Antifungal Proteins from Grains

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These exercises adapt procedures for purification and assay of antifungal proteins from grains for the undergraduate laboratory. They introduce students to concepts of protein purification and bioassay. These experiences integrate areas of microbiology and biochemistry and afford opportunities for further independent student research projects on a topical subject.

INTRODUCTION

Plants have defenses against infection. Defenses against fungi include the production of a variety of substances which are inhibitory - phenols, melanins, tannins, salicylic acid or phytoalexins, as well as proteins which can inhibit growth of fungi. Antifungal proteins have been isolated and characterized from maize seeds (Huynh, Borgezeyer and Zobel, 1986) and from barley, wheat and rye (Roberts and Selitrennikofk, 1986 a,b). These proteins are very likely a part of a complex defense system of plants (Angier, 1992). Do all grains possess these antifungal proteins as part of a defense mechanism? Are these proteins present in all tissues of the plant as it develops? A better understanding of the roles of these proteins in plant defenses may be used in attempts to improve plants using gene engineering technologies by introducing genes determining these proteins into plants which are not resistant to fungi (Moffat, 1992). This laboratory project describes the isolation, purification, characterization and bioassay of antifungal proteins from grains.

The theories and practice of these techniques are well documented (Alexander and Griffiths, 1993; Bollag and Edelstein, 1991; Boyer, 1993; Robyt and White, 1987). However, the purification of proteins with biological activity other than enzyme activity is rarely presented. Antimicrobial assays are often part of microbiology curricula, usually as antibacterial antibiotic assays using bacteria as test microorganisms. The assay described by Roberts and Selitrennikoff (1986a) is a modification of these procedures adapted for testing antifungal inhibitors. In this paper disk assay, the inhibitor(s) diffuse towards advancing mycelia growth of the test fungus. Under the conditions described, the presence of an inhibitor stops advancing growth when compared to the growth in the absence of inhibitor.

With this background, students may design experiments for independent investigations.

MATERIALS

Isolation of Proteins

Grains (barley, wheat, rye) were purchased in bulk from Constance, Ferre, and Co., Wethersfield, CT.

Electric coffee grinder
60mM acetic acid
Centrifuge
1 M Tris base
pH meter
Ammonium sulfate
Dialysis tubing
5mM sodium phosphate buffer, pH 7.0 containing 50 mM NaCl
5 mM sodium phosphate buffer, pH 7.0 containing 200 mM NaCl
10 mL column of CM Sephadex
(approximately 1 cm X 3.3 cm column) spectrophotometer
1.5 x 50 cm column of Sephacryl S-200
10 mM sodium phosphate buffer, pH 7.4 containing 125 mM NaCl
Blue dextran
Ruler
0.44 micron Millipore filters and filtration apparatus
Bioassay

**Culture:** *Tricholoma ressei* ATCC #t363 was obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA.

Potato carrot agar (Medium #335, Jong and Edwards, 1991)

0.7 cm diameter sterile paper discs

**METHODS**

**Lab I**

**Protein purification:** isolation of proteins from grains. Extracts should be handled in the same manner one would handle enzymes (Boehringer Mannheim Biochemicals, 1985). The procedures are:

1. Weigh 120 grams of plant seeds.
2. Grind to a fine powder in an electric coffee grinder.
3. Add this powder, with stirring, to 300 ml 60 mM acetic acid cooled on ice.
4. Stir in the cold (4°C) for 1 hour.
5. Centrifuge at 7400 rpm for 30 minutes.
6. Discard pellet. Neutralize the supernatant to pH 7.6 by the dropwise addition of 1 M Tris base, using a pH meter.
7. Place at 4°C overnight. (This is an appropriate stopping point.)

**Lab II**

**Ammonium sulfate fractionation.**

8. Remove any precipitate by centrifugation at 7400 rpm for 30 minutes. Save a 3ml sample of the supernatant for further analyses. Use the remainder for the next step.

9. Ammonium sulfate fractionation:
   a. Slowly, while stirring, add ammonium sulfate to a final concentration of 30% (w/v). For the weight of salt to use, consult a table of ammonium sulfate concentrations adjusted for 4oC. Remove any precipitate by centrifugation at 7400 rpm for 30 minutes. Save the pellet, label ‘0-30% ammonium sulfate fraction’ and store at -20°C (freezer).
   b. Adjust the supernatant to 55% saturation with ammonium sulfate, keeping the solution cold (on ice). The precipitate contains antifungal protein(s) activity. Collect this by centrifugation at 7400 rpm for 30 minutes. Save a 3 ml sample of the supernatant for future analysis. Label it ‘30-55% ammonium sulfate fraction’.
   c. Dissolve the precipitate in 10 mM NaCl 5 mM sodium phosphate, pH 7.0. This may require 3 to 25 ml, depending on the amount of precipitate formed. This will vary with the extraction conditions as well as with different grain extracts tested.
   d. Dialyze against 10 mM NaCl 5 mM sodium phosphate (pH 7.0) overnight with at least two changes of buffered saline. (This is an appropriate stopping point.)

**Lab III**

**Ion exchange chromatography** using carboxymethyl Sephadex (CM-Sephadex).

10. CM-Sephadex chromatography
   a. Remove the 30-55% ammonium sulfate fraction from the dialysis tubing. Save a 1 ml sample for future analysis; label appropriately and store in the freezer.
   b. Prepare a CM-Sephadex column in 10 mM NaCl 5 mM sodium phosphate, pH 7.0 and pouring it to form a column which contains 10 ml of the resin.
   c. Add the 30-55% ammonium sulfate fraction to the column. When the protein solution descends to the top of the column bed, rinse it with 20 ml of mM NaCl 5 mM sodium phosphate (pH 7.0)
solution. Remove contaminating proteins by eluting with 50 mM NaCl 5 mM sodium phosphate (pH 7.0). Monitor the 3 to 5 ml fractions by measuring absorbance at a wavelength of 280. Continue to collect fractions of 3 to 5 ml after there is a drop in absorbance at wavelength 280. Antifungal protein(s) are then eluted from the column with 200 mM NaCl 5 mM sodium phosphate (pH 7.0).

d. Determine the absorbance of the column fractions at wavelength 280.

e. Pool fractions that have a high absorbance. Set aside a 1 ml sample and store it and the remainder of the protein solutions in the freezer. Label appropriately. (This is an appropriate stopping point.)

Lab IV
Gel exclusion chromatography.

11. Gel filtration: Sephacryl S - 200

a. Prepare a 1.5 x 50 cm column of Sephacryl S - 200 equilibrated with 125 mM NaCl 10 mM sodium phosphate (pH 7.4). Standardize the column by adding about 1 ml blue dextran solution. This will check the uniformity of the column and give the void volume. The void volume is the volume in ml required to elute the blue dextran.

b. Add 1 ml of 1 mg/ml of protein fraction from CM-Sephadex chromatography to the Sephacryl column.

c. Collect fractions (3 to 5 ml) of equal volumes. The flow rate is approximately 12 ml per hour.

d. Monitor the absorbance of the fractions eluted at wavelength of 280.

e. Pool the fractions containing the peak absorption values.

f. Filter sterilize this preparation, aliquot into sterile microcentrifuge tubes, store in the freezer and label. This preparation will be examined for antifungal activity and protein content. (This is an appropriate stopping point.) This purification scheme is presented in Figure 1.

Lab V
Bioassay of antifungal activity.

In this laboratory, the amount of grain extract which inhibits a fungus will be determined. The fungus used is Trichoderma reesei.

Using sterile procedures, dilute the plant protein fraction to be tested as shown on Table 1.

Using a template, aseptically place a 0.7 cm paper disc in the center of a potato-carrot agar plate and four discs 1.2 cm from the central disc and equidistant from each other. Add 20 microliters of dilutions of plant protein fractions to the other discs.

Add 1 ml sterile PBS to a slant culture of Trichoderma reesei. Agitate vigorously using a vortex mixer (if available) or by hand to form a slightly turbid suspension of conidia. Add 20 microliters to the central disc. Incubate plates at room temperature in a moist, closed container. Inspect daily until mycelia growth from the central disc has enveloped the peripheral disk containing PBS only (control) and had formed crescents of inhibition around the discs containing inhibitor concentrations of antifungal proteins. Record all observations.

Lab VI
Protein Assays

Protein may be determined using several methods. However, the method recommended for use is the spectrophotometric method of Warburg and Christian (1941). Samples or dilutions of samples are

| Table 1. Dilution levels of plant protein fraction. |
|-----------------|--------------|-------------|
| Dilution | Microliters of extracts | Microliters of PBS |
| 1:2 | 10 | 10 |
| 1:2 | 10 | 20 |
| 1:10 | 10 | 90 |
| 1:30 | 10 | 290 |
| 1:100 | 10 | 990 |
Figure 1: Antifungal proteins purification scheme

Antifungal Proteins Purification Scheme

Grind 120 g. grain seed in electric coffee grinder to fine powder

Resuspend in 300 ml 60 mM acetic acid; stir 1 hour at 40°C

Centrifuge 7400 rpm for 30 minutes

Supematant (Sample I)
(discard)

Pellet

Adjust pH to 7.6;
Place at 40°C overnight

Centrifuge 7400 rpm 30 minutes

Supematant
0 - 30% (NH₄)₂SO₄

Centrifuge 7400 rpm 30 minutes → Pellet (discard)

Supematant
30 - 55% (NH₄)₂SO₄

Centrifuge 7400 rpm 30 minutes → Supematant (discard)

Pellet - dissolve in 3 - 25 ml 10 mM NaCl/5 mM sodium phosphate, pH 7.0;
Dialyze overnight against this buffer (PBS) (Sample II)

CM - Sephadex chromatography

10 mM NaCl/5 mM PBS → discard

5 mM NaCl/5 mM PBS → discard

200 mM NaCl/5 mM PBS (collect fractions)

Pool fractions with O.D.₂₈₀ > 0.7 (Sample III)

Gel filtration (Sephacryl S-200)

Pool fractions with O.D.₂₈₀ > 0.2 (Sample IV)

Analyze fractions for protein and for antifungal proteins by bioassay

Figure 1. Antifungal proteins purification scheme.
examined at wavelengths 280 nm and 260 nm for UV absorption and protein concentrations are estimated by use of a nomograph (Robyt and White, 1987).

Thaw the samples of fractions formed by the protein purification procedure. First, pipette 1 ml of undiluted sample into a 1 ml cuvette. If the concentration is too high, absorption may be greater than 1.00 at either (or both) of these wavelengths. In that case, dilute the sample 1:10 by pipetting 0.1 ml of the sample into 0.9 ml of water and then read absorbance at 280 nm and 260 nm. Multiply the estimation of protein concentration by the dilution factor (10, in this case) for the samples.

Record the concentration of protein in mg/ml for each fraction.

Additional Laboratories
As an additional exercise, protein purification may be analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). I have used the procedures and materials supplied by BioRad Corporation (Bio-Rad Laboratories, 220 Maple Avenue, P.O. Box 708, Rockville Center, NY 11571, USA) to perform SDS-polyacrylamide gel electrophoresis separation of the antifungal proteins in the samples. Low molecular weight standards should be separated to permit estimation of molecular sizes of isolated protein bands. The purest preparation should contain a large portion of one protein molecule (an antifungal inhibitor). The Coomassie blue staining procedure is used; samples applied to the gel contain at least 1 microgram of protein (minimum amount). Prepoured 10% gels purchased from BioRad are used.

Gels are dried using the procedure of Michael and Ford (1991). Dried gels may be copied and/or mounted directly in the laboratory notebook.

Results
The purification of antifungal protein activity from barley (*Hordeum vulgare*) seeds is summarized in Table 2. The fractions examined are indicated in the purification scheme (Figure 1). Antifungal activity is determined by bioassay. The assay of fraction IV for this preparation is shown in Figure 2. A unit of activity is defined as the smallest amount of protein from a particular fraction which causes growth inhibition of the fungus. The purification table shows that antifungal activity may be isolated by protein purification.

![Figure 2](image_url) Bioassay of antifungal activity by barley extract. Paper disks contained 25 microliters of grain extract diluted 1:30 [disk 1], 1:3 [disk 2], 1:1 [disk 3] or 25 microliters of PBS disk c. Conidia from *T. reesei* were added to the central disk. Plates were incubated at room temperature in a moist chamber for 72 hours.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total Protein (mg.)</th>
<th>Total antifungal units x 10^-3</th>
<th>% units recovered</th>
<th>Specific activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude grain extract</td>
<td>1157</td>
<td>897</td>
<td>100</td>
<td>775</td>
</tr>
<tr>
<td>2. 30-55% (NH4)2SO4</td>
<td>250</td>
<td>150</td>
<td>17</td>
<td>600</td>
</tr>
<tr>
<td>3. CM-Sephadex pool</td>
<td>38</td>
<td>88</td>
<td>9.8</td>
<td>2316</td>
</tr>
<tr>
<td>4. Sephacryl pool</td>
<td>7</td>
<td>14</td>
<td>1.6</td>
<td>2000</td>
</tr>
</tbody>
</table>

*One unit is defined as the smallest amount of protein from a fraction causing detectable growth inhibition of the fungus.
Using these procedures, students have examined different grains for protein content, and antifungal activity, examined germinated seedlings (roots and shoots) for antifungal protein content and analyzed fractions obtained by protein purification by SDS-PAGE. Other projects have examined different test fungi in bioassays. These student projects reproduce published data (Huynh, Borgmeyer and zobel, 1986; Roberts and Selitrennikoff, 1986) and extend knowledge about plant-fungi interactions (unpublished results).

These laboratory exercises provide opportunities for cooperative learning, particularly if students have differing background experiences. For example, microbiology students may assist those without training in sterile procedures. Students are motivated to develop independent research projects, once the procedures are completed successfully in the structured laboratory. These laboratories require an integration of learning, and an interdisciplinary approach to scientific investigation. Additional laboratory experiences which may be performed include the analysis of protein fractions by SDS-PAGE and more precise protein analyses. However, the protein purification and bioassay procedures as outlined here provide investigative laboratory experiences for students and skills to develop independent research projects.

Literature Cited


