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Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi

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Abstract

Arbuscular mycorrhizal fungi (AMF) produce a protein, glomalin, quantified operationally in soils as glomalin-related soil protein (GRSP). GRSP concentrations in soil can range as high as several mg g^{-1} soil, and GRSP is highly positively correlated with aggregate water stability. Given that AMF are obligate biotrophs (i.e. depending on host cells for their C supply), it is difficult to explain why apparently large amounts of glomalin would be produced and secreted actively into the soil, since the carbon could not be directly recaptured by the mycelium (and benefits to the AMF via increased soil structure would be diffuse and indirect). This apparent contradiction could be resolved by learning more about the pathway of delivery of glomalin into soil; namely, does this occur via secretion, or is glomalin tightly bound in the fungal walls and only released after hyphae are being degraded by the soil microbial community? In order to address this question, we grew the AMF *Glomus intraradices* in in vitro cultures and studied the release of glomalin from the mycelium and the accumulation of glomalin in the culture medium. Numerous protein-solubilizing treatments to release glomalin from the fungal mycelium were unsuccessful (including detergents, acid, base, solvents, and chaotropic agents), and the degree of harshness required to release the compound (autoclaving, enzymatic digestion) is consistent with the hypothesis that glomalin is tightly bound in hyphal and spore walls. Further, about 80% of glomalin (by weight) produced by the fungus was contained in hyphae and spores compared to that released into the culture medium, strongly suggesting that glomalin arrives mainly in soil via release from hyphae, and not primarily through secretion. These results point research on functions of glomalin and GRSP in a new direction, focusing on the contributions this protein makes to the living mycelium, rather than its role once it is released into the soil.

Keywords: Glomalin; Arbuscular mycorrhiza; Hyphal wall; Secretion; Soil

1. Introduction

Glomalin is a yet to be biochemically defined protein produced by arbuscular mycorrhizal fungi (AMF), measured operationally in soils as glomalin-related soil protein (GRSP; Wright and Upadhyaya, 1996; Rillig, 2004). GRSP is quantified either with a Bradford assay after autoclave-extraction from soil, or using an ELISA with the monoclonal antibody MAb32B11, produced against crushed spores of the AMF *Glomus intraradices*. GRSP can accumulate to levels of several mg g^{-1} of soil (Rillig et al., 2001) and is highly positively correlated with soil aggregate stability (Wright and Upadhyaya, 1998). GRSP is relatively long lived in soil (Rillig et al., 2001), with portions of GRSP likely in the slow turnover soil carbon pool (Rillig et al., 2003b), highlighting the structural role this compound is hypothesized to play in soil carbon dynamics.

While correlational evidence has accumulated concerning the role of GRSP in soil aggregation (Rillig et al., 2002), the function of glomalin in the biology and physiology of AMF themselves is not clearly understood. Habitat modification for improved growth of AMF hyphae may be an important factor (Rillig and Steinberg, 2002); however, GRSP has also been found in soils in which SOM is not involved in aggregate formation (Rillig et al., 2003a). This result suggests that there may be other functions for glomalin in the biology of AMF.

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That AMF apparently deposit large amounts of a proteinaceous substance into soil presents a conundrum; AMF, as obligate biotrophs, by definition should not be able to directly recapture this organic carbon and nitrogen (although AMF might be able to access some forms of organic N; Hodge et al., 2001). This conundrum could potentially be resolved if more were understood concerning the pathway of delivery of glomalin into soil. Two possible pathways for deposition of glomalin into soil by AMF mycelium have very different implications for functionality: secretion into the physical medium or environment, or incorporation into the hyphal wall and subsequent release from this structural component. If glomalin is primarily hyphal wall-bound, with secretion playing a subordinate role, then glomalin would likely have primary functionality for the AMF mycelium in the hyphal wall, as opposed to in the soil. Additionally, the effects of GRSP on soil aggregation, and its longevity in the soil, could vary greatly based on the mechanism of entry into the soil. Secretion of glomalin into the soil could imply potentially greater mobility in soil, while possibly contributing to faster breakdown through exposure to microorganisms. In contrast, incorporation of glomalin into the fungal hyphal wall likely requires subsequent microbial degradation of this complex. Understanding the incorporation and stability of glomalin in the hyphal wall could help explain the relative stability of GRSP in soil.

To test the relative contribution to delivery of glomalin into soil of secretion versus hyphal wall incorporation, we used an in vitro system of Ri T-DNA transformed carrot roots (*Daucus carota*) infected with *G. intraradices* (St Arnaud et al. 1996). We chose this artificial experimental system since there currently are no methods to assay glomalin secretion in situ (against the high GRSP background already present in soils), and this approach also excludes the effects of processing of glomalin by soil microbes which would alter glomalin production rate estimates and interfere with antibody recognition.

2. Materials and methods

2.1. Fungal material and growth conditions

Pure cultures of hyphae and spores of *G. intraradices* were obtained from in vitro culture material of colonized transformed carrot (*D. carota*) roots (St Arnaud et al., 1996). Briefly, cores were taken from preexisting cultures of carrot roots infected with *G. intraradices* and transplanted into wells in fresh plates with M-medium/Phytagel (0.4%) with 1.0% sucrose in one half of divided 100 mm diameter petri dishes (Fisher Scientific, Denver, CO). After several weeks of growth, the roots partially filled the root compartments (RC). At this time the hyphae-only compartment (HC) was filled with 10 ml of liquid M-medium without Phytagel or sucrose. Hyphae were allowed to cross the barrier while roots were trimmed back to prevent them entering the liquid in the HC. Six weeks after the hyphae had traversed the split plate barrier and grown into the HC medium, the liquid medium (7–9 ml/plate) and the fungal mycelium (1.5–3.5 mg/plate) were harvested separately from the HC. The mycelium gathered from individual plates was washed several times in distilled water, air-dried and weighed prior to further treatment.

2.2. Protein and glomalin extraction methods

Autoclave extraction of glomalin from fungal mycelium was performed using the procedure of Wright and Upadhyaya (1996). For the first cycle of autoclaving, fungal samples were placed in 20 mM sodium citrate pH 7.0 (1 ml/mg mycelium) and autoclaved at 121 °C for 30 min, centrifuged at 16,000*g* for 5 min and the supernatants completely removed. Subsequent rounds of extraction by autoclaving used 50 mM sodium citrate pH 8.0 with a 60 min autoclaving cycle at 121 °C, with the extracts removed following centrifugation after each cycle.

Glomalin extracted from mycelium of *G. intraradices* was quantified by Bradford and ELISA assay. Pure cultures of the fungus ($n=5$) varying in weight from 1.5 to 3.5 mg (dry weight) were subjected to six cycles of autoclaving (a 30 min cycle followed by five 60 min cycles) to determine if the protein is tightly bound on or in the fungal structures, or more loosely associated with the fungal surface. Samples of *G. intraradices* were air-dried and weighed before rehydrating in sodium citrate buffer. Each sample was autoclaved for 1 h in 50 mM citrate buffer pH 8.0 (1 ml/mg dry fungus), centrifuged to pellet the mycelia, and resuspended in fresh buffer for the next autoclave extraction step.

Extraction techniques originally devised for the isolation of glycoproteins from filamentous fungi and yeast cell walls (Fontaine et al., 2000) were used with modifications to determine glomalin concentrations in the *G. intraradices* hyphal walls. Hyphae and spores were placed in 2 ml microfuge tubes with 500 µl of glass beads and 1 ml of extraction buffer (see below). Samples were disrupted in a bead beater for two 4-min cycles. Sample for SDS-only extraction were places in an ice bath between bead beater cycles while those for hot SDS extraction were heated in a boiling water bath for 5 min between cycles. The extracts were centrifuged at 16,000*g* for 5 min and the supernatant containing soluble proteins removed from the hyphal wall pellet. The soluble proteins were released from the fungal mycelium using a suite of extraction buffers including: 2% hot SDS; 8 M urea; 10 mM Tris/1 mM EDTA buffer, pH 8.0; 2% acetonitrile, pH 8.0; 2% acetonitrile/0.1% trifluoroacetic acid; 0.1 M NaOH; 1 M NaOH; 2 M NaOH; or 100% trifluoroacetic acid.

Following ELISA analysis of supernatants from the previous extraction buffers, it was determined that bead-beating of mycelium followed by hot SDS-extraction

(hereafter, HSBB) was optimal for isolating glomalin in the cell wall fraction based on higher total non-immunoreactive protein yield. Non-immunoreactive proteins from fresh fungal mycelium (approx. 12 mg dry wt, pooled from six plates) were extracted using HSBB followed by rinsing of the pellet in 50 mM sodium acetate pH 6.0. The hyphal-wall pellet was divided into equal aliquots for further characterization. Hyphal-wall material was incubated twice in 1 ml of 1 M NaOH at 65 °C for 30 min to release and collect the alkali soluble material. The alkali-insoluble pellets were washed four times with 10 mM Tris (pH 7.5) buffer prior to autoclave extraction of glomalin. Hyphal-wall pellets were also washed and resuspended in 1 ml of 50 mM sodium acetate (pH 5.5) buffer and incubated with 50 μg (0.01U) laminarinase ([1,3-(1,3;1,4)-β-D-glucan 3(4)-glucanohydrolase; Sigma, St Louis, MO) for 48 h at 37 °C. After removal of the supernatant, these pellets were incubated for a second time in laminarinase for 24 h under the same conditions. Glomalin in the treated and untreated HSBB cell wall pellets was extracted by autoclaving with 50 mM sodium citrate, pH 8.0, for 30 min at 121 °C.

2.3. Protein and ELISA assays

Protein concentrations in the extracted samples were determined using Bio-Rad Protein Assay based on the method of Bradford (hereafter, Bradford assay) which utilizes an acidic solution of Coomassie Brilliant Blue G-250 dye which binds to a protein's amino acid residues (Bio-Rad Laboratories, Hercules, CA). Bovine serum albumin (BSA) (Fisher Scientific, Denver, CO) was used to prepare a standard curve for the assay. Glomalin content in samples was determined by indirect ELISA using the monoclonal antibody MAb32B11 produced against spores of *G. intraradices* following the protocol of Wright and Upadhyaya (1996).

3. Results

3.1. Secreted glomalin quantification

Each hyphal compartment had large areas of growing hyphae and spores that varied in dry weight from 1.54 to 3.42 mg per plate. ELISA readings were within detection limits and showed that the immunoreactivity accumulated in the medium from weeks 3 to 6 of the experiment (Fig. 1). Initially, and through 2 weeks incubation, the level of glomalin found in the liquid medium in the hyphal compartment of split plate cultures was below the level of detection by both the Bradford assay and ELISA. Starting at week 3, immunoreactivity was detected in several samples, and by the end of the experiment at 6 weeks, all samples had detectable levels of immunoreactivity ($n=9$). Liquid culture medium samples were below the level of detection of the Bradford assay at all time points (data not presented).

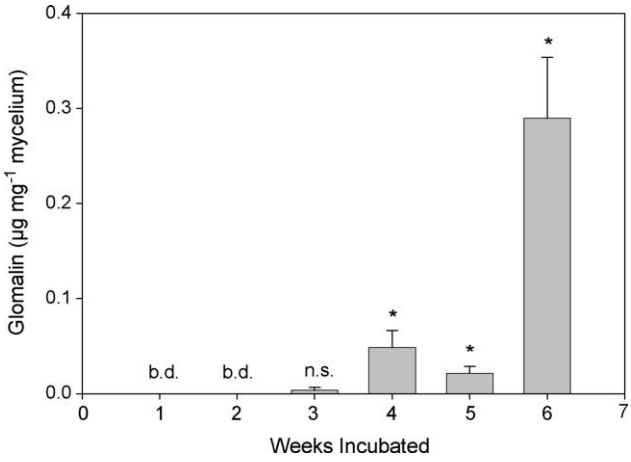

Fig. 1. Time course of glomalin (detected by ELISA with MAb32B11 in the culture supernatant) in the hyphal compartment of split plate cultures of *G. intraradices*. Immunoreactive protein was calculated as μg glomalin mg^{-1} mycelium. Error bars indicate SE of the mean ($n=9$). (b.d., below level of detection; n.s., not significantly different from zero; *, significantly different from zero; $P<0.05$).

3.2. Autoclave extraction of the mycelium

Bradford assays of the extracts showed that the amount of protein decreased after each cycle of extraction. ELISA assays of the extracts showed that the immunoreactivity also decreased after each round and at a faster rate than the Bradford results. After two rounds of autoclaving, glomalin levels had dropped to barely-detectable levels. Autoclave extraction of the dried mycelium from individual plates ($n=9$) released 1.4 μg of glomalin mg^{-1} mycelium for these 6-week old cultures. By comparison, the total amount of glomalin secreted into the culture medium during the six weeks averaged <0.3 μg mg^{-1} of mycelium (Fig. 2),

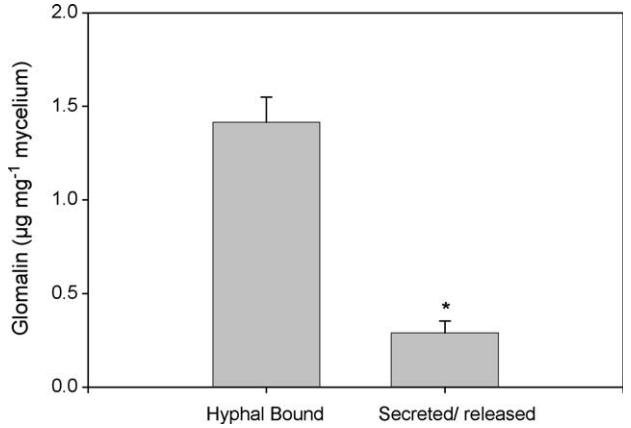

Fig. 2. Glomalin extracted from fungal mycelium and culture supernatant of *G. intraradices*. Hyphal bound: sum of glomalin recovered from mycelia after multiple rounds of extraction by autoclaving expressed as μg glomalin mg^{-1} mycelium. Secreted/released: glomalin secreted (or released through autolysis of hyphae) during 6 weeks of growth by *G. intraradices* mycelium in liquid medium, also expressed as μg glomalin mg^{-1} mycelium. Differences between the two pools were highly significant (ANOVA, $P<0.01$).

i.e. over 80% of the glomalin produced was contained in the mycelium.

3.3. Solvent and enzyme extraction of glomalin

A number of established protein extraction methods were compared for their ability to release glomalin from in vitro cultures of *G. intraradices* mycelium. Most methods released proteins as determined by protein assay and SDS-PAGE. The SDS- and urea-extracted proteins produced distinct bands in SDS-PAGE gels while all of the other extraction protocols produced a protein 'smear' when analyzed by SDS-PAGE (data not shown). In this initial study immunoreactivity, indicating the presence of glomalin, was detected only in extracts from the 4 M guanidine HCl treatment but was otherwise released from mycelium only after autoclaving in sodium citrate (Table 1). These results indicated that glomalin was tightly incorporated into a generally insoluble component of the mycelium. Several methods of extraction were subsequently combined in a stepwise fashion to map the immunoreactive signal to a specific component of the mycelium.

As previously noted, mechanical disruption of hyphae and spores with glass beads using hot SDS buffer (HSBB) yielded a number of proteins as observed by SDS-PAGE silver-stained gel analysis (data not shown). However, no immunoreactivity was observed after transfer and immunoblotting of those proteins, and the ELISA assay indicated only a very weak glomalin signal (Table 2). However, when the pellet from the HSBB extraction was subsequently autoclaved in sodium citrate buffer, the supernatant was shown to be highly immunoreactive by ELISA (Table 2), indicating the presence of glomalin in the SDS-insoluble hyphal-wall fraction. Treatment of the hyphal-wall pellet with laminarinase released glomalin at high levels and subsequent autoclaving of the enzyme-treated pellet also released glomalin. By contrast, HSBB extraction of mycelium followed by treatment in 1 M sodium hydroxide completely eliminated immunoreactivity from the pellet (Table 2). The alkaline-insoluble pellet was subsequently autoclaved in sodium citrate and the immunoreactivity of the extract was below the level of detection as determined by ELISA.

Table 1
Comparison of methods for extraction of soluble proteins (quantified by Bradford or BCA assays) and immunoreactive (IR) protein (glomalin; ELISA assay using MAb32B11) from *G. intraradices* mycelium using various methods

Extraction method	Protein	IR to glomalin
SDS (2 or 4%) w or w/o DTT	+	−
Sodium citrate 4 or 37 °C	+	−
Urea, 8 M with 4% Triton X 100	+	−
2% acetonitrile pH 8.0	+	−
2% acetonitrile/0.1% trifluoroacetic acid	−	−
0.1 M NaOH	+	−
1 M NaOH	+	−
2 M NaOH	+	−
100% trifluoroacetic acid	+	−
Tris (10 mM)/EDTA (1 mM) pH 8.0, autoclaved	−	−
Guanidine HCl, 4 M pH 5.7	+	+
Sodium citrate, autoclaved	+	+

Table 2
Glomalin content of fungal mycelial cell wall components (as determined by ELISA with MAb32B11) after various treatments and extraction sequences

Extraction method of glomalin from fungal mycelium	Glomalin $\mu g\ mg^{-1}$ mycelium[a]
Glass bead disruption	
Hot SDS extraction	2.35
Laminarinase treatment (1st) of SDS-extracted pellet	37.9
Laminarinase treatment (2nd) of SDS-extracted pellet	11.2
Autoclave extraction	
Hot SDS-extracted pellet	13.8
Hot SDS extraction/proteinase K treatment	0.0
Alkaline treatment of SDS-extracted pellet	0.0
Laminarinase (2×) treatment of SDS-extracted pellet	18.1

For details see Section 2.
[a] Approx. 12 mg mycelium from pooled in vitro samples.

4. Discussion

Using in vitro cultures of *G. intraradices* we showed that some glomalin was secreted or released from the mycelium into liquid medium, while the majority (>80%) of glomalin produced by the fungus was tightly bound in hyphae and spores. Autoclaving of the fungal mycelium released glomalin through multiple cycles, suggesting that glomalin is not simply a cytoplasmic, cell membrane, or mycelial surface-associated protein. Consistent with this idea, extraction of the fungal hyphal/spore wall with detergents, acid, base, solvents, and chaotropic agents did not release glomalin. Instead, autoclaving or enzymatic treatment after those treatments was required to extract glomalin in significant amounts. This indicates that glomalin is firmly incorporated into the hyphal wall. Proteins can be either covalently linked within the fungal cell wall or they can non-covalently associate with the wall, forming either insoluble complexes or being loosely embedded (Carlile et al., 2001; de Vries et al., 1993). Members of the Glomeromycota have soluble as well as insoluble proteins in their walls (Bonfante-Fasolo and Grippolo, 1984), which consist of cross-linked chitin (or chitosan) and β-glucan complexes (Bago et al., 1996).

There are several important implications of these results. First, they offer a resolution to the apparent conundrum that AMF, as obligate biotrophs (i.e. exclusively depending on the host for C), would secrete large amounts of a proteinaceous substance into soil. Our results strongly suggest that glomalin is, in fact, not secreted or passively

released from growing mycelium in large amounts. Hence, it is not necessary to attribute direct functionality (from the perspective of the AMF mycelium) to released glomalin or GRSP present in the soil. Instead, glomalin is contained within the hyphal and spore walls where it could fulfill physiological functions in the course of the life of the organism. This does not imply that soil glomalin does not also have beneficial effects for AMF (see Rillig and Steinberg, 2002); but these would likely be less direct effects compared to the role glomalin has as a mycelial wall component in the living mycelium. These direct effects are at present speculative, but it is possible that glomalin has a role similar to hydrophobins, relatively small proteins apparently ubiquitous among filamentous fungi (Wösten, 2001). Hydrophobins allow filamentous fungi to break the air-water interface by lowering the surface tension of water, and hydrophobins are important in hyphal attachment to surfaces. Clearly, analogous roles would also be important to AMF. Additionally, it could also be hypothesized that glomalin has a role in decreasing hyphal palatability to fungal grazers, or in the immobilization ('filtering') of pollutants at the soil-hypha interface (i.e. before entry into the fungal-plant system).

Our results further suggest that the primary delivery pathway of glomalin into soil is via hyphal turnover. Staddon et al. (2003) have suggested that hyphae of AMF, albeit not under field conditions, can turn over relatively rapidly, estimating a half-life of 5–7 days. This time frame is remarkably close to an earlier estimate of turnover of AMF mycelium based on direct microscopic observation (Friese and Allen, 1991), and we have recently shown (Steinberg and Rillig, 2003) that AMF hyphae persist for far shorter periods of time in soils than GRSP itself. Importantly, AMF hyphal lengths in soil (with the limitation that it is not generally known what percentages of these are active) can be very high, for example over 50 m g^{-1} soil in a western Montana grassland (Lutgen et al., 2003). Miller et al. (1995) reported values of 45 m g^{-1} in a prairie. Not surprisingly, AMF have been estimated to be the recipient of between 4 and 20% of the total plant photosynthate (Graham, 2000). The apparently high turnover, coupled with the great abundance of the mycelium, lend support to the model that GRSP could accumulate in soils to the commonly measured levels of several mg g^{-1} soil via hyphal turnover and release of glomalin from dead mycelia. Given the harsh extraction conditions necessary to release glomalin, the latter is most likely mediated by a microbial community associated with AMF hyphae (and likely does not primarily occur through physico-chemical processes like leaching).

A caveat of our study was that it was carried out in an in vitro culture system, i.e. in the absence of soil. However, this experimental design was necessary since it would be very difficult to measure potential or de novo glomalin secretion in situ, i.e. in the soil. It is also likely that we did underestimate the ratio of wall-bound to secreted/released glomalin in this system. First, hyphae were repeatedly rinsed prior to extraction, and loosely wall-attached material might have been lost. Secondly, there is evidence for intrinsic turnover of hyphae in in vitro cultures (e.g. branched absorbing structures; Bago et al., 1998); hence part of the pool we described as released/secreted may in fact have been derived from hyphal autolysis (with glomalin contained in walls/cells subsequently accumulating in the culture medium).

Notwithstanding the persistent lack of biochemical characterization of glomalin, our study has shown that this substance is tightly bound within the hyphal wall of AMF, rather than primarily released or secreted into the medium. This observation opens up new areas of research into the roles of glomalin in the ecophysiology of AMF, and sheds light on a hitherto unexplored problem, namely the delivery pathway of glomalin into soil.

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