of several types of fungi *in vitro* (Mauch et al. 1988). In addition, it has been demonstrated that over-expression of these genes in transgenic plants provides resistance to some fungi (Broglie et al. 1991, Lin et al. 1995).

Multiple forms of β-1,3-glucanase have been characterized in different plants such as in tobacco, cotton, and chickpea plants, under various circumstances (Kauffmann, et al. 1987, Bucheli et al. 1985, Cabello et al. 1994). Different enzymatic activities, physical properties, and cellular compartmentation have been reported for the various isoforms. Presently, no work has been done to study β-1,3-glucanases in strawberry plants, to distinguish different isozymes, and to determine the activity of the enzyme. This paper presents preliminary work on establishing assay conditions to determine the enzymatic activity of β-1,3 glucanases in the strawberry plant and on distinguishing different isoforms using native polyacrylamide gel electrophoresis (PAGE).

III. Methods and Materials

Enzyme preparations

Leaves from healthy strawberry plants (*Fragaria x ananassa* Dutch. Cultiva Chandler) were harvested and frozen at –70° C. Leaves were ground to a fine powder using a mortar and pestle in liquid nitrogen. Samples were immediately extracted by homogenizing 1 g of ground leaves in 10 mls of cold extraction buffer (25 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$; 0.5 M NaCl; 10% (w/w) polyvinyl polypyrrolidone (PVPP); 1 mM phenylmethyl-sulfonyl fluoride (PMSF)) using a homogenizer (Dupont Instruments® Sorvall Omni-Mixer on high speed setting) 4 times at 1 minute intervals waiting 3 minutes between intervals to avoid heating the sample. The samples were centrifuged at 20,000 g for 15 minutes. Streptomycin sulfate was added to the supernatant to a final concentration of 15 g/L. Samples were centrifuged again at 20,000 g for 15 minutes. Solid ammonium sulfate was added to the supernatant to 80% saturation and proteins were precipitated at 4 °C for 60 minutes. Samples were centrifuged at 20,000 g for 20 minutes and the pellet was resuspended in extraction buffer without NaCl (1/50 of original volume). After clarification by centrifugation (Eppendorf centrifuge, 10 minutes), the extract was used directly for enzyme assays and identification of isoforms on native PAGE.

Protein determination

Protein concentration was determined using the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.

Enzyme assays

Neocuproine test

Activity of β-1,3, glucanase was initially determined using laminarin (Sigma) as a substrate by the neocuproine test (Dygert et al.1965). The standard assay contained 1 µl enzyme extract (with a protein concentration of 1 µg/µl) and 0.35 ml laminarin (0.3 % solutions for Trials 1-4) in Na-acetate buffer (0.1 M, pH 5.0). The reaction mixture was incubated for 45 minutes at 37 °C. Enzyme alone and substrate alone were used as controls. Various amounts of glucose (15 µg, 25 µg, 50 µg, 75 µg) were used as standards. Reactions were stopped by incubating samples on ice. Equal volumes (4 ml) of Solution A (40 g anhydrous Na_2CO_3, 16 g glycine, 0.450 gram $CuSO_4 \cdot 5H_20$ in 1L dH_2O) and freshly prepared Solution B (0.12 g neocuproine·HCl (Sigma) in 0.1 L dH_2O) were added to all samples. Samples were heated in a boiling water bath for 12 minutes. After boiling, samples were allowed to cool to room temperature for 30 minutes. Samples were diluted to 10 mls and their absorbances at 450 nm were measured against water using a spectrophotometer. Readings were completed within 30 minutes. Activities were calculated as nkat mg^{-1} protein with one nkat defined as the enzyme activity catalyzing the formation of 1 nmol of glucose equivalents in 1 second under the assay conditions.

Modified neocuproine test (Boller and Mauch, 1988)

Preparation of substrate, reduced laminarin (2% w/v)

To eliminate the problem of high substrate blank due to the relatively short chain length of laminarin, the terminal groups of the laminarin chains were reduced with sodium borohydride ($NaBH_4$). Briefly, 6 g of laminarin was dissolved in 150 ml water and stirred at 80 °C for 20 min. One g of solid sodium borohydride was added and stirring continued at 80 °C for 60 min. The solution was cooled to 40 °C and the pH was adjusted to 5.5 with glacial acetic acid. A mixed-bed ion exchange resin (Amberlite MB3) was added and the mixture stirred for 30 min to remove salts. The mixture was

filtered and the ion exchange beads were washed by resuspending in 100 ml dH_20. Both filtrates were combined and the volume was adjusted to 300 ml to obtain a 2 % solution of reduced laminarin. Aliquots were frozen at -20°C.

Removal of reducing sugars in enzyme extract.

To reduce the high enzyme blank due to high level of reducing sugar in the enzyme extract, the enzyme extract was passed over a Sephadex G-25 column using 10 mM sodium acetate, pH 5.0, 10 mM β-mercaptoethanol as a column buffer.

Protein concentration of the pooled protein-containing fractions was determined again. Pooled fractions were referred to as "column-processed" extract.

β-1,3-glucanase essay

Reaction mixtures (250 µl) were prepared containing 50 µl of 100 mM sodium acetate buffer (pH 5.5), 20 µl of enzyme extract, 50 µl of 2 % reduced laminarin, and column buffer to bring total volume to 250 µl. The following blanks (with their abbreviations for reference) were also prepared for each enzyme preparation (E): substrate blanks (SB), containing column buffer instead of enzyme extract; reagent blanks (RB),containing column buffer instead of enzyme extract and 50 µl water instead of the laminarin solution, reagent blanks with internal standard (RI), containing column buffer instead of enzyme and 50 µl of 3 mM or 5 mM glucose instead of laminarin; and enzyme blanks (EB), containing 50 µl water instead of laminarin. Reactions were mixed and incubated at 37 °C for 30 minutes. Reactions were stopped by returning mixtures to ice. Equal volumes (3 mls) of Solution A and B from the neocuproine test (See above) were added to each sample and all samples were heated in a boiling water bath for 12 minutes. Reactions were cooled for 30 minutes to room temperature. Samples were diluted to 10 mls. Absorbances at 450 nm (A_{450}) were measured against water using a spectrophotometer. The amount of reducing sugar formed in the assay is calculated as

$$\text{nmol sugar} = \left[\frac{A_{450} \text{ of E} - A_{450} \text{ of EB}}{A_{450} \text{ of RI} - A_{450} \text{ of RB}} - \frac{A_{450} \text{ of SB} - \text{A450 of RB}}{A_{450} \text{ of RI} - \text{A450 of RB}} \right] \times \text{nmol of glucose in internal standard}$$

The activity was calculated in nkat mg^{-1} protein with 1 nkat defined as the enzyme activity catalyzing the formation of 1 nmol of glucose equivalents in 1 second under the assay conditions

Native PAGE

Anodic polyacrylamide resolving gels (15%, 1.0 mm thick) were prepared by mixing 5 ml 30 % acrylamide (acrylamide/Bis; 30:0.8), 3.675 ml dH_2O, 1.25 ml 3.0 M Tris-Cl, pH 8.8, 0.075 ml 10 % ammonium persulfate, 10 µl TEMED. The stacking gel included 0.625 ml 30 % acrylamide (acrylamide/Bis; 30:0.8), 3.72 ml dH_2O, 0.625 ml 1 M Tris-Cl (pH 6.8), 0.038 ml 10% ammonium persulfate, 8 µl TEMED. The gels were run at constant voltage (100V through stacking gel and 200 V through resolving gel) on a Hoeffer® Mighty Small Electrophoresis unit.

β-1,3-glucanase detection on native PAGE

To stain for β-1,3-glucanases, the PAGE gels were washed with water, incubated with 0.05 M sodium acetate (pH 5.0) for 5 min. Gels were then incubated at 40 °C for 45 min in a mixture containing 75 ml of 0.05 M sodium acetate (pH 5.0) and 1 g of laminarin dissolved in 75 ml of water by heating in a boiling water bath. The gels were then incubated in a mixture of methanol, water and acetic acid (5:5:2) for 5 min., washed with water, and stained with 0.15 g of 2,3,5-triphenyltetrazolium chloride (Sigma) in 100 ml of 1.0 M NaOH in a boiling water bath until red bands appeared (about 10 min).

IV. Results

A. Enzyme activity using unmodified neocuproine test

β-1,3-glucanase activity was detected in the leaves of the strawberry plant according to the unmodified neocuproine test of Dygert, et al. (1965). An average activity of 19.14 ± 9.45 nkat mg^{-1} protein was calculated (Table 1). Within trials, it was observed that the increase in the volume of the enzyme sample and the concentration of the laminarin substrate solution corresponded to a decrease in activity (data not shown). This led to attempts to reduce the background from the enzyme extract and substrate.

Table 1: β-1,3-glucanase activities (nkat mg^{-1} protein) in strawberry leaves. The value of trials 1 was obtained from 4 samples; the values of trials 2, 3, 4 were obtained from 6 samples)

Trial	Activity
1	17.73 + 10.17
2	23.37 + 9.02
3	20.13+ 5.86
4	24.14 + 6.18
Average activity	21.67+7.59

B. Enzyme activity using modified neocuproine test (Boller and Mauch)

β-1,3-glucanase activity was detected in strawberry leaves using the modified neocuproine test according to Boller and Mauch (1988). The average activity was determined to be 22.03 ± 6.59 nkat mg^{-1} protein (Table 2).

In certain trials, precipitation was observed to be present in the column-processed enzyme extract. Samples were centrifuged and the activity of the supernatant was tested. Protein concentration was determined again for these samples.

Table 2: β-1,3-glucanase activity (nkat mg-1 protein) in strawberry leaves. The value for trial 1 was obtained from 4 samples; 6 samples each for trials 2 and 4; 9 samples for trial 3.

Trial	Activity
1	30.76+4.81
2	21.56+1.42
3	20.05+1.51
Average Activity	23.29+5.13

C. Detection of β-1,3-glucanase isoforms using PAGE

Anodic PAGE gels were run to separate β-1,3-glucanase isozymes in the crude extract and in the column processed extract that was passed through the molecular sieve column. The β-1,3-glucanase isozymes were stained with 2,3,5-triphenyltetrazolium chloride on the PAGE gels. There was one dominant β-1,3-glucanase isozyme detected in the crude extract and two in the column processed extract (Figures 1 and 2). The intensity of the bands increased with an increase in the amount of enzyme extract used.

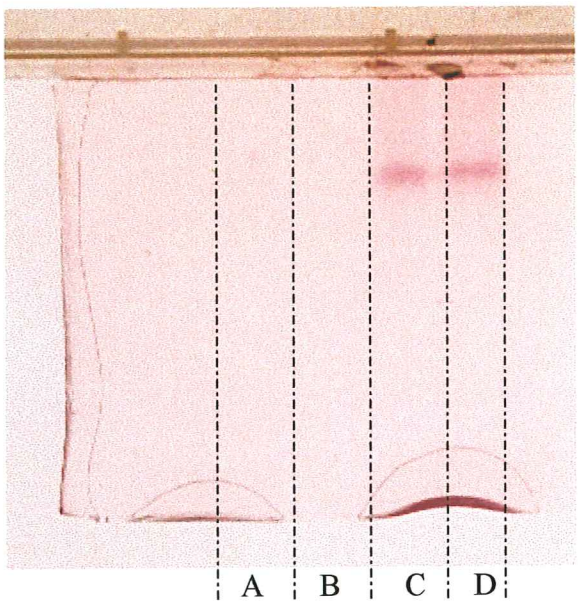

Figure 1: Detection of acidic β-1,3-glucanase isozymes after anodic native polyacrylamide gel electrophoresis of crude leaf extract from strawberry leaves. Crude enzyme extracts (1 μg in lanes A and B, 5 μg in lanes C and D) were loaded on 15% PAGE gels and stained for β-1,3-glucanase isoforms according to procedure.

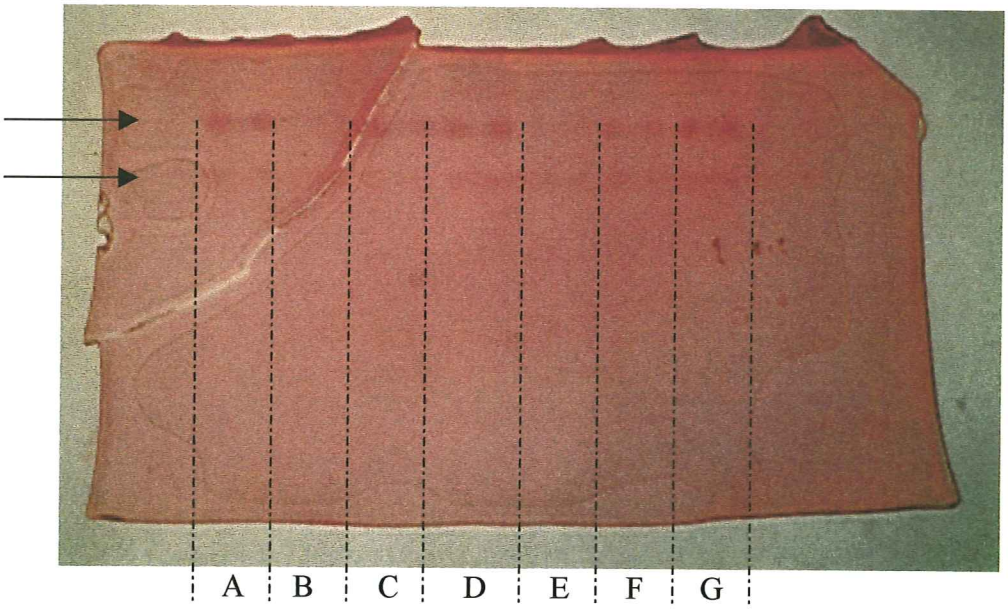

Figure 2: Detection of acidic β-1,3- glucanase isoforms after amodic native polyacrylamide gel electrophoresis of column-processed extract from strawberry leaves. Column processed extract (10 ug protein in lanes A, D, and G, 5 ug protein in lanes C and F, and 2 ug protein in lanes B and E) were loaded on 15% PAGE gels and stained for β-1,3-glucanase isoforms according to procedure.

V. Discussion

β-1,3-glucanase activity was detected in the leaves of healthy strawberry plants (*Fragaria x ananassa* Dutch. Cultiva Chandler). Using the original neocuproine test described by Dygert et al. (1965), the β-1,3-glucanase activity was determined to be 19.14 ± 9.45 nkat mg^{-1} protein. As the background from the enzyme and substrate blanks were quite high using this method, attempts were made to lessen the background and possibly reduced the standard deviation of the activity measurements.

One source of high background is the relatively short chain length of the substrate laminarin. Previous studies have reported chains lengths of 16 to 20 glucose units (Black et al, 1951). Thus for approximately 16 to 20 units of glucose attributed to the hydrolytic action of β-1,3-glucanase, the production of one glucose unit should not be attributed to the enzymatic action of β-1,3-glucanase. To eliminate this background, the terminal end of laminarin was reduced with sodium borohydride and used in the modified neocuproine test. In addition, since crude plant extract contain high levels of reducing sugar, the crude plant extract was passed through a molecular sieve column to remove the sugars. This column processed extract was then further analyzed for β-1,3-glucanase activity in the modified neocuproine test and for the detection of isozymes on PAGE.

Determination of β-1,3-glucanase activity using the modified neocuproine reaction yielded an average activity of 23.293 ± 5.13 nkat mg^{-1} protein. This activity is similar to that determined by first method of 21.67 ± 7.59 nkat mg^{-1} protein. Though the variation in the activity was decreased using the modified test, the standard deviation of the activities is still significantly large indicating that further work is necessary to better standardize the assay conditions. In particular, one source of variation is due to the precipitation of proteins in the crude and dialyzed extract even though reducing agents (such as β-mercaptoethanol) were present in the solution to prevent aggregation. Further purification and establishing better solvent conditions (salt concentration, pH) may help resolve this problem.

Though no reported activity values for β-1,3-glucanase isolated from strawberry was found in literature, the activity reported here is comparable to the activities of β-1,3-glucanases found in other plants. Activities of 5, 23, 1100, 1300 nkat mg^{-1} protein were

reported for the four isozymes found in tobacco; 25 nkat mg^{-1} protein for grapefruit flavedo; 56 and 443 for groundnut leaves; and 0.6 for chickpea plants have been reported (Kauffmann et al. 1987, McCollum et al. 1997, Roulin and Buchala 1995, and Cabello et al. 1994).

The determination of the number of isozymes of β-1,3-glucanases in strawberry leaves was accomplished by staining for activity after separation on an anodic native PAGE gel. Whereas only one band was detected in the crude extracts, two bands (one intense band and one less noticeable band) were detected in the dialyzed extract indicating the presence of at least two isozymes in strawberry (Figures 1 and 2). As the crude extract and dialyzed extract were from different samples of strawberry leaves, it is not possible to attribute the difference in number of isozymes detected to variation in enzyme extraction technique. Further analysis of crude and dialyzed extracts of each sample needs to be done for clarification on this point. For both extracts, the intensity of the band staining increased with an increase in the amount of enzyme sample loaded.

No report on the number of β-1,3-glucanase isozyme in strawberry was found in literature. However, numbers of β-1,3-glucanase isozymes were reported for other plants. Four isozymes were reported in the tobacco, two were reported in grapefruit flavedo, four were reported in chickpeas, and two in barley (Kauffmann et al.1987, McCollum et al.1997, Cabello et al. 1994, Ignatius et al. 1994).

This paper has demonstrated the presence of β-1,3-glucanase activity in the leaves of strawberry plant and distinguished at least two isozymes of β-1,3-glucanase. Preliminary measurements of the cumulative activity of β-1,3-glucanase isozymes using the neocuproine test (19.14 ± 9.45 nkat mg^{-1} protein) and the modified neocuproine test (22.03±6.59 nkat mg^{-1} protein) are close in agreement. However, variation in the measured activities indicates that further attempts to standardize assay conditions are required for a more accurate characterization of purified β-1,3-glucanase isozymes.

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