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COMPARISON OF GLOMALIN AND HUMIC ACID IN EIGHT NATIVE U.S. SOILS

K. A. Nichols¹ and S. F. Wright²

Two important extractable fractions of soil organic matter (SOM) are humic acid (HA) and glomalin-related soil protein (glomalin). Optimizing the purity of each fraction is necessary to correlate fraction quantity and molecular characteristics with soil quality. Manipulation of extraction sequence and controlled precipitation of HA were used to evaluate co-extraction of HA and glomalin. Eight bulk soil samples (0 to 10 cm depth) were collected from four U.S. states (Colorado, Nebraska, Maryland, and Georgia). In Experiment 1, glomalin extraction (50 mM citrate, pH 8.0, at 121°C) was followed by HA extraction (0.1 N NaOH at room temperature), and Experiment 2 used the reciprocal sequence. Experiment 2 HA was precipitated stepwise at pH levels 2.5, 2.0, and 1.0. Gravimetric weight, Bradford-reactive soil protein (BRSP), and immuno-reactive soil protein (IRSP), along with percentages of C, N, H, and Fe, were used to compare glomalin and HA. The HA fraction from Experiment 2 contained 2-fold greater amounts of BRSP than HA from Experiment 1 and showed that pre-extraction of glomalin improved the purity of HA. The glomalin fraction from Experiment 1 contained 1.5 times the BRSP of glomalin from Experiment 2 and was twice the gravimetric weight. BRSP and gravimetric weight were concentrated in HA that precipitated at pH 2.5 or 2.0 and percentage IRSP was significantly higher in the pH 2.5 precipitate. The results indicate that glomalin should be extracted first and examined as a biomolecule separate from the humic acid mixture. Percentages C, H, N, and Fe in glomalin varied across soils and experiments. In seven soils, the changes in Fe percentage in glomalin from Experiment 1 to 2 were significantly correlated with the changes in glomalin weight and %C. Iron in glomalin from Experiment 1 was related to soil pH and clay content, whereas soil organic C was positively and significantly correlated with Experiment 1 glomalin BRSP and IRSP. In Experiment 1, a recalcitrant pool of glomalin was released by treating soil with NaOH, suggesting that a fraction of glomalin is difficult to remove from soil and glomalin extraction efficiency could be improved. Refinements to extraction and purification protocols such as pretreatment of soils with HCl and sequential extraction can facilitate studies on organic matter structure and function. (Soil Science 2005;170:985-997)

Key words: Organic matter, humic substances, soil carbon, glomalin.

LUCIDATING the quantities and compositions of extractable soil organic fractions will help clarify processes involved in soil stabilization and C storage. Humic substances (HS), which include humic acid (HA), fulvic acid (FA), and humin, are formed by the decomposition of plant and animal debris, microfauna, biowastes, and other organic materials in the soil (Burdon, 2001; Hayes and Clapp, 2001). Soil quality factors attributed to HS include improved buffering capacity, increased moisture retention, and enhanced spring warming. HS also reportedly...
function as reservoirs of plant-available micro-
nutrients, bind metals to alleviate both heavy
metal toxicity and deficiency, bind clays and other
small organic molecules to form aggregates, and
act as electron shunts in microbial and abiotic
redox reactions (Burdon, 2001; Fan et al., 2000;
Analytical techniques such as nuclear mag-
netic resonance (NMR), gas chromatography-
mass spectroscopy, and thermochemolysis have
been applied to HS to identify functional groups
and match function with chemistry (Hatcher
et al., 2001). These techniques confirm that
other molecules, such as amino acids, carbo-
hydrates, and lipids frequently are co-extracted
with HS (Hatcher et al., 2001, Kingery et al.,
2000; Simpson, 2001). These associated organic
molecules—proteins, carbohydrates, and lipids—
have chemical compositions that may provide
buffering capacity, assist in redox reactions, and
bind organic matter, metals, and clays—functions
that have been attributed to HS (Burdon, 2001).

Humic acid is the abundant, alkaline-
soluble, and acid-insoluble dark brown to black
extractable component of HS. Whether HA is
a super-mixture of low molecular weight mole-
cules (Hayes and Clapp, 2001, MacCarthy, 2001)
or a high molecular weight complex (Schulten
and Schnitzer, 1997) has not been resolved.
Studies of HA quantity, structure, and function
begin with material extracted nonspecifically
from soil using NaOH or sodium pyrophos-
phate at room temperature (RT) (Swift, 1996).

Glomalin-related soil protein, referred to in
this manuscript as glomalin, is a nonspecifically
extracted, alkaline-soluble soil C pool that is
linked to arbuscular mycorrhizal (AM) fungi
(Rillig, 2004, Wright et al., 1996). This red-
brown glycoprotein does not have co-extracted
or attached tannins (Rillig et al., 2001). Accord-
ing to 1H NMR experiments, glomalin has a
consistent organic structure across soils and in
sterile glomalin-free sand pot cultures that is
different from HA (Nichols, 2003). The gloma-
lin extraction protocol consists of incubating soil
in 20 to 50 mM sodium citrate at an alkaline pH
and 121°C for 1-hour intervals (Wright and
Upadhyaya, 1996). This is in contrast with RT
extraction of HA (Swift, 1996). Glomalin
precipitates from solution at pH 2.5 to 2.0
(Wright and Upadhyaya, 1998), a pH level where a
high protein fraction of HA has been isolated (Hayes
and Clapp, 2001; Hayes and Graham, 2000).

Glomalin appears to have properties and func-
tions similar to fungal hydrophobins (Nichols,
2003; Nichols and Wright, 2004; Wright
and Upadhyaya, 1996), which are small, self-
aggregating, hydrophobic proteins found on
hyphae of many types of fungi, including
ectomycorrhizal fungi (Wessels, 1997). Hydro-
phobins protect hyphae from solute loss, allow
hyphae to grow through soil and maintain turgor
pressure while crossing air- or water-filled pores
(Wessels, 1997).

Glomalin sloughs from hyphae (Wright,
2000) and accumulates in soils (Rillig et al.,
2001; Wright and Upadhyaya, 1996). Accumu-
lation is thought to result from the insolubility,
hydrophobicity and high Fe content of the
molecule. Iron concentrations of 0.8 to 8.8%
(Wright and Upadhyaya, 1998), may protect
glomalin from degradation by as proposed for
the role of Fe in organic matter (Hassink and
Whitmore, 1997) and may increase the thermal
stability and antimicrobial properties of glomalin
(Paulsson et al., 1993).

The objectives of the current work were to
(1) determine if extraction-protocol-defined
fractions of both glomalin and HA can be iso-
lated from the same soil sample, and if so, can
they be isolated separately or are they co-
extracted; (2) test sequential extraction protocols
and stepwise precipitation to isolate glomalin and
HA if co-extraction occurs; (3) compare C, N,
H, Fe, and protein concentrations of glomalin
and HA; and (4) compare amounts of glomalin
and HA to soil properties, such organic C, clay,
P, and Fe concentrations that may affect glomalin
and HA accumulation and function.

MATERIALS AND METHODS

Soils

Bulk soil samples (0 to 10 cm depth) were
collected with a shovel at two sites in each of
four states: Maryland (MD), Nebraska (NE),
Colorado (CO), and Georgia (GA) (Table 1).
Soil was freshly collected at each site except for
the NE soils that had been stored at RT for 3
years. Each site had native vegetation.

Soils were air-dried and sieved to collect
material < 2 mm. Selected soil characteristics are
shown in Table 1. Cation exchange capacity,
P, P, sand, and clay concentrations were
measured by the Soil Testing Laboratory at the
University of Maryland. Phosphorus concentra-
tion was measured with a colorimetric assay on
solution extracted from soil using the Mehlich I
method (Mehlich, 1953). Soil pH was measured
in 1:1 (wt/vol) 0.01 M CaCl₂ solution. Soil texture was measured using the pipette method (Day, 1965). Total organic C, N, and H was measured by combustion with a Perkin-Elmer Series II C, H, N, S/O 2400 Analyzer (Shelton, CT) on soil treated with 0.1 N HCl.

Iron was extracted from soil by a modified Aqua Regia (McGrath and Cunliffe, 1985) procedure and quantified by AA (atomic absorption). Briefly, concentrated HNO₃ was added to the sample and heated to 85 to 90 °C (a temperature high enough to cause evaporation but not boiling) for 2 hours. Next, concentrated HCl (1:3 HNO₃: HCl) was added followed by incubation at 60 °C for 1 hour. After hydrolysis, samples were decanted through a Whatman 1 filter into a volumetric flask and brought to volume with deionized water (dH₂O). Iron concentration was measured with a Varian atomic absorption spectrometer (AA-400, Palo Alto, CA) with deuterium background correction.

Extraction of Glomalin

Glomalin was extracted with 50 mM sodium citrate, pH 8.0, at 121 °C for 1 hour (Wright and Upadhyaya, 1999). Samples were centrifuged and the supernatant was decanted and saved. The procedure was repeated until the supernatant was straw-colored (up to 3 more times). Supernatants from each 1-hour extraction cycle were combined and centrifuged. A 1-mL subsample was removed for protein assays (see below), and the remainder was flocculated at pH 2.0 to 2.5 by slowly adding 1 N HCl, the solution was placed on ice for 45 minutes, and the precipitate was pelleted by centrifuging. The pellet was dissolved in a minimum amount of 0.1 M NaOH and immediately dialyzed against dH₂O in dialysis tubing with molecular weight cutoff of 8,000 to 12,000 Daltons (D). Proteolysis of glomalin by exposure to 0.1 M NaOH was not detected by ninhydrin (unpublished data). Water in the dialysis chamber was changed at least 5 times with 8- to 12-hour incubation periods each time. Dialyzed material was centrifuged and the supernatant was collected and freeze-dried. All centrifuging was carried out at 6,850g for 10 minutes.

Extraction of Humic and Fulvic Acids

The International Humic Substances Society method described by Swift (1996) was used to extract HA and FA. Modifications to the method were in sample size (2 g instead of 50 g) and in the purification steps. Briefly, soil was
pre-incubated in 1 N HCl followed by a multi-step extraction procedure: (i) extraction with 0.1 N NaOH at RT under N₂ overnight; (ii) centrifuging to collect the supernatant; (iii) acidification of the supernatant; (iv) precipitation of HA overnight; and (v) separation of HA (precipitate) from FA (supernatant) by centrifuging. The NaOH extraction followed by acidic separation was repeated until the solution was almost clear (two more times) to assure that all humic and fulvic acids were extracted. Protein was not detected in FA by the Bradford soil protein assay (see below), so there was no further analysis of this fraction. All centrifuging was carried out at 6,850g for 10 minutes.

Insoluble solid particles were removed from HA by redissolution in a minimum volume of KOH under N₂, addition of KCl (until [K⁺] > 3 M), and centrifuging at 10,844g to remove suspended solids. HA was precipitated with HCl. After settling overnight, samples were centrifuged and the supernatant was discarded. Precipitated HA was suspended in an HCl/HF solution, incubated overnight, and collected by centrifuging at 6,850g. The supernatant was discarded. The HCl/HF treatment was repeated twice. Residual acid was removed by repeatedly washing the precipitate with dH₂O and centrifuging at 10,844g for 3 minutes.

Precipitated HA was redissolved in a minimal measured volume of 0.1 N NaOH. A subsample (0.5 ml) was removed for protein assays (see below), and the remaining solution was acidified rapidly to precipitate HA. Acid was removed from the precipitate by centrifuging at 10,844g and washing with dH₂O. The precipitate was freeze-dried.

**Experiment 1: Citrate Extraction Followed by Sodium Hydroxide Extraction**

Five 2-g samples per soil were citrate-extracted for glomalin followed by NaOH extraction of HA. Residual soil (soil remaining after sequential extraction of glomalin followed by HA) from all soils except Pawnee was re-extracted with citrate to determine whether the NaOH treatment to extract HA facilitated the release of a recalcitrant pool of glomalin (R. glomalin).

**Experiment 2: Sodium Hydroxide Extraction Followed by Citrate Extraction**

Humic acid was extracted from five, 2-g samples per soil, and the remaining soil was extracted with citrate.

**Stepwise Precipitation of Glomalin in HA**

A subsample of the purified and freeze-dried HA from each soil from Experiment 2 was extracted with citrate to assess co-extraction of glomalin. Trace amounts of citrate-insoluble material were collected by centrifuging at 10,844g, and the supernatant was titrated in steps: (1) pH 2.5 (HA2.5), (2) pH 2.0 (HA2.0), and (3) pH 1.0 (HA1.0). At each step, the precipitate was collected by centrifuging at 6,850g after a 30-minute incubation on ice. After step 3, the supernatant was discarded. Each precipitate was redissolved in 0.1 N NaOH and dialyzed against water in dialysis tubing with molecular weight cutoff of 500 D. After dialysis and centrifuging at 6,850g for 10 minutes, the supernatant was collected and freeze-dried. A subsample was collected for the protein assays (see below) by dissolving the freeze-dried fractions in dH₂O at neutral pH.

**Protein Assays**

Bradford reactive soil protein (BRSP) and immunoreactive soil protein (IRSP) (Rillig, 2004) concentrations were measured on subsamples (collected as described above) of glomalin and HA from Experiments 1 and 2, R. glomalin from Experiment 1, and the three fractions of HA collected at different pH levels in Experiment 2.

A modified Bradford protein assay (Wright et al., 1996) was used to measure BRSP concentration using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), which detects proteins >3,000 to 5,000 D (Bio-Rad Protein Assay, LIT33 Rev C). IRSP was measured by enzyme-linked immunosorbent assay (ELISA), as described by Wright and Upadhyaya (1998) with modifications in the enzyme and color developer. ExtrAvidin (Sigma-Aldrich, Inc.) phosphatase was used instead of peroxidase. Wells were rinsed with Tris [Tris (hydroxy-methyl) aminomethane]-buffered saline with Tween 20 (polyoxyethyleneorbitan monolaurate) before adding the color developer, p-nitrophenyl phosphate in diethanolamine buffer (Wright, 1994). Absorbance was read at 405 nm after 15 minutes. Test samples were compared with a standard curve produced by dilutions of highly immunoreactive glomalin extracted from a temperate soil under native grasses. Percent immunoreactivity was calculated as amount of IRSP divided by amount of BRSP multiplied by 100.
Gravimetric Weight

All purified and freeze-dried samples—glomalin, HA, H. glomalin, HA 2.5, HA 2.0 and HA 1.0—were weighed to the nearest 0.1 mg. Gravimetric weights were reported as g kg⁻¹ soil. Soil before (Initial Soil) and after (Residual Soil) extraction was dried at 70 °C, weighed, ground with a mortar and pestle, and stored under vacuum in a desiccator.

Elemental Composition

Extracted, freeze-dried organic matter fractions—glomalin, HA, H. glomalin, HA 2.5, HA 2.0 and HA 1.0—and Initial and Residual Soil were analyzed for percentages C, N, and H. Iron was measured on glomalin, HA, H. glomalin, and on soil before extraction. Carbon, N, and H contents were measured by combustion with a Perkin-Elmer Series II C, H, N, S/O 2400 Analyzer. Iron concentration was measured by AA on samples hydrolyzed using the Aqua Regia procedure (see above). When hydrolysis was incomplete, the nonhydrolyzed material was collected by centrifuging at 10,844g and the dry weight was subtracted from the weight of the original sample before calculating percentage Fe.

Statistical Analysis

Gravimetric weight values were corrected for subsamples removed for protein measurement and/or secondary extractions. Means and standard error of the mean were calculated for all soils combined. All mean values were compared across experiments for all five fractions (three from Experiment 1 and two from Experiment 2) or between the three HA fractions from Experiment 2 that were precipitated at three pH levels. All means comparisons were made at the α ≤ 0.05 level by analysis of variance, using restricted maximum likelihood) after the residuals met the assumptions for normality and homogeneity of variance. When needed, data were transformed to meet the assumptions.

Pearson product-moment correlation coefficients (r) were calculated for gravimetric, BRSP and IRSP weight, and percentages C and Fe in glomalin or HA from Experiment 1 and soil concentrations of organic C, clay, Fe and P and pH level, and in a comparison of changes between Experiments 1 and 2 in glomalin %Fe, %C, and gravimetric weight. Data were checked for a normal distribution and transformed when necessary before analysis. All statistical analyses were performed using SAS software, version 8 (SAS Institute, 1999) or Statistix 8 Analytical Software (Tallahassee, FL).

RESULTS AND DISCUSSION

Glomalin and HA Isolation

Extraction-defined glomalin and HA fractions were isolated from all eight soils in both extraction sequences. Extraction of glomalin in Experiment 2 and isolation of H. glomalin after extraction of HA in Experiment 1 confirmed that glomalin is a different fraction from HA and is primarily in the “insoluble” humin fraction.

The fraction defined as H. glomalin was detected after comparing Residual Soil C values for Experiment 1 (data not shown) with Residual Soil C values from Experiment 2. High C values for Experiment 1 Residual Soil suggested that a fraction of organic matter, possibly glomalin, remained in soil even after extensive extraction with citrate and NaOH. Re-extraction of Experiment 1 Residual Soils from seven sites with hot citrate yielded the BRSP and IRSP fraction classified as H. glomalin (Table 2 and Fig. 1). The R. glomalin pool may be an encapsulated fraction of glomalin that could accumulate in soil and is only released from soil after treatment with NaOH. Further work is needed to define this glomalin fraction.

Percentages of C, H, and N in the Residual Soil decreased from the Initial Soil values by an average of 50, 80, and 33%, respectively, in Experiment 1 and 69, 80, and 66%, respectively, in Experiment 2 (Table 3), indicating that at least half of the organic matter was extracted using these procedures.

Glomalin and HA Co-Extraction Related to Extraction Sequence

Protein Values

Mean BRSP values for glomalin in Experiment 1 were higher than for any of the other fractions from Experiment 1 or Experiment 2 (Table 2). Humic acid BRSP concentrations showed significantly higher mean BRSP values in Experiment 2 than Experiment 1. When the extraction sequence maximized HA, BRSP in the glomalin fraction increased in the Sampson, Wymore, and Pawnee soils by 1, 59, and 27%, respectively, but decreased by 60 to 69% in the other five soils. The largest (3- to 4-fold) differences between BRSP in HA for the two experiments were in Maryland and Georgia.
Table 2
Bradford-reactive soil protein and immunoreactive soil protein concentrations in glomalin, humic acid (HA), and recalcitrant glomalin (R. glomalin) extracted from eight native US soils when glomalin concentrations were maximized by extracting glomalin first (experiment 1) or when HA values were maximized by extracting HA first (experiment 2).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fraction</th>
<th>Soils</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total protein (g kg⁻¹ soil)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDA</td>
<td>MDB</td>
</tr>
<tr>
<td>1</td>
<td>Glomalin</td>
<td>2.53</td>
<td>1.61</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>0.41</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>R. glomalin</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>2.94</td>
<td>1.98</td>
</tr>
<tr>
<td>2</td>
<td>Glomalin</td>
<td>0.78</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>1.74</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>2.52</td>
<td>2.06</td>
</tr>
<tr>
<td></td>
<td>Glomalin</td>
<td>1.41</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>HA</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>R. glomalin</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0.13</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Glomalin</td>
<td>0.39</td>
<td>0.22</td>
</tr>
</tbody>
</table>

*NA, Samples without enough material for analysis; ND, samples not collected.

bMean ± SE. Different letters within a column for each assay indicate significant differences (P > F = 0.0014 for total protein and <0.0001 for immunoreactive protein) according to REML after a log transformation to meet the assumptions of normality and homogeneity of variance.

Soils. In Experiment 1, the glomalin fraction contained significantly more BRSP than the HA fraction (Prob > F = 0.0009). In Experiment 2, a significant difference between glomalin and HA BRSP could not be determined (Prob > F = 0.7016).

![Fig. 1](image-url)
## Table 3

Values for elemental analysis [C, Fe, N and H] of glomalin, humic acid (HA), and recalcitrant glomalin (R. glomalin) extracted from eight native US soils in experiment 1: Citrate extraction of glomalin before NaOH extraction of HA or experiment 2: citrate extraction of glomalin after NaOH extraction of HA. *b*<br><br>**%** | **Fraction** | **MDa** | **MDb** | **Sampson** | **Haxtun** | **Pacolet** | **Cecil** | **Wymore** | **Pawnee** | **Means**<br>---|---|---|---|---|---|---|---|---|---|---<br>C | Soil - initial | 3.1 | 2.7 | 2.8 | 0.7 | 3.9 | 3.0 | 2.1 | 2.4 | 2.6±0.3<br>C | Exp. 1 glomalin | 26.5 | 24.6 | 40.7 | 31.0 | 36.9 | 37.3 | 15.1 | 15.4 | 28.4±3.5<br>C | Exp. 1 residual | 39.7 | 37.1 | 47.7 | 46.5 | 41.9 | 42.8 | 45.9 | NA | 43.1±1.5<br>C | Exp. 2 glomalin | 40.0 | 39.1 | 48.8 | 44.2 | 39.0 | 40.1 | 47.6 | 46.1 | 43.1±1.4<br>C | Exp. 1 HA | 55.0 | 55.0 | 53.7 | 54.6 | 39.6 | 53.7 | 32.7 | 51.3 | 49.4±3.0<br>C | Exp. 2 HA | 46.0 | 50.9 | 52.1 | 53.6 | 38.7 | 52.8 | 51.6 | 48.9 | 49.3±1.4<br>C | Exp. 1 soil - residual | 1.1 | 1.3 | 0.9 | 0.4 | 1.6 | 1.0 | 1.5 | 1.1 | 1.3±0.2<br>C | Exp. 2 soil - residual | 1.1 | 0.9 | 0.5 | 0.2 | 1.3 | 0.8 | 0.9 | 0.9 | 0.8±0.1<br>N | Soil - initial | 0.4 | 0.6 | 0.6 | 0.2 | 0.5 | 0.7 | 0.5 | 0.6 | 0.5±0.1<br>N | Exp. 1 glomalin | 2.3 | 2.1 | 3.9 | 2.7 | 4.1 | 2.9 | 1.5 | 1.4 | 2.6±0.4<br>N | Exp. 1 residual | 3.2 | 3.5 | 4.1 | 7.3 | 4.7 | 2.7 | 2.8 | NA | 4.1±0.6<br>N | Exp. 2 glomalin | 2.7 | 2.9 | 3.9 | 3.3 | 3.2 | 2.1 | 3.1 | 2.8 | 3.0±0.2<br>N | Exp. 1 HA | 3.4 | 3.8 | 4.6 | 4.5 | 3.6 | 3.8 | 2.3 | 3.0 | 3.6±0.3<br>N | Exp. 2 HA | 3.5 | 4.0 | 4.8 | 4.5 | 3.6 | 3.1 | 4.3 | 3.8 | 4.0±0.2<br>N | Exp. 1 soil - residual | 0.1 | 0.0 | 0.1 | NA | 0.3 | 0.2 | 0.2 | 0.2 | 0.1±0.0<br>N | Exp. 2 soil - residual | 0.0 | 0.0 | 0.1 | 0.2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1±0.0<br>H | Soil - initial | 0.8 | 0.7 | 0.6 | 0.1 | 1.3 | 0.5 | 0.5 | 0.5 | 0.6±0.1<br>H | Exp. 1 glomalin | 3.7 | 3.4 | 4.6 | 3.5 | 4.7 | 4.6 | 2.5 | 2.4 | 3.7±0.3<br>H | Exp. 1 residual | 4.7 | 4.6 | 6.0 | 4.6 | 5.4 | 5.3 | 4.4 | NA | 5.0±0.2<br>H | Exp. 2 glomalin | 4.5 | 4.5 | 5.5 | 4.6 | 4.4 | 4.5 | 3.9 | 3.5 | 4.4±0.2<br>H | Exp. 1 HA | 5.5 | 5.5 | 5.9 | 5.6 | 4.5 | 6.1 | 3.0 | 3.4 | 5.0±0.4<br>H | Exp. 2 HA | 4.5 | 4.9 | 5.3 | 5.1 | 4.0 | 5.1 | 4.6 | 3.9 | 4.7±0.2<br>H | Exp. 1 soil - residual | 0.3 | 0.3 | 0.2 | 0.1 | 0.9 | 0.4 | 0.4 | 0.4 | 0.4±0.1<br>H | Exp. 2 soil - residual | 0.4 | 0.3 | 0.1 | NA | 0.6 | 0.1 | 0.2 | 0.2 | 0.2±0.1<br>Fe | Exp. 1 glomalin | 6.1 | 6.4 | 0.7 | 1.7 | 3.8 | 3.2 | 6.0 | 5.0 | 4.1±0.8<br>Fe | Exp. 1 residual | 2.3 | NA | NA | NA | 1.9 | 12.3 | NA | NA | 5.5±3.4<br>Fe | Exp. 2 glomalin | 1.9 | 1.8 | 0.2 | 0.3 | 1.5 | 3.1 | 0.3 | NA | 1.3±0.4<br>Fe | Exp. 1 HA | 3.0 | 1.2 | 0.1 | NA | 1.0 | 4.1 | 1.2 | 0.1 | 1.3±0.5<br>Fe | Exp. 2 HA | 0.1 | 0.1 | 0.4 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 | 0.8±0.7<br><br>C, N, and H values for soil before (initial) and after extractions (residual) are shown. *aND, not determined.<br>bNA, sufficient material to perform the analysis was not extracted.<br>cMean ± SE in a column followed by different lowercase letters are significantly different at α = 0.05 for separate comparisons of glomalin and HA across experiments.<br>Immunoreactive soil protein concentrations followed a trend similar to BRSP concentrations (Table 2). Mean values were significantly higher in the glomalin fractions than in the HA fractions from both experiments. The HA fraction had slightly higher IRSP concentrations in Experiment 2 than in Experiment 1 for all soils except the Sampson, Pacolet, and Cecil soils. In both experiments, IRSP values were significantly greater in the glomalin fractions than in the other fractions (Prob > F = 0.0030 and <0.0001, respectively). Glomalin is a protein and therefore is excluded as a component of pure HA (Burdon,
In these experiments, BRSP concentrations showed that both the glomalin and HA fractions contained proteinaceous material. The ELISA, which measures IRSP, uses an antiglomalin monoclonal antibody to measure glomalin concentration (Wright et al., 1996; Wright and Upadhyaya, 1996). IRSP was present in both glomalin and HA from both experiments. However, both BRSP and IRSP values indicated that when glomalin was extracted first (Experiment 1), the HA fraction contained less BRSP and IRSP than when HA was extracted first (Experiment 2).

Previous research has shown that some glomalin is soluble in an alkaline solution at RT (Wright and Upadhyaya, 1996). In addition, both HA and humic contain nonhumic substances such as lipids, carbohydrates, and proteins (Burdon, 2001; Hatcher et al., 2001; Hayes and Clapp, 2001; Kingery et al., 2000; MacCarthy, 2001; Rice, 2001; Simpson, 2001), which may resist humification by physical encapsulation in humic-mineral complexes (Hassink and Whitmore, 1997) or within soil aggregates (Bird et al., 2002; Degens, 1997).

Gravimetric Weights

Gravimetric weight of glomalin and HA varied among soils and across experiments (Fig. 1). When glomalin extraction was maximized (Experiment 1), weights were 7 to 19 g kg$^{-1}$ for glomalin and 0.4 to 7 g kg$^{-1}$ for R. glomalin (Fig. 1). Mean glomalin weight (13.4 g kg$^{-1}$) was significantly greater than R. glomalin (3.0 g kg$^{-1}$) or HA (3.1 g kg$^{-1}$) weights (Prob > $F < 0.0001$). Maximizing extraction of HA over glomalin (Experiment 2) resulted in HA weight increases of 62 to 92% in all soils, except Wymore (27% decrease) and Pawnee (10% decrease). Mean weight of HA (9.1 g kg$^{-1}$) in Experiment 2 was not significantly different from glomalin (6.2 g kg$^{-1}$) (Prob > $F = 0.2911$). Glomalin from Experiment 1 had a significantly greater weight than all other fractions from both experiments, except HA in Experiment 2 (Prob > $F < 0.0001$).

These results showed that when glomalin was extracted first, the combined weight of HA and glomalin was 19.5 g kg$^{-1}$ soil (Experiment 1) compared with 15.3 g kg$^{-1}$ soil when HA was extracted first (Experiment 2). Glomalin contributed 69% vs 41% to the total weight for Experiments 1 and 2, respectively, indicating that more glomalin appeared to be co-extracted with HA when HA is extracted first (Experiment 2).

Elemental Concentrations

Concentrations of C, H, N, and Fe in glomalin, R. glomalin, and HA extracted from soils in the same geographic region were similar, but there were wide variations across geographic regions (Table 3). HA had higher C, N, and H percentages but lower Fe percentages than the glomalin fractions across soils and across experiments indicating differences between HA and glomalin molecular structure. Elements in HA were consistent across experiments, except for
the Wymore soils, and significant differences could not be determined. In glomalin, there were significant differences in C, H, and Fe from Experiments 1 and 2.

Carbon and H percentages in glomalin were significantly higher in Experiment 2 than Experiment 1, whereas %Fe was significantly higher in Experiment 1 than in Experiment 2 (Table 3). A significant, negative linear relationship exists between the change in Fe percentage from Experiment 1 to Experiment 2 and the change in %C, whereas a positive relationship exists between the change in %Fe and gravimetric weight (Fig. 2). A pure glycoprotein is composed primarily of C, N, and O, with some possibility of S. In glomalin-free sand pot cultures, glomalin collected from hyphae has C, N, and H percentages of 40.2, 5.7, and 6.5%, respectively (Nichols, 2003). Based on the chemical composition of carbohydrates and proteins, much of the remaining 47.6% of hyphal glomalin should be O. Previous research has shown that glomalin accumulates Fe and other metals, such as Al and Cu, depending on solution or soil concentration of the elements and AM fungal species (Gonzalez-Chavez et al., 2004; Nichols, 2003). Iron concentration of glomalin collected from hyphae (discussed above) is 0.2%, so the remaining 47.4% is O, possibly other metals and ash. The gravimetric weights and %C, N, and H of glomalin from Experiments 1 and 2 indicated that Experiment 1 glomalin contains a high concentration of ash material (Table 3 and Fig. 1).

Although there was a significant difference in %C between Experiments 1 and 2, the ratio of %C to the sum of C, N, and H percentages in Experiment 1 glomalin of 0.816 is similar to 0.853 for Experiment 2 (Table 3). This comparison indicated that the %C in glomalin from both experiments would be similar if ash material were removed. In addition, the ratio of glomalin Experiment 1 BRSP to Experiment 2 BRSP of 2.030 (Table 2) is similar to the ratio of 1.882 for C, N, and H weight (percentage of C, N, and H times gravimetric weight) between Experiment 1 and 2 (Table 3, Fig. 1). These comparisons indicated that the percentages of C, N, and H from both experiments reflected the amount of protein (i.e., glomalin) in the samples, and that almost all of the C, H, and N in glomalin extracts was proteinaceous material.

The higher Fe content in Experiment 1 glomalin compared with Experiment 2 glomalin may have resulted from a combination of two factors. The first factor was pretreatment of the soil in Experiment 2 with HCl before both the HA and glomalin extractions. This treatment would have removed any free or loosely bound Fe from the soil and organic matter. The second factor results from the theoretical structure and properties of glomalin. Hydrophobicity associated with native glomalin is thought to come primarily from hydrophobic amino acids (Nichols, 2003; Nichols and Wright, 2004; Wright and Upadhyaya, 1996) (also unpublished data). On hyphae and soil aggregates, these hydrophobic groups should be oriented to the surface similar to the way amino acids are oriented in fungal hydrophobins (Wessels, 1997). When exposed to high heat for prolonged periods of time, such as during the glomalin extraction procedure, proteins unfold (Creighton, 1993). When a protein cools, it refolds into a thermodynamically stable state. In an aqueous solution, such as citrate, this will be a conformation where hydrophilic groups are oriented to the surface and hydrophobic groups are folded inside (Creighton, 1993). When this happens in glomalin, Fe that is bound to native glomalin would fold into the protein and would not be removed by treatment with HCl or any other competitive chelator (Nichols, 2003; Nichols and Wright, 2004). Lower IRSP values due to the loss of the epitope for the antibody following exposure to high temperature provide further evidence for the conformational changes in glomalin during extraction (Nichols, 2003; Nichols and Wright, 2004; Wright and Upadhyaya, 1996).

Recalcitrant glomalin was higher in C, N, H, and Fe percentages than either Experiment 1 or 2 glomalin (Table 3). The high C, N, and H percentages were speculated to be because the ash content of R. glomalin would be low if previously extracted glomalin and HA co-extracted ash. The R. glomalin fraction possibly is an older fraction of glomalin and one that has accumulated higher concentrations of Fe contributing to its recalcitrance. Also, although soils were treated with HCl before extraction of R. glomalin, the treatment occurred after exposure to the high temperature citrate extraction conditions used to extract the initial Experiment 1 glomalin fraction. This may have kept the Fe in R. glomalin resulting in high Fe values. Further work is necessary to determine how R. glomalin is related to humin. There is also a need to find other extractants might be more effective in releasing all of the glomalin present in a soil.
Stepwise Precipitation of HA to Separate Glomalin and HA

Glomalin was not successfully separated from Experiment-2 HA by citrate extraction of the HA fraction. The stepwise precipitation of HA at pH levels 2.5, 2.0, and 1.0 indicated that it may be possible to separate glomalin and HA by titration between pH 2.5 and 2.0. BRSP and gravimetric weight were concentrated in HA fractions that precipitated at pH levels 2.0 and 2.5, mostly 2.0, whereas significantly higher percentage IRSP was in the HA 2.5 fraction (Table 4).

The HA fraction isolated between pH 2.5 and pH 2.0 by Hayes and Clapp (2001) is distinctly different from the other HA fractions collected at other pH levels. According to $^{13}$C NMR spectroscopy, this fraction consists mostly of aliphatic hydrocarbon residues and is high in both amino acids (14%) and carbohydrates (6.7%) but low in aromatic groups (Hayes and Clapp, 2001). Glomalin contains amino acid and carbohydrate residues, is low in aromatic groups, and is speculated to contain some hydrophobic, aliphatic groups (Nichols, 2003). This further suggests that the NaOH extraction method used for HA co-extracts glomalin or a glomalin-like molecule. To obtain isolated glomalin and HA fractions, glomalin should be extracted before HA.

These results concur with the conclusion by Hayes and Clapp (2001) and MacCarthy (2001) that it is currently not possible to definitively isolate pure humic acid and assure that no non-humic substances are coextracted. However, pretreatment of the soil with HCl or a competitive chelator followed by sequential extraction of glomalin and HA and stepwise precipitation may assist isolation and separation of organic matter constituents for future analysis.

Impact of Soil Properties on Glomalin and Humic Acids

Soils examined in these experiments varied in textural and chemical characteristics (Table 1). Soil pH varied from acidic (4.4 for Cecil) to near neutral (6.7 for Sampson), and there were wide variations among soils in organic C, cation exchange capacity, Fe, and P.

Soil organic C was significantly and positively correlated with glomalin BRSP and IRSP concentrations (Table 5). Iron concentrations in glomalin were significantly correlated with soil pH and clay content, whereas Fe in HA was correlated with soil pH. Percentage C in glomalin was correlated with percentage Fe in glomalin, HA IRSP, and percentage C in HA. Humic acid HA weight was correlated with percentage Fe in the soil as well as percentage C in HA. Percentage Fe in HA was related to pH and soil P. Soil P was correlated with HA BRSP.

The results indicate that glomalin and HA concentration is affected by soil properties. Organic matter concentration is correlated with high clay content because Fe- and Al-(hydr) oxides may create bridges between organic matter and clay minerals forming organo-mineral complexes (Degens, 1997; Hassink and Whitmore, 1997). These complexes protect organic matter.

### TABLE 4

<table>
<thead>
<tr>
<th>HA fraction MDa MDb Sampson Haxtun Pacolet Cecil Wymore Pawnee Meanb</th>
<th>Weight (g kg⁻¹ soil)</th>
<th>%BRSP</th>
<th>%IRSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2.5</td>
<td>1.15 1.11 0.53 0.23 9.69 0.04 1.59 0.40 1.8 ± 1.1b</td>
<td>17.43 21.05 15.08 1.65 23.9 ± 11.3b</td>
<td>12.21 11.80 18.67 6.21 9.31 3.21 11.45 16.76 11.1 ± 1.8b</td>
</tr>
<tr>
<td>pH 2.0</td>
<td>6.10 3.51 3.65 2.14 0.10 6.18 3.46 4.99 3.8 ± 0.7b</td>
<td>81.58 77.78 84.64 95.74 0.45 95.34 65.52 92.83 74.2 ± 11.1b</td>
<td>6.14 6.53 5.69 5.51 3.60 4.17 6.91 11.18 6.2 ± 0.8b</td>
</tr>
<tr>
<td>pH 1.0</td>
<td>0.28 0.16 0.02 0.07 0.21 0.13 0.18 0.51 0.2 ± 0.1b</td>
<td>1.00 1.17 0.28 2.61 0.59 2.32 3.94 3.07 1.9 ± 0.5b</td>
<td>2.74 7.10 5.77 2.78 1.51 2.08 0.43 5.14 3.4 ± 0.8b</td>
</tr>
</tbody>
</table>

*NA, Samples without enough material for analysis.

bMean ± SE in a column and within an experiment followed by different lowercase letters are significantly different at $\alpha = 0.05$ according to REML.
TABLE 5
Correlation coefficients (r) comparing gravimetric (weight), total protein (BRSP), and immunoreactive protein (IRSP) weights and percentage iron (Fe) of glomalin and humic acid (HA) from experiment 1 (glomalin extracted before HA) to soil edaphic factors — pH and concentrations of organic carbon (C), iron (Fe), phosphorus (P), and clay.

<table>
<thead>
<tr>
<th>Variables</th>
<th>BRSP</th>
<th>IRSP</th>
<th>C</th>
<th>Fe</th>
<th>Weight</th>
<th>BRSP</th>
<th>IRSP</th>
<th>C</th>
<th>Fe</th>
<th>Clay</th>
<th>pH</th>
<th>Organic</th>
<th>Fe</th>
<th>P</th>
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<tbody>
<tr>
<td>Weight</td>
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<td>0.24</td>
<td>-0.17</td>
<td>0.15</td>
<td>-0.39</td>
<td>-0.30</td>
<td>-0.30</td>
<td>-0.07</td>
<td>-0.54</td>
<td>0.18</td>
<td>0.35</td>
<td>-0.25</td>
<td>-0.50</td>
<td>-0.20</td>
</tr>
<tr>
<td>BRSP</td>
<td>0.66&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.47</td>
<td>-0.02</td>
<td>0.06</td>
<td>0.30</td>
<td>0.52</td>
<td>0.00</td>
<td>0.45</td>
<td>-0.07</td>
<td>-0.49</td>
<td>0.72&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.13</td>
<td>0.05</td>
<td></td>
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<tr>
<td>IRSP</td>
<td>0.41</td>
<td>0.24</td>
<td>-0.32</td>
<td>0.14</td>
<td>0.31</td>
<td>-0.21</td>
<td>0.16</td>
<td>0.33</td>
<td>-0.39</td>
<td>0.77&lt;sup&gt;†&lt;/sup&gt;</td>
<td>-0.31</td>
<td>-0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.73&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.15</td>
<td>-0.46</td>
<td>0.01</td>
<td>0.87&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>-0.75&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.20</td>
<td>-0.59</td>
<td>0.15</td>
<td>0.30</td>
<td>-0.54</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>0.15</td>
<td>-0.04</td>
<td>-0.74&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.52</td>
<td>0.20</td>
<td>0.82&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>-0.65&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.23</td>
<td>0.34</td>
<td>-0.46</td>
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<table>
<thead>
<tr>
<th>Variables</th>
<th>BRSP</th>
<th>IRSP</th>
<th>C</th>
<th>Fe</th>
<th>Weight</th>
<th>BRSP</th>
<th>IRSP</th>
<th>C</th>
<th>Fe</th>
<th>Clay</th>
<th>pH</th>
<th>Organic</th>
<th>Fe</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>0.80&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.02</td>
<td>0.87&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>-0.42</td>
<td>0.36</td>
<td>0.08</td>
<td>0.18</td>
<td>0.86&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRSP</td>
<td>0.48</td>
<td>0.57</td>
<td>-0.55</td>
<td>0.39</td>
<td>0.18</td>
<td>0.54</td>
<td>0.59</td>
<td>0.67&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRSP</td>
<td>-0.35</td>
<td>0.07</td>
<td>-0.42</td>
<td>0.29</td>
<td>0.42</td>
<td>-0.16</td>
<td>0.54</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>C</td>
<td>0.33</td>
<td>0.58</td>
<td>-0.09</td>
<td>0.09</td>
<td>0.83&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.17</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>-0.22</td>
<td>-0.86&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.16</td>
<td>0.01</td>
<td>-0.70&lt;sup&gt;‡&lt;/sup&gt;</td>
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</tbody>
</table>

Clay                                 -0.30 | 0.37 | 0.43 | -0.15 |
pH                                    -0.52 | -0.12| 0.39 |
Organic C                             0.06 | 0.34 |
Fe                                    0.07 |

<sup>*</sup> All values were tested for a normal distribution and log or sine transformed when necessary.
<sup>*, †, ‡</sup> Denote significance at 0.10, 0.05 and 0.01, respectively.
from decomposition and are speculated to be responsible for the formation of humin (Hayes and Clapp, 2001; Rice, 2001). Multiple soil factors such as soil water, soil texture, P, Fe, and pH may influence the production of glomalin by influencing AM fungal growth. Further work will be required to show the relationship between Fe and clay content in soils, clay mineralogy, and glomalin and HA accumulation.

CONCLUSIONS

Both glomalin and HA fractions may be isolated from the same soil sample. The impact of extraction sequence on glomalin and HA BRSP, IRSP, gravimetric weight, and elemental composition followed similar trends across soils and geographic regions despite wide variations in actual values. Extraction of HA before extraction of glomalin is likely to co-extract glomalin, but protein-free HA was not achieved even by first extracting glomalin with citrate. Extractants other than the nonspecific citrate and NaOH solutions and modifications to the extraction protocol (such as pretreatment of the soil with HCl and removal of ash material from extracts) should be investigated for more thorough extraction, purification, and separation of glomalin and HA. Glomalin content of soils is related to amounts of soil organic C and clay content.

ACKNOWLEDGMENTS

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REFERENCES


Wright, S. F., and A. Upadhyaya. 1999. Quantification of arbuscular mycorrhizal fungi activity by the glomalin concentration on hyphal traps Mycorrhiza. 8:283–285.