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RESEARCH ARTICLE

Double-network gels and the toughness of terrestrial slug glue

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ABSTRACT

The terrestrial slug *Arion subfuscus* produces a defensive secretion that is sticky and tough, despite being a dilute gel. It is unusual in having high stiffness for a gel, yet retaining the high extensibility typical of mucus. In tensile tests, it sustains an average peak stress of 101 kPa, and fails at an average strain of 9.5. This gives the gel toughness; it requires much greater strain energy to fracture than most gels. This toughness may arise from a double-network type mechanism. In this mechanism, two separate, interpenetrating networks of polymers with different properties combine to give toughness that can be several orders of magnitude greater than either network individually. Native gel electrophoresis suggests that *A. subfuscus* glue consists of two networks: a network of negatively charged proteins ranging in M_r from 40×10^3 to 220×10^3 that can be dissociated by hydroxylamine and a network of heparan sulfate-like proteoglycans. The two networks are not tightly linked, though proteins of M_r 40×10^3 and 165×10^3 may associate with the carbohydrates. Targeted disruption of either network separately, using enzymatic hydrolysis, disulfide bond breakage or imine bond disruption completely disrupted the glue, resulting in no measurable toughness. Thus, the two networks separately provide little toughness, but together they work synergistically to create a tough material, as predicted in the double-network mechanism.

KEY WORDS: Adhesion, *Arion*, Cross-linked, Stiffness, Extensibility, Proteoglycans, Strain energy

INTRODUCTION

The terrestrial slug *Arion subfuscus* (Draparnaud 1805) produces an unusual glue that is a strong adhesive despite being a dilute gel. This glue is secreted from its dorsal surface in response to threats (Smith, 2006). The glue contains more than 95% water, yet it can produce shear strengths over 100 kPa (Smith, 2006). Notably, the glue appears to have an unusually high toughness for a gel. Most other gels are either weak and highly deformable, like common forms of mucus, or stiff and easily fractured, like gelatin or agar. *Arion subfuscus* glue is much stiffer than typical mucus (Smith, 2006), yet it retains high extensibility and does not undergo brittle fracture like most other stiff gels. A high stiffness combined with high extensibility leads to a tougher material. In other words, it can markedly increase the energy required to cause fracture. Toughness is an essential component of a good adhesive (Smith et al., 1999b).

The mechanism by which the glue achieves this toughness has not been investigated. Previous work focused on the factors that make gastropod adhesive gels much stiffer than other forms of mucus (Smith et al., 1999a; Smith and Morin, 2002; Pawlicki et al., 2004; Werneke et al., 2007; Bradshaw et al., 2011; Braun et al., 2013). There are two main types of cross-links: direct metal cross-links involving calcium, magnesium and possibly iron (Werneke et al., 2007; Braun et al., 2013), and cross-links that derive from metal-catalyzed oxidation (Bradshaw et al., 2011; Braun et al., 2013). There is indirect evidence that the latter are imine bonds created when amino acids, such as lysine, are oxidized to create carbonyls, which are susceptible to nucleophilic attack from primary amines (Braun et al., 2013). The result would be labile bonds that link the higher molecular weight proteins of the glue into a network (Braun et al., 2013). Metals also act directly; when metals are removed, the glue loses all of its integrity and strength (Werneke et al., 2007; Braun, et al., 2013). Direct metal cross-links may link the proteins together (Werneke et al., 2007), but they may also link the negatively charged polysaccharides. Most terrestrial gastropod mucus is composed of sulfated polysaccharides (Denny, 1983). Such large polysaccharides typically tangle to form a loose network (Smith, 2002). They may also be reinforced with metal ions. Calcium and sulfate are the most common ions that are bound within *A. subfuscus* glue, and the calcium balances the negative charge of the sulfate roughly stoichiometrically (Braun et al., 2013). Thus, calcium and other divalent metals may link sulfated polysaccharides electrostatically or by coordinate bonds.

Because there may be two cross-linked systems in the glue, it is possible that they work synergistically as described by the double-network mechanism. In a double-network hydrogel, two separate networks of polymers work together to produce toughness that is several orders of magnitude higher than each can produce individually (Gong, 2010; Haque et al., 2012). While the original double-network gels were based on a very specific chemistry and mode of synthesis, later work showed that the same general principles applied to gels made of different components. Typical double-network gels have several essential features. First, the two networks interpenetrate, as opposed to being tightly connected to each other. Second, one network must be markedly stiffer with a high cross-link density, while the other is highly deformable with a low cross-link density (Webber et al., 2007; Gong, 2010; Haque et al., 2012). During fracture, the bonds in the stiff network rupture, but for the crack to spread, the more ductile second network must be ruptured as well. This requires extensive deformation; as the polymers of the second network are stretched they distribute stress to a large volume, causing extensive damage to the rigid network (Haque et al., 2012). This is commonly explained in terms of sacrificial bonds and hidden length; the first network provides a high density of sacrificial bonds that must be broken, and the second network's extensibility provides hidden length that ensures that this damage continues through a large strain (Haque et al., 2012). The creation of a large volume of damage rather than a simple fracture plane requires far more energy (Brown, 2007). Thus, the material is tougher. The increases in toughness relative to single networks can be of the order of a thousandfold; thus, the effect of the two networks is not merely additive, it is synergistic (Haque et al., 2012).

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It was recently suggested that *A. subfuscus* glue could derive toughness from a double-network type of mechanism (Smith, 2013). It has unusually high toughness, and it appears to have two systems of cross-linked polymers. It is based on a highly deformable, polysaccharide mucus and also proteins that seem to cause high stiffness. The proteins may form a network that does not merely stiffen the gel but contributes in a fundamentally different manner by leading to a double network. This study tested the hypothesis that the proteins and carbohydrates work together in a double-network type of mechanism. Specifically, we tested two predictions. The first is that there are separate protein and carbohydrate networks. The second is that the two networks work together synergistically to achieve high toughness, rather than having separate, additive contributions. This was tested by separately disrupting the protein and carbohydrate networks.

RESULTS

Testing for the presence of protein and carbohydrate networks

In native gel electrophoresis, the negatively charged proteins in the glue typically failed to enter the gel, resulting in staining at the bottom of the well, with a fraction of the protein entering and creating diffuse staining at the top (Fig. 1A,B). Second dimension SDS-PAGE showed that the region at the top of the lane contained a mixture of all the proteins from asmp-40 through to asmp-220, and occasionally some of the proteins identified as asmp-15. Note that proteins are identified as asmp (*A. subfuscus* mucus protein) followed by the relative molecular mass $\times 10^{-3}$ (Bradshaw et al., 2011). On a 5% native gel, these proteins ran together with less mobility than a ferritin standard (450 kDa) when they were even able to enter the gel (Fig. 1A). These findings are consistent with different buffer systems and gel concentrations. Normally in native gel electrophoresis followed by second dimension SDS-PAGE, one would expect proteins to resolve as spots in a rough diagonal extending from the top left to the bottom right. Instead, all the slug glue proteins are at the left of the second dimension gel indicating that they ran together at the top of the native gel. This typically indicates the presence of a large complex that is broken up by SDS (Wittig and Schägger, 2005). Furthermore, they appear as streaks, indicating that they did not resolve into bands on the native gel. In contrast, when the positively charged proteins were analyzed, these entered the native gel and resolved into sharp, dark bands (Fig. 1B). Second dimension SDS-PAGE showed that these bands corresponded to proteins in a group identified collectively as asmp-15, with the upper band containing proteins that migrate with an apparent mass slightly higher than 15×10^3 and the lower band containing proteins that migrate closer to 15×10^3 (Fig. 1B). Blue native gel electrophoresis was used to identify proteins that failed to enter the gel because they were hydrophobic and thus normally lacked sufficient charge to migrate into the gel. On the blue native gel, one band resolved, which was identified by second dimension SDS-PAGE as asmp-57. All the other proteins ran the same as on other gels. Isoelectric focusing showed that most of the proteins in the glue had isoelectric points (pI) between 6 and 7, except one band that was above 9.3 and another near 4.6.

The inability of negatively charged proteins to enter native gels and the absence of distinct bands was consistent even in the presence of the following treatments: 10 mmol l^{-1} EDTA, 5 mmol l^{-1} DTT and 1% Tween-20 (Fig. 2A) as well as 1 mol l^{-1} NaCl (data not shown). The only treatment that consistently disrupted the protein complexes enough to allow proteins to migrate as distinct bands was hydroxylamine (Fig. 2B). The effect of

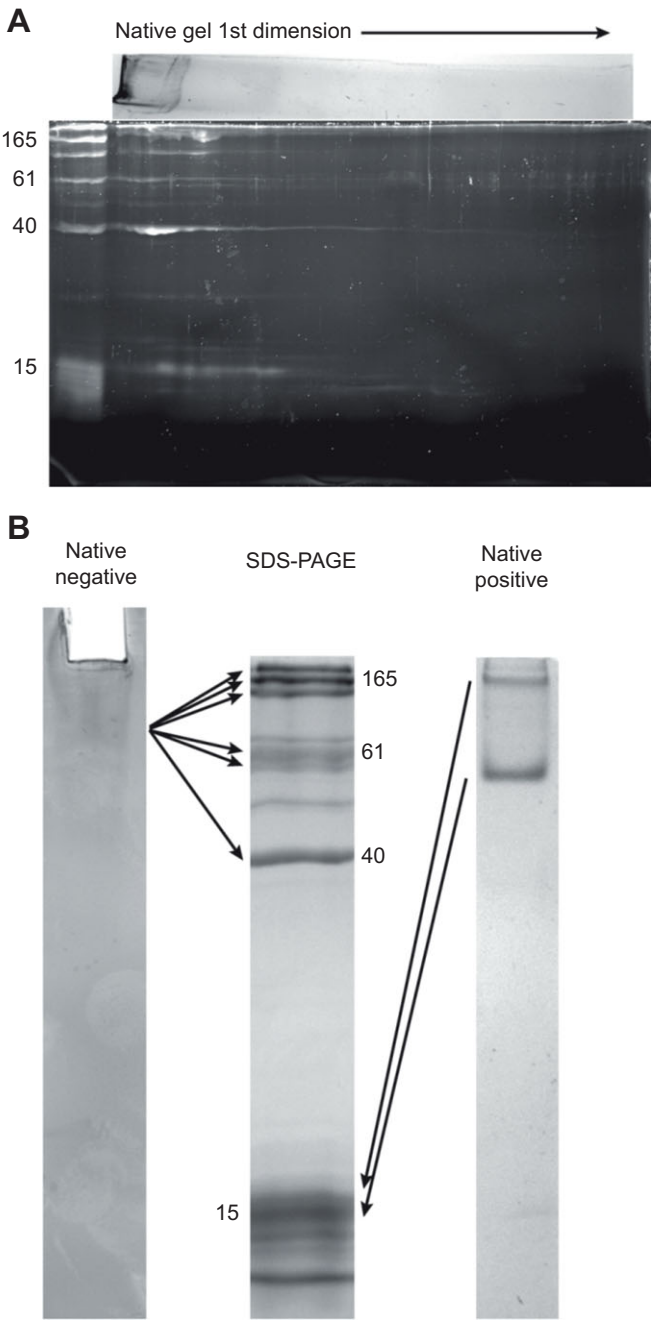

Fig. 1. Native gel electrophoresis of *Arion subfuscus* glue samples followed by second dimension SDS-PAGE to identify bands or complexes. (A) Glue run in one lane of a 5% native Tris-acetate gel (pH 7.5) as a first dimension then immediately loaded across the top of a 12.5% SDS gel, which was run as a second dimension (stained with Sypro Ruby). The native lane that is shown is a separate lane that was stained with Coomassie Blue R-250; the original lane was not stained before loading. The far-left lane on the SDS-PAGE gel contains an extract of all the proteins in the glue for reference. The stained region at the top of the native gel contains most of the proteins in the glue; these were separated by SDS-PAGE, but on the native gel travel together with lower mobility than a ferritin standard (450 kDa, not shown). (B) Summary of the results of two-dimensional electrophoresis, showing 10% native gels with standard polarity (cathode at top) to resolve negatively charged proteins (left) and reversed polarity to resolve positively charged proteins (right). Arrows indicate where proteins from the native gel ran in second dimension SDS-PAGE (center). M_r (values given in thousands) of select proteins is indicated on the SDS-PAGE gel; 30 µl of sample were loaded in each lane. Gels were stained with Coomassie Blue R-250.

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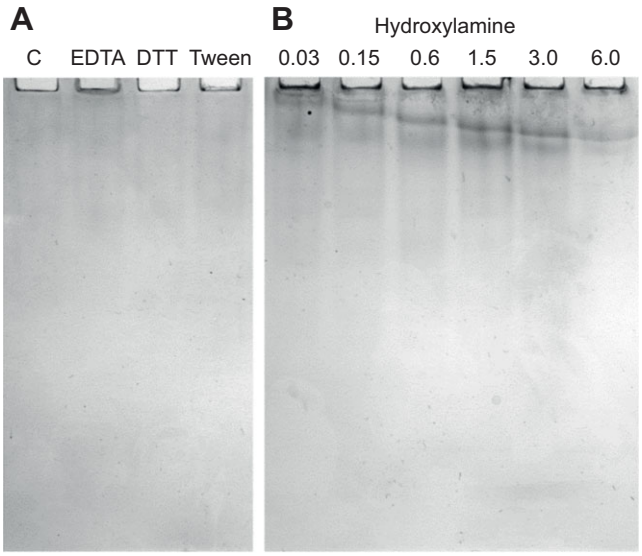

Fig. 2. Native gel electrophoresis of *A. subfuscus* glue samples after treatment with different reagents to disrupt aggregation. The 10% Tris-acetate gels (pH 7.5) were run with standard polarity (cathode at top) in order to resolve negatively charged proteins. (A) Proteins failed to enter the gel when dissolved with the following treatments (from left): control (C), 10 mmol l^{-1} EDTA, 5 mmol l^{-1} DTT and 1% Tween. (B) The dose-dependent effect of hydroxylamine allowing proteins to enter the gel and resolve as bands. Samples were dissolved in Tris-acetate with (from left): 0.03, 0.15, 0.6, 1.5, 3, 6 mol l^{-1} hydroxylamine. The gels were stained with Coomassie Blue R-250.

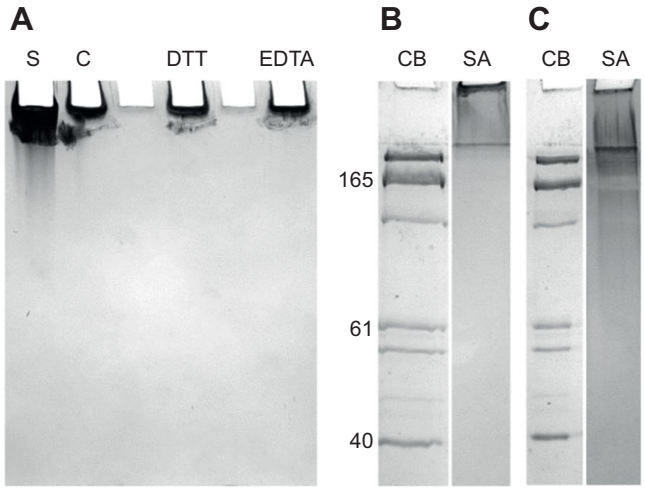

Fig. 3. Staining of *A. subfuscus* glue samples to detect strongly polyanionic polymers on native and SDS gels. (A) Native gel electrophoresis with standard polarity (cathode at top). All samples were dissolved in Tris-acetate, pH 7.5, with treatments as follows (from left to right): sonicated (S), control with no treatment (C), 5 mmol l^{-1} DTT and 10 mmol l^{-1} EDTA. Lanes 3 and 5 are empty. The gel was stained with Stains-All to detect polyanionic polymers. (B) SDS-PAGE (7.5% total acrylamide) of a non-sonicated glue sample stained with Coomassie Blue R-250 (CB) and Stains-All (SA). M_r (values given in thousands) of selected proteins is indicated to the left. (C) SDS-PAGE (7.5%) of a sonicated sample stained with Coomassie Blue R-250 and Stains-All. A 30 µl sample was loaded in each lane.

hydroxylamine was dose dependent, with the strongest bands forming at 1.5 mol l^{-1} hydroxylamine, and some disappearance of bands at 6 mol l^{-1}. Second dimension SDS-PAGE showed that the primary protein present in the darker band was asmp-40. The other main proteins in the glue were also seen in smaller quantities in extracts from this region of the native gel, similar to the results shown in Fig. 1.

Stains-All staining for polyanions on native gels showed strong staining when samples were sonicated, with the polyanions able to enter the gel and migrate a short distance (Fig. 3A). In some gels loaded with lower sample concentrations, these ran as a distinct band. Without sonication, there was much less intense staining, and it was present only where the sample was loaded on the gel. DTT and EDTA did not change the intensity of this band or its ability to enter the gel (Fig. 3A). The staining did not co-localize with any proteins, as there were no clear protein bands on this type of gel, whether the sample was sonicated or not. Analysis of positively charged polymers on native gels showed occasionally much fainter Stains-All staining associated with asmp-15 (data not shown). In SDS-PAGE, most of the Stains-All staining was at the top of the stacking gel, whereas all the Coomassie-stained proteins resolved normally (Fig. 3B). Sonication caused the Stains-All positive polymers to smear through the top of the SDS gel, but did not affect the Coomassie-stained proteins (Fig. 3C).

Testing for interactions between proteins and carbohydrates

Most of the carbohydrates were strongly anionic and bound to the sepharose Q-beads in the batch ion exchange. In two separate trials, 74% and 75% of the carbohydrates bound to the Q-beads at pH 4.6. A similar or higher percentage was bound at pH 5.1 and 6.2, but there was substantial clumping of the beads as the pH increased. The bound fractions turned a reddish-pink hue in the orcinol assay, as opposed to the dark brown that characterizes neutral sugars.

Only two of the proteins in the glue, asmp-40 and asmp-165, co-eluted with the negatively charged carbohydrates (Fig. 4). The rest were easily separated from the carbohydrates. All of asmp-165 co-eluted with the carbohydrates, and more than half of asmp-40. The results were the same with and without reducing agents or sonication. These accounted for 41% and 27% of the total amount of

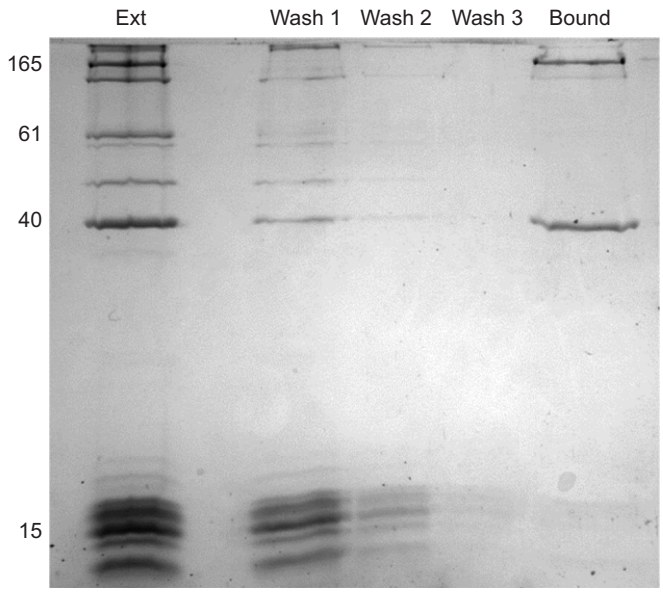

Fig. 4. SDS-PAGE of fractions after ion exchange showing proteins that co-elute with polyanionic carbohydrates. From left, original extract (Ext), unbound material that eluted with the first, second and third washes, and bound material that eluted after the addition of 1 mol l^{-1} NaCl. The buffer was 20 mmol l^{-1} piperazine pH 4.6. M_r (values given in thousands) of select proteins is indicated to the left; 30 µl was loaded in each lane and the gel was stained with Coomassie Blue R-250.

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protein in two separate trials (Fig. 4). At the higher pH values, a small percentage of other proteins began to bind.

Contributions of protein and carbohydrate networks to gel mechanics

In tensile tests on unsoaked samples, stress increased linearly with strain up to a strain of 3.8±0.6 (mean±s.e.m., *N*=8) reaching a peak stress of 101±15 kPa (Fig. 5). Note that this is engineering strain, and corresponds to an extension that is 3.8 times the initial length. This was followed by yield and extended deformation. The average stress in this plateau region was 48±3 kPa. This extension continued up to a final strain of 9.5±0.9. The average elastic modulus in the initial region was 30±5 kPa. The stiffness combined with extensive deformation led to an average toughness of 538±93 kJ m^{-3}.

Freezing and thawing the samples did not have much effect on their mechanical properties. When repeatedly frozen and thawed test strips were compared with paired, unfrozen controls from the same sample, the average values for peak stress, yield strain and total strain were all within 20% of the control averages (*N*=3). The average values for toughness of frozen and unfrozen samples were within 5% of each other.

Samples treated with 1.5 mol l^{-1} hydroxylamine (*N*=4), 25 mmol l^{-1} Tris (2-carboxyethyl) phosphine (TCEP; *N*=3) or proteinase K (*N*=11) to disrupt the protein network were either liquified or extraordinarily deformable with a stiffness that was too low to be measured on the tensometer, which had a resolution limit of roughly 1 kPa for this system. In contrast, the paired controls retained substantial toughness (*N*=23) (Fig. 6). The proteinase-treated samples could often stretch to strains of 20–30 before failure, gradually thinning into a fine, viscous thread. The effect of hydroxylamine was dose dependent, with a detectable effect occurring at 0.075 mol l^{-1}, and complete loss of integrity occurring between 1 and 1.5 mol l^{-1}. At these concentrations, the samples were either a slightly viscous liquid or soft enough to be drawn into a transfer pipette. When samples were treated with heparinase II to eliminate the carbohydrate network, the glue was fragmented into tiny particles (*N*=3).

The hydroxylamine treatment and the treatments that reduced disulfide bonds appeared to be more specific for the protein network

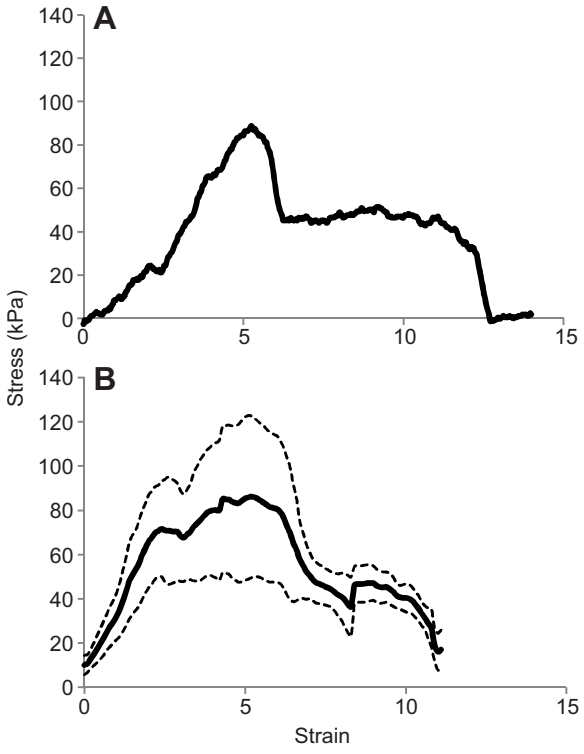

Fig. 5. Stress–strain curves of untreated *A. subfuscus* glue. (A) Sample data showing one of eight tensile tests. (B) Average data for eight tests with 95% confidence intervals (dashed lines). Strips of glue, 1×1 mm, were analyzed. Stress is engineering stress and strain is engineering strain. A strain of 10 indicates that the extension was 10 times the original length.

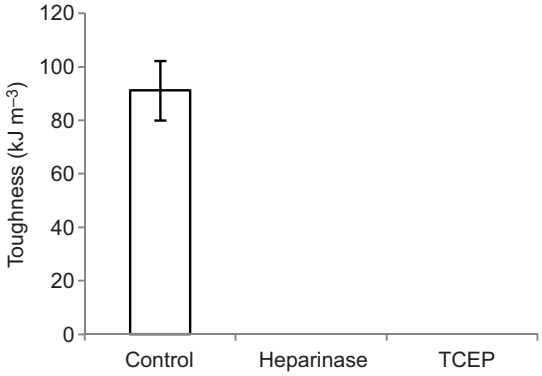

Fig. 6. Toughness of soaked, intact glue versus glue with only the protein network or only the carbohydrate network. Heparinase II was used to disrupt the carbohydrates, leaving the proteins intact; 25 mmol l^{-1} TCEP was used to disrupt the proteins, leaving the carbohydrates intact. Toughness was measured as the strain energy necessary to cause failure, by integrating the area under the stress–strain curve. Values are means±s.e.m. Paired controls for all the treatments in this study were similar, so the data for all controls are lumped (*N*=23). The heparinase- (*N*=3) and TCEP-treated (*N*=3) samples did not have measurable toughness. This was also true of hydroxylamine- and protease-treated samples.

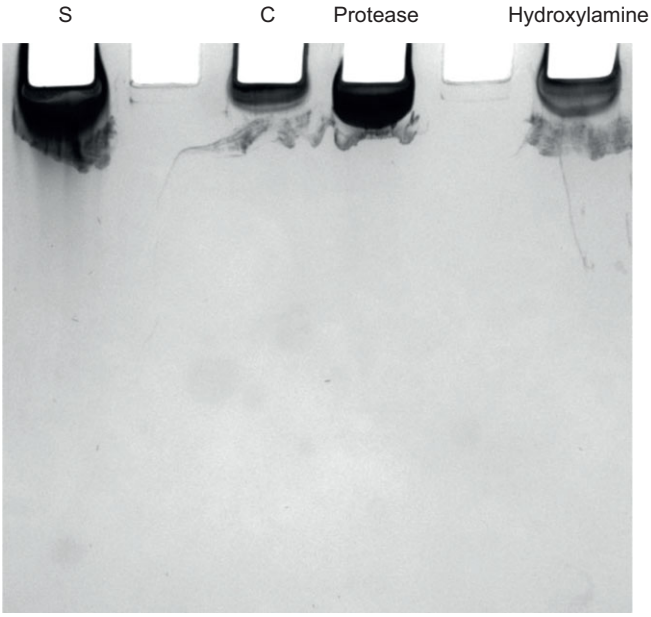

Fig. 7. Staining of *A. subfuscus* glue samples to detect strongly polyanionic polymers in native gels after proteinase and hydroxylamine treatment. A Tris-acetate (pH 7.5) native gel with standard polarity was used to resolve negatively charged proteins and polysaccharides. The gel was stained for polyanions with Stains-All. Lanes are (from left): sonicated (S), unsonicated control (C), unsonicated glue treated with proteinase K (6 U ml^{-1}) and unsonicated glue treated with 1.5 mol l^{-1} hydroxylamine. Lanes 2 and 5 are empty; 30 µl of sample was loaded in each lane.

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than the proteinase. SDS-PAGE confirmed that proteinase K completely eliminated all the proteins normally seen in the glue, but it also allowed more polyanionic carbohydrates to enter the native gel (Fig. 7). In contrast, disulfide reduction and hydroxylamine treatments had minor effects, if any, on the banding pattern of the polyanionic carbohydrates. There was only a slight increase in mobility of a relatively faint Stains-All positive band when hydroxylamine was added, and no change upon disulfide bond reduction (Fig. 7, Fig. 3A). These treatments did have marked effects on the protein network. The effect of hydroxylamine on proteins was clear from native gels, which showed that it allowed some negatively charged proteins to run as distinct bands (Fig. 2B). The proteins themselves did not appear changed, as they ran the same way on SDS-PAGE as controls. In contrast, reduction of disulfide bonds caused all of the proteins from asmp-40 to asmp-220 to increase in apparent mass (Fig. 8). Asmp-15 was not affected by disulfide reduction (data not shown). Interestingly, asmp-165 did not seem to be present in significant quantities on SDS-PAGE when the proteins were not reduced. Finally, heparinase was highly specific to the carbohydrate network, completely eliminating the Stains-All positive band in the native gel while causing no change to the proteins on SDS-PAGE.

DISCUSSION

Arion subfuscus glue is tough because it is relatively stiff for a dilute gel, yet it has great extensibility. It can stretch roughly 10 times its original length before fracturing. The initial elasticity is responsible for a surprisingly large extension then, after yield occurs, an extended period of gradual tearing takes place, absorbing more energy. This pattern is similar to the results of tensile tests on some synthetic double-network gels. These synthetic gels yielded at strains of roughly two, followed by extensive necking, resulting in a stress plateau and extension to a failure strain of roughly 15 (Haque et al., 2012).

The experiments suggest that *A. subfuscus* glue gains this toughness by a double-network type mechanism. It consists of a network of heavily sulfated heparinase-sensitive proteoglycans and a network of proteins. These two networks work together synergistically to create much greater toughness than they can separately. Disrupting either network causes near-complete loss of the glue's integrity rather than weakening it proportionally. Nevertheless, while it is consistent with the basic principles of a double network, there are significant differences from the double network described by Gong (2010), aside from the type of polymers used. First, the networks depend on reversible bonds, rather than permanent covalent bonds. Second, the two networks are not fully separate. There are some proteins bound to the carbohydrates. Finally, there are other differences in the composition and proportion of the different networks. Overall, these differences suggest that while *A. subfuscus* glue operates on the same principles as the double network, it is a different variant of that type of gel.

The presence of reversible bonds makes the glue more similar to recent synthetic, tough hydrogels. Researchers have been able to improve on the original double networks by using reversible bonds as sacrificial bonds in the stiffer network. Hydrophobic interactions, ionic interactions and metal-based coordinate bonds have all been used successfully to dissipate energy in hydrogels, while allowing self-healing after the stress is removed (Henderson et al., 2010; Haque et al., 2011; Sun et al., 2012, 2013).

The fact that the two networks are not fully separate is also consistent with recent work on high-performance hydrogels. For example, Sun et al. (2012) used a stiff alginate network covalently linked to a deformable polyacrylamide network. Even the original double-network gels could be strengthened by some connections between networks (Gong, 2010). Another group created triblock co-polymers in a single network, using metal ions as sacrificial bonds (Henderson et al., 2010), while another used a single polyampholyte gel (Sun et al., 2013). In all cases, the essential feature was that an extensible polymer provided hidden length, and there was a high density of weaker sacrificial bonds to provide energy dissipation. This general pattern has been seen in a number of solid biomaterials as well, with examples such as nacreous gastropod shell (Smith et al., 1999b), bone (Fantner et al., 2005), whelk egg capsule (Rapoport and Shadwick, 2007; Miserez et al., 2009), mussel byssus (Harrington et al., 2009; Krauss et al., 2013) and caddisfly silk (Ashton et al., 2013; Ashton and Stewart, 2015).

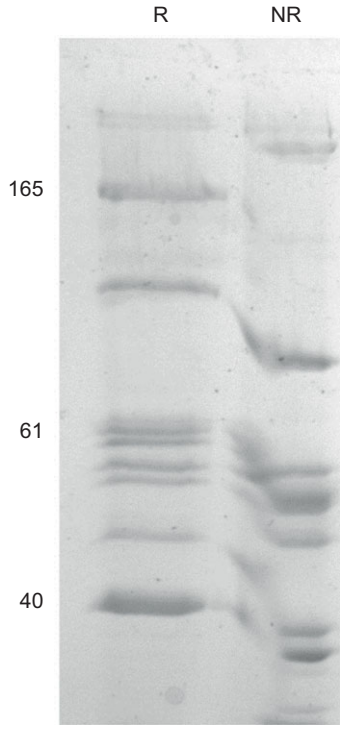

Fig. 8. The effect of disulfide bond reduction on the apparent mass of proteins from *A. subfuscus* glue. Samples were run on 7.5% SDS-PAGE. Lane 1 is reduced (R) while lane 2 is not reduced (NR). The effect of the reducing agent in lane 1 spreads to the edge of lane 2, showing which bands correspond in the two lanes. M_r (values given in thousands) of select proteins is indicated on the left.

The nature of the two networks in *A. subfuscus* glue

The primary evidence for protein and polysaccharide networks comes from native gel electrophoresis. This is a direct and widely used method of detecting protein complexes (Krause, 2006; Miernyk and Thelen, 2008; Wittig and Schägger, 2009). In native gels, the negatively charged proteins from slug glue mostly fail to enter the gel, and the proteins that do enter the gel run together in the top part of the gel. This is consistent with the formation of large complexes of at least 1000 kDa that are stably but non-covalently linked (Schägger and Jagow, 1991). The polysaccharides fail to enter native and SDS gels, indicating that they are in large complexes that cannot be disrupted by SDS. For both networks, there are certain conditions that can disrupt the networks so that the component polymers enter the electrophoresis gel and resolve as

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bands, but in their native state they appear linked into such large networks that they cannot enter the gels.

The carbohydrates are likely to be complexed as proteoglycans. Proteoglycans are often so large that they entangle into physical networks to form gels at low concentrations (Smith, 2002). This would explain why they can only enter native and SDS gels when sonicated, which would fragment such large complexes (Sajdera and Hascall, 1969). Also, the proteinase treatment led to polyanionic carbohydrates entering the gel, which is consistent with loss of the protein core of a proteoglycan. The efficacy of heparinase II in digesting the carbohydrates suggests that they are in the form of heparin or heparan sulfate. These glycosaminoglycans often associate in the form of proteoglycans (Kjellen and Lindahl, 1991).

The negatively charged proteins from asmp-40 to asmp-220 also appear to form complexes that are too large to enter native gels, leading to most of the protein staying at the top of the gel, with small fragments dissociating and entering the gel to form a faint smear as evidenced by the presence of all of these proteins near the top of the native gel. These are robust networks that stay together even in the presence of EDTA, DTT, high salt and non-ionic detergents. They do not depend on permanent covalent bonds, because SDS disrupts the network. The finding that hydroxylamine leads to the appearance of distinct bands on native gels suggests that it disrupts oxidatively derived cross-links that are essential to this network, as suggested by Braun et al. (2013). The protein most affected by this treatment was asmp-40, which formed the darkest band on the native gel. The fact that the other proteins did not appear to dissociate to the same extent suggests that other bonds may also be involved. In the case of asmp-40, previous work found that it behaved as if it formed a giant complex that may have been due to oxidative cross-linking with itself (Smith et al., 2009).

Not all of the proteins are part of the network. Interestingly, most of the positively charged proteins, identified collectively as asmp-15, are easily separated from this network, running as distinct bands on native gels. This further suggests a catalytic role for these proteins as opposed to a structural role (Smith, 2013). These proteins were found to be characteristic of the glue and to have non-specific gel-stiffening activity (Pawlicki et al., 2004). Alternatively, their positive charge may lead them to interact with polyanionic carbohydrates. Nevertheless, if there were such interactions, they are not likely to be strong as these proteins were easily separated from the carbohydrates. In addition, asmp-57 may not be part of the network. This protein can be resolved using blue native electrophoresis, where the dye Coomassie Blue G-250 is used to give a slight negative charge to otherwise hydrophobic proteins.

In ion exchange, asmp-40 and asmp-165 co-eluted primarily with the carbohydrates rather than the other proteins. This may indicate that they are more closely connected to the carbohydrate network than the protein network. The co-elution of asmp-40 with the carbohydrates may indicate that it binds to them, but this could be a physical tangling interaction between very large complexes, since a substantial amount of asmp-40 did wash off with the other proteins. Asmp-165 co-elutes more strongly with the carbohydrates, possibly indicating a closer interaction. This is not consistent with previous work that found that asmp-165 was heavily oxidized and appeared to associate with the other high molecular weight proteins in the glue (Bradshaw et al., 2011), though that study did not distinguish between asmp-165 and asmp-220. Further work will be needed to clarify the exact connections between polymers in the glue. It is also possible that these proteins have some ability to interact with the beads, despite their overall positive charge at this pH.

Overall, while there are interactions between the protein and carbohydrate networks, the networks are not tightly interconnected. Most of the protein easily separates from the carbohydrates, and there is minimal co-localization of proteins and polyanionic carbohydrates in native gels. Treatments that allow the carbohydrates to enter the gel do not lead to a corresponding increase in proteins entering the gel, and vice versa. This argues against the hypothesis that the carbohydrates form the backbone of a gel that is directly cross-linked by proteins.

How does each network contribute to toughening?

In a typical double-network gel, there is a soft, deformable network that makes up the majority of the material, and a stiff, highly cross-linked network (Gong, 2010). In slug glue, the proteoglycans are likely to provide a deformable network. Most highly deformable mucus is based on such tangling, carbohydrate-rich networks (Denny, 1983; Smith, 2002). They can extend to large strains, but typically have very low stiffness. This is consistent with the results of disrupting the protein network; the remaining material typically formed a viscous, highly deformable material whose stiffness was too low to be measured in the tensometer. Despite the low stiffness, the double-network mechanism allows the carbohydrates to play a crucial role in creating toughness. This is interesting in light of the fact that proteoglycans are widespread in the extracellular matrix of tissues, most notably in cartilage. These tissues typically consist of a network of collagen fibers embedded in a proteoglycan matrix. The collagen fibers are viewed as being the primary factor governing the material's strength. In contrast, the proteoglycans are viewed as space-filling materials that contribute primarily to swelling pressure and resistance to compression, rather than resistance to fracture (Yanagishita, 1993; Roughley and Lee, 1994). The results for slug glue suggest that such large carbohydrates may play a much larger role in toughening materials than expected.

If the carbohydrates in slug glue constitute the deformable network, then the proteins probably form the stiff, highly cross-linked network. The evidence that the protein network is essential for gel toughness is based on three different treatments aimed at disrupting it: hydroxylamine, TCEP and proteinase K. The fact that all three treatments had a similar effect is important, because the treatments were less specific than the heparinase, and thus may have disrupted the carbohydrate network to some extent as well. The protease clearly affected the carbohydrate network, as evidenced by the increase in polyanionic carbohydrates in the native gel after protease treatment. As carbohydrates are linked to a protein core in proteoglycans, it is likely that the protease also disrupts that network. Thus, the protease treatment alone does not provide a strong test of the double-network mechanism. Disulfide bond reduction is more specific to proteins in the protein network. It clearly affects the structure of all the proteins in the network, and there was no evidence from native gels that disulfide reduction impacted the carbohydrate network. Nevertheless, some proteoglycans can be linked together into larger complexes by disulfide bonds, and it is possible that those complexes were broken into smaller groups that were still too large to enter the gel. The hydroxylamine treatment should have been more specific to the proteins, as it was aimed at disrupting specific bonds in that network. Analysis of native gels showed minimal effects on the carbohydrate network, and the main effect was to allow proteins to run as distinct bands in native gels. At concentrations of 1.5 mol l^{-1}, however, it could have had other effects. Nevertheless, given that all three treatments caused a similar, total loss of toughness, it is likely that the protein network is equally essential to the glue. This would

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not be surprising, as simple proteoglycan networks would be expected to have minimal toughness on their own; such gels typically serve a lubricating function (Smith, 2006). It cannot be stated for certain, though, because it is possible that all three treatments had different side effects that impacted the carbohydrate network in some way. In summary, there is strong evidence that the carbohydrate network is essential for dramatically increased toughness of the glue, and there is solid but not definitive evidence that the proteins are similarly essential.

In the proposed model, the carbohydrate network provides the hidden length and the protein network provides the sacrificial bonds. The difficulty with this interpretation is that the calcium ions are likely to associate with the carbohydrate network purely based on charge considerations. These ions are excellent candidates to provide sacrificial bonds because they form relatively labile electrostatic or coordinate bonds and are present at a high concentration (Braun et al., 2013). Some synthetic super-tough hydrogels use calcium and alginate for sacrificial bonds (Sun et al., 2012). In those gels, however, it should be noted that alginate forms inherently much stiffer networks than proteoglycans. Similarly, caddisfly silk uses calcium to form sacrificial bonds and thus increase toughness (Ashton et al., 2013; Ashton and Stewart, 2015). However, some of the proteins in slug glue are also metal binding (Werneke et al., 2007), and perhaps they use calcium for such bonds. Proteins often form stable coordinate interactions because they can have specialized metal-binding regions. Alternatively, oxidatively derived imine bonds are labile and may provide sacrificial bonds. A final possibility is that the proteins may have internal bonds that are disrupted as the proteins stretch, as was found for mussel byssus (Harrington et al., 2009), or changes in secondary structure that absorb energy as the proteins extend, as seen in whelk egg capsules (Miserez et al., 2009). They may also have rubbery elasticity like elastin (Wainwright et al., 1976). It is intriguing that the glue has such a long, apparently elastic region before the initial yield (Fig. 5). Rubbery elasticity is a major factor in the toughness of spider silk, allowing stiffness with a high extensibility (Gosline et al., 1984). Some combination of these effects could be at play in slug glue. In order to gain a clearer picture, structural information about the proteins in the glue is essential. In addition, cyclic mechanical tests to determine the extent of energy dissipation versus pure elasticity will be informative.

MATERIALS AND METHODS

Sample collection

Slugs (*A. subfuscus*) were collected in the forest surrounding Ithaca, NY, USA. Glue was obtained by gently rubbing the dorsal side of the slugs with a thin, metal spatula (Werneke et al., 2007). The glue from each slug was stored separately at −80°C, and thawed for each experiment.

Testing for the presence of protein and carbohydrate networks

Native gel electrophoresis was used to determine whether the proteins formed complexes. This method is widely used to identify protein–protein interactions and protein complexes, especially with complexes that may involve a number of different proteins (Krause, 2006; Miernyk and Thelen, 2008; Wittig and Schägger, 2009). This simple method minimizes false positives because it identifies interactions that are present *in vivo*, rather than interactions that are possible *in vitro* (Miernyk and Thelen, 2008). It is also well suited for studying stronger interactions. In native gels, protein–protein interactions would lead to the formation of either large complexes that would run as higher molecular weight bands or complexes so large that they would not be able to enter the gel and resolve into distinct bands. The components of these complexes can then be resolved by running them on a second dimension of SDS-PAGE (Miernyk and Thelen, 2008). In contrast, proteins that do not interact with each other should form distinct bands on a native gel.

The procedure for native gel electrophoresis was derived from the protocols of Hames (1990). Several different buffer systems and pH values were tested for native gel electrophoresis. Tris-acetate, Tris-glycine and Tris-Cl were all tested, spanning a range of pH values from 7 to 8.8. Both continuous and discontinuous gels were tested. The results were similar in all cases, but continuous Tris-acetate gels (40 mmol l^{-1} Tris, 20 mmol l^{-1} acetate, pH 7.5) gave consistently clear results, and were thus used for all further experiments. Gels with total acrylamide concentrations of 5%, 7.5%, 10% and 12.5% were tested (19:1 acrylamide:bisacrylamide). Several trials were also performed with blue native PAGE, where Coomassie Blue G-250 is included in the sample and running buffer to increase the negative charge on hydrophobic proteins and thus improve their resolution (Wittig and Schägger, 2009). For most of the proteins in slug glue, which are acidic and water soluble, the blue dye did not change the results, so most samples were analyzed without dye in the buffer (i.e. using clear native PAGE), which works well for water-soluble proteins (Wittig and Schägger, 2005, 2009).

In preparation for electrophoresis, glue was soaked for 2 h at room temperature or overnight at 4°C in 200 mmol l^{-1} Tris, 100 mmol l^{-1} acetate (pH 7.5), at a concentration of 30 mg glue ml^{-1} buffer. The glue was then homogenized with a rotor-stator, sonicated for less than 10 s on ice unless otherwise specified, and centrifuged at 14,000 ***g*** for 10 min. Subsequently, the dissolved extract was combined 1:1 (v:v) with 80 mmol l^{-1} Tris, 40 mmol l^{-1} acetate (pH 7.5) with 20% glycerol. For blue native gels, Coomassie Blue G-250 (0.02%) was added to this extract, and to the cathode buffer of the electrophoresis apparatus (Schägger and von Jagow, 1991). PAGE was then performed using a 5% or 10% continuous Tris-acetate pH 7.5 gel with a reservoir buffer of 40 mmol l^{-1} Tris, 20 mmol l^{-1} acetate, pH 7.5. Gels were run for 40 min at 40 mA using normal polarity (cathode at the top), or for 60 min using reverse polarity of the voltage terminals (anode at the top). Unless otherwise stated, all gels were stained with Coomassie Blue R-250 overnight and imaged using a ChemiDoc Gel Imager (Bio-Rad, Hercules, CA, USA). Note that all experiments were replicated two or more times.

For many of the gels, discontinuous SDS-PAGE (Laemmli, 1970) was used as a second dimension to separate complexes and identify the proteins present in bands or stained regions of the native gel (Miernyk and Thelen, 2008). This was done either by cutting a lane from the native gel immediately after running and laying it horizontally in a large well across the top of an SDS gel, or by excising regions of the native gel that were known to have bands, extracting the proteins from that region and loading them on standard SDS-PAGE. To aid in band excision in the latter approach, an adjacent lane with the same loading was stained with an abbreviated protocol taking only 1–2 h. This was used as a guide to cut bands from the unstained neighboring lanes. The unstained lanes were kept on ice covered with plastic wrap while the guide lane was stained. After excision, the gel slices were heated at 80°C for 15 min in 150 µl of SDS-PAGE sample buffer (0.125 mol l^{-1} Tris-Cl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol). The buffer extract was then loaded on a 12.5% gel (19:1 acrylamide: bisacrylamide) with wells that could accommodate 100 µl of sample. When an entire lane was loaded across the top of the SDS gel, thicker gels were used (1.5 versus 1 mm thick) to facilitate loading, and SDS-PAGE sample buffer filled the remainder of the well surrounding the gel strip. This was allowed to equilibrate for 20 min before electrophoresis. Second dimension SDS gels were stained with Sypro Ruby (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol.

Because the native gels indicated that many of the proteins were forming complexes that were too large to enter the gels, different treatments were used to try to disrupt those complexes. Separate 30 mg ml^{-1} samples of glue were soaked overnight in 40 mmol l^{-1} Tris, 20 mmol l^{-1} acetate (pH 7.5) alone or with 10 mmol l^{-1} EDTA, 5 mmol l^{-1} DTT, 1% Tween-20, 1 mol l^{-1} NaCl or 0.03–7.5 mol l^{-1} hydroxylamine. Before adding sample to hydroxylamine solutions, the pH was adjusted back to 7.5 with hydrochloric acid. All samples were run on normal polarity native electrophoresis gels and Coomassie stained.

Stains-All was used to visualize negatively charged carbohydrates on native gels. This cationic dye binds to strong polyanions, such as sulfated

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polysaccharides (Goldberg and Warner, 1997). Samples were prepared in the same way as those analyzed for protein, except that some samples were not sonicated, as that can fragment large, polysaccharide-rich molecules (Sajdera and Hascall, 1969). To test common factors that might affect carbohydrate networks, samples were soaked overnight at 4°C in 40 mmol l^{-1} Tris, 20 mmol l^{-1} acetate buffer with 10 mmol l^{-1} EDTA or 5 mmol l^{-1} DTT. The staining procedure of Campbell et al. (1983) was used, but with 0.025% Stains-All overnight.

Testing for interactions between proteins and carbohydrates

The carbohydrates were isolated and any proteins that were bound to them were identified. The isolation procedure was based on ion exchange, but as dissolved glue samples tend to clog chromatographic columns (Smith et al., 2009), a batch ion exchange assay was developed. Positively charged beads were used to bind the sulfated polysaccharides at a pH that would give the proteins a positive charge. Any proteins that were present in the bound fraction were presumably attached to the carbohydrates.

The isoelectric points of the proteins were first analyzed by isoelectric focusing (IEF). Samples were dissolved to 20 mg glue ml^{-1} buffer in 50 mmol l^{-1} Hepes, pH 7.7, with homogenization and brief sonication. They were then mixed with an equal volume of sample buffer (9 mol l^{-1} urea, 2% CHAPS, 5% 2-mercaptoethanol). The sample was run on a gel containing 4% total acrylamide (19:1 acrylamide:bisacrylamide) with 1% Triton X-100 and 2% ampholytes (FlukaBrand pH 3–10), the upper buffer was 40 mmol l^{-1} sodium hydroxide and the lower buffer was 0.085% phosphoric acid. The gel was pre-run at 300 V for 15 min before samples were loaded. The gel was stained using Coomassie Blue R-250/copper sulfate (Righetti et al., 1990).

The batch ion exchange assay was performed using Q Sepharose Fast Flow beads (Q-beads) (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Approximately 0.5 ml of Q-beads was used in a 1.7 ml microcentrifuge tube. The loading buffer was 20 mmol l^{-1} piperazine adjusted to the final pH with hydrochloric acid. As all the main proteins had pI values above 6, three different loading buffers were tested: pH 6.2, 5.1 and 4.6. The glue was dissolved in this buffer (20 mg glue ml^{-1}) by soaking it for 1 h then homogenizing, sonicating and centrifuging it as before. Before loading the sample, the beads were washed with loading buffer. For each wash, 1 ml of loading buffer was added to the Q-beads and they were mixed by repeated inversion on a rotator for 5 min. The beads were then centrifuged at 700 ***g*** for 1 min and the supernatant was discarded. The process was repeated for a total of three washes before 1 ml of sample was loaded and mixed for 30 min. The beads were washed again three times with loading buffer and the supernatant was collected each time. Finally, the bound material was eluted with loading buffer containing 1 mol l^{-1} NaCl. This fraction was compared with the original extract, and with the material that did not bind and came off with the first wash. SDS-PAGE, and colorimetric assays for protein (Bradford, 1976) and carbohydrate (Kennedy and Pagliuca, 1994) were used to analyze all fractions. The orcinol assay for carbohydrate was performed at 510 nm instead of 420 nm (Smith et al., 1999a). This assay works best for neutral sugars, but also detects acidic sugars, though not amino sugars (Smith and Morin, 2002). The batch ion exchange was repeated on samples dissolved in loading buffer containing 5 mmol l^{-1} DTT to determine whether disulfide bonds were involved in the proteins' ability to interact with carbohydrates.

Mechanical characterization of the glue

Samples for mechanical testing were cut from homogeneous glue samples collected from individual slugs. When a sample was collected from a slug, the viscous secretion accumulated on the spatula and was allowed to set into an irregular ball that was then stored. For the mechanical tests, samples of at least 5 mm diameter were selected. Samples were taken from the −80°C freezer and allowed to warm until they were just barely frozen. In this soft-frozen state, they were easily cut by a razor blade but did not deform and did not adhere to surfaces. It was necessary to repeatedly place the sample on dry ice in order to maintain it in a sufficiently frozen state for manipulation. To cut the test strips, a single planar sheet was first cut from the middle of the frozen glue ball by two parallel razor blades joined together so that their edges were 1 mm apart. Uniform rectangular strips were then cut from this sheet using the same conjoined razor blades. The resulting test strips were 1×1 mm in cross-section and roughly 5 mm long. For experiments comparing different treatments, the two most clearly homogeneous strips from a given sample were selected and used. For all such treatments, one strip would be soaked in a buffer containing the treatment, and the other would be soaked in the same buffer without the treatment, thus giving paired controls for every trial.

Stress–strain curves were measured in a simple tensometer using uniaxial tensile loading. The tensometer was designed for simplicity and ease of working with soft samples rather than high precision because of the difficulty working with small gel strips, many of which had been soaked for extended periods. The soaked samples were swollen and deformable, rendering high-precision characterization of their dimensions impractical. A Labquest linear force transducer (force range ±10 N; Vernier, Beaverton, OR, USA) was clamped in place with a small (20 mm) alligator clamp fixed to the sensor. The sample rate was set at 50 samples s^{-1} and a 0.2 s running average was calculated giving a resolution of roughly 1 kPa for the gel strips used in this system. A second alligator clamp was suspended from thread above the other clamp and connected via a pulley and gearing system to a 9 V electrical motor. The motor was geared down to pull at a constant 0.2 cm s^{-1}. Glue samples were clamped between the alligator clips. Strain was calculated from the elapsed time and strain rate. Toughness was calculated by trapezoidal integration of the stress–strain curve.

Because of the difficulty in measuring the unstressed dimensions of soaked samples, all values of stress and toughness are with respect to the original, unsoaked dimensions. Soaking typically caused significant swelling, increasing the diameter by a factor of roughly 2–3. The effect was similar in treated and untreated samples when the treated samples were not completely disrupted. Also, values are in terms of engineering stress, i.e. the stress was not adjusted to account for changes in cross-section at increased strain. When samples were soaked, the deformation due to the sample's own weight and the clamping made it difficult to get a precise measure of the initial length of an unstressed sample. Thus, all soaked samples were clamped with roughly 2 mm of test strip between the clamps and that value was used as the initial length. For samples that had not been soaked, it was possible to measure the initial, unstressed length more precisely with calipers. Finally, if a sample failed at the site of clamping as a result of tearing or slipping, the data for that trial were discarded. These trials were apparent because failure typically occurred in the middle of the sample at large strains, and it was possible to observe whether it occurred at the clamp, or whether the sample pulled completely free of one clamp.

To verify that freezing did not have a large effect on samples, three separate samples were cut into pairs of strips, with one strip from each tested immediately at room temperature, and the other repeatedly frozen on dry ice and thawed, with a total of three freeze–thaw cycles. These samples were then tested at room temperature. The strips were roughly 1×1 mm, but it was difficult to create test strips of precise dimensions in this test, as they were at room temperature when they were prepared.

Contributions of protein and carbohydrate networks to gel mechanics

To test the relative contributions of protein and carbohydrate networks, each was selectively disrupted by different treatments, and then mechanical tests were performed. The carbohydrate network was disrupted enzymatically. The protein network was disrupted by hydroxylamine; this is a nucleophile that can compete for carbonyls and thus disrupt the bonds that appear important for cross-linking the proteins in the glue (Braun et al., 2013). In addition, a general protease was tested, though that approach was not as specific because many polysaccharides are known to be part of protein–polysaccharide complexes such as proteoglycans (Denny, 1983; Kjellen and Lindahl, 1991). Reduction of disulfide bonds was also performed in order to disrupt protein function.

Heparinase II was used to digest the carbohydrates. This enzyme digests the main carbohydrate in the mucus of another terrestrial slug, *Lehmannia valentiana* (Li and Graham, 2007), and was found to be effective in preliminary trials on *A. subfuscus* glue. Strips from three samples were soaked overnight at 22°C in 100 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-Cl, pH 7.5, 1.5 mmol l^{-1} calcium chloride with or

without 16 U ml^{-1} of recombinant *Bacteroides* Heparinase II (New England Biolabs, Ipswich, MA, USA). Mechanical tests were performed as described above unless the sample was liquified by the treatment. Native gel electrophoresis with Stains-All was used to confirm digestion of the carbohydrates.

Hydroxylamine was used to break up the protein network. Four separate trials were performed where a single sample was divided into 5 mg parts and each was soaked overnight at 4°C in 20 mmol l^{-1} Tris-Cl, pH 8, with different concentrations of hydroxylamine (0.03, 0.15, 0.6, 1, 1.5, 3, 7.5 mol l^{-1}) or a control without hydroxylamine. The pH was adjusted to 8 as necessary with hydrochloric acid. For this test, sample mechanics were observed qualitatively. Similar trials were performed with the tensometer on separate samples and controls, though samples had not been cut into precise strips for these preliminary experiments. For these, the hydroxylamine concentrations ranged from 0.01 to 0.6 mol l^{-1}. Higher concentrations did not have sufficient integrity to be tested reliably with the tensometer. To test that hydroxylamine was disrupting the protein network without strong side effects, separate sets of samples in the same solutions were homogenized and analyzed with native gel electrophoresis and SDS-PAGE as described previously.

Proteinase K was also used to eliminate the protein network. Strips from 11 samples were soaked in 20 mmol l^{-1} Tris-Cl, pH 8, 0.01 mmol l^{-1} calcium chloride, 0.3% glycerol at 22°C overnight with or without 6 Units ml^{-1} of Proteinase K (from *Tritirachium album*, IBI Scientific, Peosta, IA, USA). Mechanical tests were performed as described above. Native gel electrophoresis with Stains-All was used to see if the treatment affected the carbohydrates.

Proteins were also disrupted by breaking disulfide bonds with 5 mmol l^{-1} DTT or 25 mmol l^{-1} TCEP. Both were in 20 mmol l^{-1} Tris-Cl (pH 8), which was used for the control. For TCEP, the FOCUS protein reduction–alkylation kit was used (G-Biosciences, St Louis, MO, USA) following the manufacturer's protocol but without alkylation. Mechanical tests were performed as described above. To characterize the effect of reduction on the proteins, dissolved samples of glue were run on 7.5% and 12.5% gels (total acrylamide) using SDS-PAGE sample buffer with or without mercaptoethanol. When reduced and non-reduced samples were run in adjacent lanes, the effect of the mercaptoethanol spread to the neighboring lane, causing a continuous gradient from reduced to not-reduced, so that it was possible to identify the reduced band that corresponded to each non-reduced band.

Competing interests

The authors declare no competing or financial interests.

Author contributions

A.M.W., S.R.R., H.S.G. and C.C.H. each were responsible for several key experiments. They helped refine the methods, conducted the experiments, analyzed the data and helped draft sections of the article. A.M.W. conducted many of the mechanical measurements, S.R.R. conducted the biochemical characterizations of the glue structure, H.S.G. conducted experiments involving protein reduction and those involving interactions between proteins and carbohydrates, and C.C.H. conducted the experiments using enzyme treatments. A.M.S. proposed the research idea, was involved in the design, execution and interpretation of the experiments, and helped draft and revise the article.

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