

# GLOMALIN EXTRACTION

(Rillig, 2004; Rosier *et al.*, 2007; and Rillig, 2003; Wright *et al.*, 1996; Wright and Jawson, 2001; Wright, Nichols, & Schmidt, 2006; Wright & Upadhyaya, 1996; & Wright & Upadhyaya, 1998)

## Introduction

Any of the following procedures may be used to remove glomalin from field soil, roots, mesh (horticultural or nylon) strips or bags, or pot culture media (sand or crushed coal) depending on the objectives of the experiment. Each procedure has a brief introduction explaining what fraction or pool of glomalin is represented by a given procedure. The extract solution then may be used in further analyses (e.g. ELISA, Bradford total protein assay and dot blot assay). Caution must be used in the current analysis of glomalin since the extraction protocol may co-extract other soil proteins, humic substances, or polyphenolics. Caution must be used when conducting the ELISA and Bradford total protein assays on the extracted material. In the Bradford total protein assay, sodium pyrophosphate cross-reacts with the Bradford dye reagent and must be added to the standard as described in that procedure. Also, the Bradford assay measures total protein with a dye that reacts with any protein >3000 daltons and with polyphenolics. Therefore, these values should be listed as Bradford-reactive soil protein (BRSP) as described in Rillig, 2004 and should not be relied upon as exact glomalin values. For the ELISA, immunoreactivity may be reduced due to prolonged exposure to high temperature.

**Note: It is recommended, in any of the procedures listed below, to use at least three duplicate samples from each soil sample, depending on field sampling protocols used and resources. It is also recommended to separate the coarse fraction (i.e. roots, sand, and gravel) from the soil weight. A method for separating the coarse fraction is provided below.**

# Sodium Pyrophosphate Extraction

## Introduction

This method for glomalin extraction utilizes sodium pyrophosphate at a higher concentration and pH level to extract glomalin. Research has demonstrated that this procedure may extract more glomalin than the total protein procedure and may remove some of the more recalcitrant fraction of glomalin rather than having to conduct a longer sequence of extractions (Wright, Nichols, and Schmidt, 2006). The recalcitrant glomalin fraction was identified after extracting soil samples using the total protein extraction procedure followed by extraction with sodium hydroxide for humic acid and then another extraction with sodium citrate (Nichols and Wright, 2005). This procedure does not separate out a glomalin fraction by age but rather is an operationally-defined glomalin pool which many represent more of the total amounts of glomalin in the soil. Continuing to analyze this pool may give some valuable information in regards to function of glomalin on fungal hyphae or soil aggregates.

**Note: When utilizing sodium pyrophosphate to extract glomalin, sodium pyrophosphate cross-reacts with the Bradford dye reagent and must be added to the standard (see Bradford total protein procedure with pyrophosphate modification).**

**Note: It is recommended, in any of the procedures listed below, to use at least three duplicate samples from each soil sample, depending on field sampling protocols used and resources. It is also recommended to separate the coarse fraction (i.e. roots, sand, and gravel) from the soil weight. A method for separating the coarse fraction is provided below.**

## Materials

Autoclave\*

Autoclavable round-bottom centrifuge tubes (50 ml) (Nalgene tubes work the best)

Caps for centrifuge tubes (small holes may need to be punched into the caps to relieve pressure during autoclaving)

Centrifuge tube rack

Centrifuge capable of at least 3000 xg with appropriate adaptors for 50 mL tubes

50 ml screw-capped tubes for storing extract

100 mM sodium pyrophosphate, pH 9.0

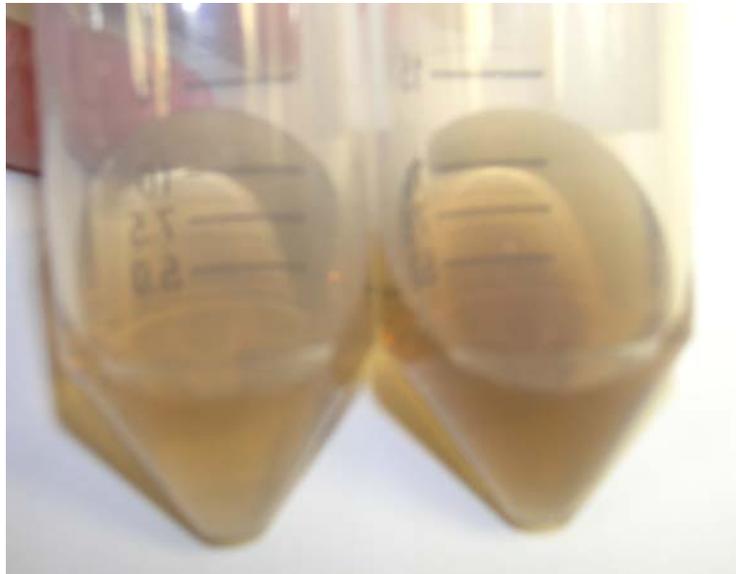
Graduated cylinder

Microtiter tubes

\*An alternative to using an autoclave is to use a pressure cooker. This methodology was tested and proved to be possible (Wright and Jawson, 2001).

## Methods

- 1) Place 1.0 to 2.0 g of soil in a centrifuge tube with 8 ml 100 mM sodium pyrophosphate.
- 2) Cap tubes and vortex to have appropriate soil:solution contact.
- 3) Autoclave for 60 min at 121°C.
- 4) Centrifuge at 5000 xg for 10 min after samples are cool to the touch. (Centrifugation is just to pellet the soil particles and may be conducted at any speed from 3000-10000 xg. Higher speeds or longer duration will reduce the amount of clay contaminating the extract.)
- 5) Remove the supernatant containing the protein by pouring into screw-capped tubes and store\* at 4°C.
- 6) Repeat steps 2-4 until the extract is straw-colored (Fig. 1).
- 7) Measure total volume of extract with a graduated cylinder and transfer 1 mL to a microtiter tube.\* Pyrophosphate does cross-react slightly with the Bio-Rad dye reagent used in the Bradford assay. Therefore, you must add pyrophosphate to the buffer solution for the standards at the same concentration as the sample (i.e. a background correction for the pyrophosphate) (see Bradford total protein procedure with the pyrophosphate modification).



**Figure 1. The color of the extract solution is used as an indicator of when the extraction is complete. The sample on the left is done whereas the sample on the right should be run at least one more time. An analysis of each extract solution from each 1-hr increment collected separately showed that on average about 65% of the total amount of protein is extracted in the first two 1-hr increments and about 88% is extracted by the fourth increment with amounts declining rapidly with each subsequent increment. Therefore, although the color may be hard to interpret making it difficult to determine when the extraction is complete, stopping too early will not have a large impact on the data and going too long will only dilute the sample. However, if the sample is light colored initially, extracting too many times may dilute the sample too much to allow for analysis by the Bradford total protein or ELISA assays. In this case, the samples may be concentrated by evaporation or centrifugation.**

- \* Protein may be stored in this manner for 2-4 weeks while the other analyses are conducted. Samples must be observed for the growth of contaminants, at which point samples can no longer be used. If desired, 1-mL subsamples can be transferred from extraction tubes to microtiter tubes (centrifuge at 10,000 rpm for 3 minutes). These subsamples are easy to transport and perform protein and ELISA analyses from rather than working with extraction tubes. The microtiter tubes are sold in boxes containing 96 tubes corresponding with 96-well plates and with multichannel pipettors. The extract may also be transferred to eppendorf tubes instead of the microtiter tubes and treated in a similar manner, but these tubes do not work well with multichannel pipettors.

# Easily-Extractable Glomalin (EEG) Extraction

## Introduction

The easily-extractable glomalin (EEG) method for glomalin extraction utilizes more gentle conditions (one 30-min or 60-min increment of autoclaving using 20 mM sodium citrate, pH 7.0) than any of the other methods. The 30-min extraction may be used to collect protein identified in the EEG pool or for the ELISA standard. However, the 60-min extraction is used to extract the total glomalin (TG) fraction from a sand or sand:coal based potting medium in pot culture experiments. In the literature, this fraction is identified as easily-extractable glomalin (EEG) and was initially speculated to contain freshly produced glomalin (Wright and Upadhyaya, 1998). More recent research has shown that following incubation of soil samples, glomalin moves in and out of the TG and EEG pools regardless of incubation time (Steinberg and Rillig, 2003). This indicates that this procedure does not separate out a glomalin fraction by age but rather is just a different operationally-defined glomalin pool. Continuing to analyze this pool may give some valuable information in regards to function of glomalin on fungal hyphae or soil aggregates. Also, the EEG procedure typically gives the most immunoreactive fraction and may be important in determining conditions to stabilize glomalin for better contact with the epitope for the MAb32B11 antibody.

**Note: It is recommended, in any of the procedures listed below, to use at least three duplicate samples from each soil sample, depending on field sampling protocols used and resources. It is also recommended to separate the coarse fraction (i.e. roots, sand, and gravel) from the soil weight. A method for separating the coarse fraction is provided below.**

## Materials

Autoclave\*

Autoclavable round-bottom centrifuge tubes (50 ml) (Nalgene tubes work the best)

Caps for centrifuge tubes (small holes may need to be punched into the caps to relieve pressure during autoclaving)

Centrifuge tube rack

Centrifuge capable of at least 3000 xg with appropriate adaptors for 50 mL tubes

50 ml screw-capped tubes for storing extract

20 mM sodium citrate (citric acid, tri-sodium salt dihydrate), pH 7.0

Graduated cylinder

Microtiter tubes

\*An alternative to using an autoclave is to use a pressure cooker. This methodology was tested and proved to be possible (Wright and Jawson, 2001).

## Methods

- 1) Place 1.0 to 2.0 g of soil in a centrifuge tube with 8 ml 20 mM sodium citrate. (Smaller samples may be extracted as long as a similar weight to volume ratio is used. Larger samples may also be extracted, such as the root ball or potting media from pot cultures, by completely covering the sample.)

- 2) Cap tubes and vortex to have appropriate soil:solution contact.
- 3) Autoclave for 30-60 min. at 121°C. [A 30 min extraction may be used on soil to collect protein identified in the easily-extractable glomalin (EEG) pool or for the ELISA standard. However, the 60 min extraction is used for pot cultures as a total protein assay.]
- 4) Centrifuge at 5000 xg for 15 min immediately after extraction. (Centrifugation is just to pellet the soil particles and may be conducted at any speed from 3000-10000 xg).
- 5) Remove the supernatant that contains the protein and store at 4°C\*.
- 6) Measure total volume of extract with a graduated cylinder and transfer 1 mL to a microtiter tube.\*

\* Protein may be stored in this manner for 2-4 weeks while the other analyses are conducted. Samples must be observed for the growth of contaminants, at which point samples can no longer be used. If desired, 1-mL subsamples can be transferred from extraction tubes to microtiter tubes (centrifuge at 10,000 rpm for 3 minutes). These subsamples are easy to transport and perform protein and ELISA analyses from rather than working with extraction tubes. The microtiter tubes are sold in boxes containing 96 tubes corresponding with 96-well plates and with multichannel pipetters. The extract may also be transferred to eppendorf tubes instead of the microtiter tubes and treated in a similar manner, but these tubes do not work well with multichannel pipetters.

# Total Glomalin (TG) Extraction

## Introduction

The total glomalin (TG) extraction method for glomalin extraction utilizes harsher conditions (repeated 1-hr increments of autoclaving using 50 mM sodium citrate, pH 8.0) than the easily extractable glomalin (EEG) procedure but is less harsh than the sodium pyrophosphate procedure. In the literature, this fraction was initially identified as the total amount of glomalin extractable from the soil and was stated to contain older fractions of glomalin whereas the EEG solution contained freshly produced glomalin (Wright and Upadhyaya, 1998). More recent research has shown that neither of these theories were correct (Nichols and Wright, 2005; Steinberg and Rillig, 2003). A more recalcitrant fraction of glomalin was removed from soil by following this extraction with an extraction with sodium hydroxide for humic acid and then another extraction with sodium citrate (Nichols and Wright, 2005). Also, glomalin will move in and out of the TG and EEG pools regardless of incubation time (Steinberg and Rillig, 2003). This indicates that this procedure does not separate out a glomalin fraction by age but rather is just a different operationally defined glomalin pool. Continuing to analyze this pool may give some valuable information in regards to function of glomalin on fungal hyphae or soil aggregates or within the different glomalin pools.

**Note: It is recommended, in any of the procedures listed below, to use at least three duplicate samples from each soil sample, depending on field sampling protocols used and resources. It is also recommended to separate the coarse fraction (i.e. roots, sand, and gravel) from the soil weight. A method for separating the coarse fraction is provided below.**

## Materials

Autoclave\*

Autoclavable round-bottom centrifuge tubes (50 ml) (Nalgene tubes work the best)

Caps for centrifuge tubes (small holes may need to be punched into the caps to relieve pressure during autoclaving)

Centrifuge tube rack

Centrifuge capable of at least 3000 xg with appropriate adaptors for 50 mL tubes

50 ml screw-capped tubes for storing extract

50 mM sodium citrate (citric acid, tri-sodium salt dihydrate), pH 8.0

Graduated cylinder

Microtiter tubes

\*An alternative to using an autoclave is to use a pressure cooker. This methodology was tested and proved to be possible (Wright and Jawson, 2001)

## Methods

- 1) Place 1.0 to 2.0 g of soil in a centrifuge tube with 8 ml 50 mM sodium citrate. (Smaller samples may be extracted as long as a similar weight to volume ratio is used. Larger samples may also be extracted by completely covering the sample.)
- 2) Cap tubes and vortex to have appropriate soil:solution contact.

- 3) Autoclave for 60-90 min at 121°C. (The 60-min extraction is typical.)
- 4) Centrifuge at 5000 xg for 10 min immediately after extraction. (Centrifugation is just to pellet the soil particles and may be conducted at any speed from 3000-10000 xg. Higher speeds or longer duration will reduce the amount of clay contaminating the extract.)
- 5) Remove the supernatant containing the protein and store at 4°C.\*
- 6) Repeat steps 2-4 until the extract is straw-colored (Fig. 1).
- 7) Measure total volume of extract with a graduated cylinder and transfer 1mL to a microtiter tube.\*

\* Protein may be stored in this manner for 2-4 weeks while the other analyses are conducted. Samples must be observed for the growth of contaminants, at which point samples can no longer be used. If desired, 1-mL subsamples can be transferred from extraction tubes to microtiter tubes (centrifuge at 10,000 rpm for 3 minutes). These subsamples are easy to transport and perform protein and ELISA analyses from rather than working with extraction tubes. The microtiter tubes are sold in boxes containing 96 tubes corresponding with 96-well plates and with multichannel pipettors. The extract may also be transferred to eppendorf tubes instead of the microtiter tubes and treated in a similar manner, but these tubes do not work well with multichannel pipettors.

## Coarse Fraction Separation (Optional)

### Introduction

This procedure is optional but is recommended to remove the large roots, sand, and gravel from the weight of the soil sample.

### Materials

53 um sieve  
other sieves of the appropriate size  
pre-weighed weigh boats

### Methods

1. Rinse soil pellet over a sieve with a mesh size that is equal to the smallest aggregate size or for bulk soil use the 53 um mesh. You may need to vortex or dry the pellet prior to pouring over the sieve to assist in the removal of the sample from the extraction tube.
2. Wash the sample on the sieve using forced water and a rubber policeman, if necessary, to break up the pellet and insure that only sand, gravel, and plant debris remain on the sieve without fine soil particles.
3. Collect the coarse material (sand, gravel, and plant debris) remaining on the sieve by rinsing it into a pre-weighed weigh boat.
4. Dry the coarse material at 70 to 90°C. Weigh the dried material and subtract this value from the original soil sample weight.\*\*

\*\* When analyzing the results, the standard error (SE) of sample weight following subtraction of the coarse fraction should be  $<0.05$ . In some cases, when a lot of root debris, sand, or gravel is present in these samples (especially in the larger aggregate size fractions or deeper soil samples), the SE may be as high as 1.0.