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# The effect of molluscan glue proteins on gel mechanics

J. M. Pawlicki[1], L. B. Pease[1], C. M. Pierce[1], T. P. Startz[1], Y. Zhang[2] and A. M. Smith[1,*]

[1]*Department of Biology, Ithaca College, Ithaca, New York 14850, USA* and [2]*Department of Materials Science and Engineering, Cornell University, Ithaca, New York 14850, USA*

*Author for correspondence (e-mail: asmith@ithaca.edu)

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## Summary

**Several molluscs have been shown to alternate between a non-adhesive trail mucus and a similar gel that forms a strong glue. The major structural difference between the two secretions is the presence of specific proteins in the adhesive mucus. The present study identifies similar proteins from the glue of the slug *Arion subfuscus* and the land snail *Helix aspersa*. To investigate the role played by these proteins in adhesion, the proteins were isolated from the adhesive mucus of different molluscs and added to commercial polymer solutions. The effect was observed qualitatively, and quantified using a dynamic rheometer. The isolated proteins triggered gelling or visible stiffening of agar, pectin and polygalacturonic acid. The effect was stronger on more negatively charged polymers. The effect of the proteins was concentration dependent with an optimal concentration of 1–1.5 mg ml$^{-1}$, and was weakened when their structure changed. Other proteins and carbohydrates found in the adhesive mucus had no clear mechanical effect on gels. These findings show that the addition of these proteins to large, anionic polymers plays a central role in the formation of a glue from a mucus-like secretion. Such a mechanism may be common among invertebrates, and it may guide biomimetic approaches in the development of glues and gels.**

Key words: mollusc, slug, *Arion subfuscus*, land snail, *Helix aspersa*, glue, gel, adhesion, mucus, periwinkle, *Littorina irrorata*.

## Introduction

Animals depend on the unusual mechanics of gels for a wide variety of disparate tasks (Denny, 1989). Most commonly, mucous gels serve as a lubricating and protecting layer. Many molluscs, however, use similar gels to glue themselves to the substratum when they are inactive. Limpets use such gels to glue themselves to rocks amid violent wave surge. Similarly, a thin line of gel along the lip of a marsh snail's shell can firmly attach it to the top of a wet marsh grass stem, despite the windswept movements of the grass. These gels, like most mucous secretions, consist of a dilute network of polymers, usually containing more than 95% water (Smith, 2002). One would not expect a dilute gel that is typically a lubricant to form strong attachments. In order to achieve such strength, it seems likely that the adhesive gel differs significantly in structure from other forms of mucus.

Smith et al. (1999) and Smith and Morin (2002) showed that the adhesive and non-adhesive forms of mucus from these animals are structurally similar except for the presence of specific proteins in the glue. These will be referred to as glue proteins. The difference can be striking, with the glue proteins making up roughly 10–50% of the secretion in the animals studied. The presence of such quantities of these proteins in the adhesive mucus suggests that they play a key role in adhesion. Specifically, we hypothesize that the proteins act on other polymers to stiffen the gel. One possibility is that the proteins cross-link the other polymers in the gel. This would have a large impact on the gel's mechanical strength (Denny, 1983; Smith, 2002). They may cross-link the large polymers of the normal, non-adhesive mucus, or similar large polymers. Alternatively, the proteins may serve as enzymes to catalyze a cross-linking reaction, or otherwise modify the polymers in the gel. It is also possible that the proteins themselves form an adhesive bond independent of the other polymers in the gel. Finally, the presence of these proteins may only be incidentally related to adhesion.

To determine the role of these proteins, their effect on different gels was measured. We isolated the glue proteins from the adhesive mucus of marsh periwinkles and two other species, terrestrial slugs and land snails. We added these proteins to commercial polymers that can gel or form highly viscous solutions. We predicted that the glue proteins would increase the stiffness and viscosity of gels formed by large polysaccharides. These experiments provide qualitative and quantitative evidence that the identified proteins play a direct role in adhesion.

## Materials and methods

The effect of molluscan glue proteins was tested on different commercial polymers. In this paper, the commercial polymers will be referred to as gel-forming polymers, as they provide

the ground material or structural backbone of the gels, which the glue proteins may act upon. These polymers provided several important advantages over the natural polymers found in the mucus. While it might be directly relevant to add the glue proteins to the non-adhesive mucus and observe changes, the fact that the non-adhesive mucus is already a gel greatly complicates the process of uniform mixing. Pouring a proposed cross-linker on the surface of a gel will probably not change the mechanics throughout the gel, and mixing could destroy the giant polymers that give mucus its unique mechanics. Alternatively, one could purify the large gel-forming polymers from mucus and mix them with the glue proteins, but in practice, it is difficult to isolate sufficient quantities for mechanical testing. To make a gel, a polymer concentration of roughly 20 mg $ml^{-1}$ is necessary, and 0.5 ml would be needed for each test. By using commercial polymers, one can mix the components in solution and observe gel formation, and the amount of material available for testing is not limiting. Furthermore, one can test polymers with different chemical structures to determine the specificity of the glue proteins.

### *Isolation of glue proteins*

The glue proteins from the marsh periwinkle *L. irrorata* (Say) were tested first. Smith and Morin (2002) showed that both the adhesive and non-adhesive mucus from these snails contain MDa-size carbohydrates. In addition, the adhesive mucus has two proteins of 41 and 36 kDa that appear to be related. These proteins make up roughly half of the organic material in the adhesive mucus. Samples of the adhesive mucus were collected as described by Smith and Morin (2002) from roughly 200 periwinkles kept in a 29 gallon aquarium with recirculating artificial seawater. Samples were dried and stored at –80°C until use.

The 41 and 36 kDa glue proteins were isolated by gel filtration chromatography. For each separation, several mg of dried sample were dissolved in 2 ml of buffer containing 8 mol $l^{-1}$ urea, 0.5 mol $l^{-1}$ NaCl, 0.5% Triton X-100 and 20 mmol $l^{-1}$ phosphate, pH 7.4. Samples were heated to 80°C, vortexed and sonicated until they dissolved. They were then loaded onto a 1.6 cm × 60 cm Sephacryl S-400 column (Pharmacia Biotech, Sweden) with a column buffer of the same components, but containing urea at a concentration of 6 mol $l^{-1}$ instead of 8 mol $l^{-1}$. In earlier trials, lower concentrations of urea, Triton and sodium chloride were tried, but these did not give such consistent results. Fractions from the column were assayed by the Bradford assay for protein and the orcinol–sulfuric acid assay for carbohydrates (see Smith and Morin, 2002). This column cleanly separated the MDa-size carbohydrates from the smaller proteins (Fig. 1). As the only significant proteins present in the protein peak were the 41 and 36 kDa glue proteins (Smith and Morin, 2002), further purification steps were not taken. The protein peak was pooled and dialyzed exhaustively against phosphate-buffered saline (PBS; 20 mmol $l^{-1}$ phosphate, 130 mmol $l^{-1}$ NaCl, pH 7.4) or, in some cases, distilled water. There was no detectable difference in results between samples dialyzed in either

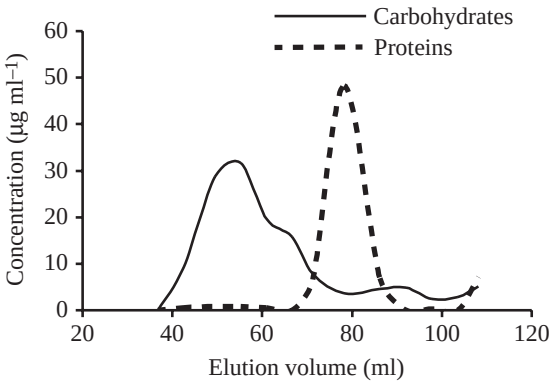


Fig. 1. Elution profile of *L. irrorata* adhesive mucus on a sephacryl S-400 column. The protein peak corresponds to a molecular mass of roughly 10–100 kDa and the carbohydrate peak corresponds to roughly 1000 kDa, based on calibration of the column with BSA (66 kDa), ferritin (440 kDa) and blue dextran (2000 kDa).

way. The dialysis bags were then placed on high molecular mass polyethylene glycol chips to concentrate the protein by osmosis. Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the methods of Laemmli (1970) and Hames (1990) confirmed that the samples contained the 41 and 36 kDa proteins and little else. This procedure yielded 1–2 ml of these proteins at a concentration of roughly 1 mg $ml^{-1}$. Attempts to increase the amount isolated were often complicated by the difficulty in dissolving more than a few mg of dried glue per ml of buffer and because of possible aggregation at the top of the column. One might expect this given that the polymers function by forming gels at low concentrations.

### *Qualitative effects of* L. irrorata *glue proteins*

The effect of *L. irrorata* glue proteins on gel-forming polymers was assessed qualitatively. After dialysis, solutions of glue protein were added to dried gel-forming polymers, mixed, allowed to set in microcentrifuge tubes, then removed for observation and comparison to controls. Controls were the same as the treatments, except with bovine serum albumen (BSA) instead of glue proteins. To ensure that the only difference between treatments and controls was the type of protein added, the BSA was dissolved in the bathing solution from the last dialysis bath used on the glue protein sample. Thus, it should have had the same concentration of ions, the same pH, and the same amounts of trace contaminants. This is particularly important since traces of urea and detergent may interfere with gelling. In addition to BSA, other control proteins were tested, including gelatin, ovalbumin and non-fat dried milk. The presence or absence of BSA, or any of these control proteins, had no clear effect on gels. Nevertheless, every trial using glue proteins was compared with a paired control using the same concentration of BSA.

The gel-forming polymers that were tested included agar, pectin (apple and citrus), polygalacturonic acid and guar gum. Phillips and Williams (2000) provide detailed information on

these polymers. All of these polymers are roughly MDa-size polysaccharides except for agar, which has subunits of roughly 100–150 kDa. Agar, guar gum and pectins are composed of different subunits, while pectins are similar to polygalacturonic acid, except that they are partially methylated. Unlike the other polymers, guar gum is neutral and its mechanics depend solely on intermolecular tangling. The others are capable of gelling by different mechanisms.

Several different concentrations of gel-forming polymer were tested for each gel. The concentrations that worked best, and were thus used in later trials, were 2% for guar gum, polygalacturonic acid or pectin, and 0.5 or 0.6% for agar. Dried samples were weighed out on an electronic balance that was accurate to the nearest 0.01 mg. The dissolved proteins were added with a digital pipettor. Care was taken to ensure that the concentration of gel-forming polymers varied by less than 1% among samples (i.e. 2% gels may have actually been 1.98–2.02%). For the pectin samples, calcium chloride (to 50 mmol $l^{-1}$) was added to assist gelling. Similarly, hydrochloric acid (to 20 mmol $l^{-1}$) was added to polygalacturonic acid samples to help dissolve the polymer. Agar was heated to 100°C for 2 min before being allowed to set.

Samples were placed on a flat surface and compared visually. They were categorized as being either primarily liquid, gelled slightly into loose clumps, or firmly gelled, retaining the shape of their container. Statistical tests were not performed on these qualitative experiments. Instead, it was concluded that the glue proteins had an effect on a gel if they produced a repeatable change between the aforementioned states in each of at least four trials.

To provide further support that any effects were due to the presence of the glue proteins, tests were performed to see if the effect depended on their concentration and their structure. First, different concentrations of glue proteins were tested on 0.6% agar or 2% polygalacturonic acid. The glue protein concentrations typically ranged from 0.1 to 3 mg $ml^{-1}$, with only a few higher concentrations due to the difficulty of getting sufficient material. The concentration was manipulated by diluting concentrated samples with the bathing solution from the last dialysis step. As described above, the same concentration of BSA was used as a control. Overall, 15 trials were performed with agar, and 21 with polygalacturonic acid.

To test the effect of the structure of the glue proteins, some trials were performed with 1% dithiothreitol (DTT) added to 0.6% agar and 1.3 mg $ml^{-1}$ of glue protein or BSA. Reduction of disulfide bonds changes the structure of the glue proteins, based on samples run on SDS-PAGE (Smith and Morin, 2002). Thus, if the proteins' structure is important for their effect, then DTT should weaken or eliminate that effect. This test was repeated with two different samples.

### *Identification of glue proteins in other species*

Since both limpets and marsh periwinkles have specific proteins associated with adhesion, it is likely that this is a common method of controlling gel mechanics among molluscs. Thus, two other molluscs capable of strong adhesion were studied: the slug *Arion subfuscus* (Draparnaud) and the land snail *Helix aspersa* Müller. The dorsal surface of the slug produces copious amounts of highly sticky, orange mucus when disturbed. This mucus is secreted quickly, often as a liquid, and stiffens within seconds into a sticky, rubbery mass. In contrast, the land snail *H. aspersa* secretes a mucus that dries to a hard film for a long-term adhesion (Barnhart, 1983). Because of the size of this snail and its predictable behavior, it is easier to collect sufficient quantities of sample for quantitative testing.

Adhesive samples were collected from roughly 100 land snails and 30 slugs kept in terraria. Land snails survived well for over a year with periodic feedings of lettuce, chicken feed and crushed oyster shells. The slugs survived for weeks in humid containers with grass and chicken feed. Adhesive mucus was collected from the slugs by rubbing their dorsal surface with a metal spatula. Non-adhesive trail mucus was collected by allowing the slugs to crawl over a glass surface and scraping the glass behind them, being careful to avoid contamination from the adhesive mucus produced by their backs. Despite this, contamination was a continual problem. It was difficult to collect pure trail mucus without inducing the slug to attempt to stick to the glass, or to produce mucus from its back that slid off onto the glass. Such contamination was not a problem with land snails. Dried adhesive mucus was collected from land snails that were firmly attached to the terrarium wall. Snails were detached and the glue was scraped off the glass with a razor blade and carefully removed from the shells with forceps. After this, trail mucus was collected by allowing the snails to crawl in a damp plastic tub for roughly 1 h, draining the excess water, then rubbing the mucus off the surface of the tub with a rubber spatula.

Trail and adhesive samples were then compared by SDS-PAGE. Equal amounts of trail mucus and adhesive mucus were dissolved in sample buffer (0.125 mol $l^{-1}$ Tris-Cl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 1.6 mol $l^{-1}$ urea). Polyacrylamide gel concentrations of 10% and 15% were used. Coomassie Blue-stained gels were photographed using a digital camera and analyzed using Kodak 1D software (Rochester, NY, USA) to identify the molecular mass and net staining intensity of the bands above background. To identify proteins correlated with adhesion, six samples of trail and adhesive mucus from slugs were compared, and four samples of trail and adhesive mucus from land snails.

### *Qualitative effects of* H. aspersa *and* A. subfuscus *glue proteins*

Proteins characteristic of the adhesive mucus of *H. aspersa* and *A. subfuscus* were isolated by gel filtration, dialyzed and concentrated as described previously. The effect of these proteins on the degree of gelation of gel-forming polymers was compared visually to the effect of other fractions from the column. For *H. aspersa*, the effects of the glue proteins and of the MDa-sized polymers from early fractions were tested at 1 mg $ml^{-1}$ on 0.6% agar. These experiments were performed twice using material from different column runs. For *A.*

*subfuscus*, four separate experiments were performed, using material from different column runs. For each column run, fractions were pooled into five groups and each was adjusted to 0.3 mg ml$^{-1}$ and tested on 2% citrus pectin (one set of experiments) or adjusted to roughly 0.1 mg ml$^{-1}$ and tested on 0.6% agar (three sets of experiments).

*Quantitative effects of* H. aspersa *glue proteins*

Quantitative measurements of the effect of glue proteins from *H. aspersa* were made using a dynamic rheometer (Ares, TA Instruments, New Castle, DE, USA). The storage and loss moduli of the gels were determined, thus giving a measure of the elastic and viscous contributions to the gels' mechanics (Denny, 1983). The elastic contribution will often be referred to as the stiffness. To be an effective glue, either a high stiffness, a high viscosity or both are necessary to prevent flow within the adhesive (Wake, 1982). Thus, these are useful measures for characterizing gel mechanics. It is important to note, however, that the overall adhesive strength also depends upon interfacial adhesion and the ability to dissipate energy by deformation (Gay, 2002).

Samples were tested in shear between 25 mm parallel plates at room temperature. They were tested at a strain of 5% and a frequency of 10 rad s$^{-1}$. Based on repeated measurements of materials with a constant modulus, the precision of the rheometer was within 0.5–1 Pa.

Agar (0.5%) and citrus pectin (2%) were tested since they responded well and were easy to manipulate. Powdered polymers were mixed with either glue proteins or with BSA in dialysis solution. The glue proteins were tested at concentrations ranging from 0.1 to 2 mg ml$^{-1}$. Agar samples were loaded on the rheometer while still liquid and analyzed for 10 min. Since the modulus increased for several minutes, then leveled off as the agar gelled, the peak values for each trial were recorded. Pectin samples were fully gelled when loaded and 5–10 measurements were taken per sample. These were consistent and showed no increasing trend, so the average values were recorded. Values for agar were compared using non-parametric statistics, as the variation did not follow a normal distribution.

To determine the importance of charge, other gel-forming polymers were also tested quantitatively. In these experiments, the glue proteins were used at a concentration of 1 mg ml$^{-1}$. The effect of the glue proteins on 2% citrus pectin and 2% apple pectin was compared. Similarly, the effects on 0.6% agar and 0.2% agarose were compared and the effects on 2% carboxymethylcellulose (high viscosity grade) and 2% methylcellulose (high viscosity grade) were compared. In each pair, the gel-forming polymers were structurally similar except that the former was more negatively charged than the latter.

## Results

*Qualitative effects of* L. irrorata *glue proteins*

*L. irrorata* glue proteins triggered gelling or visible stiffening of agar (Fig. 2A), citrus pectin and polygalacturonic acid (Fig. 2B). The effect on citrus pectin and polygalacturonic acid was almost immediate upon mixing, and it did not change noticeably over time. There appeared to be a weak, inconsistent effect on apple pectin and guar gum. Apple pectin was slightly thickened, while guar gum thickened to its final viscosity more rapidly, though it reached roughly the same final consistency as the controls.

The effect of the glue proteins was concentration-dependent. For both agar and polygalacturonic acid, the firmest gels were formed with roughly 1–2 mg ml$^{-1}$ of glue proteins. With polygalacturonic acid, all 13 trials with 1–3 mg ml$^{-1}$ of glue proteins showed a clear effect. At lower concentrations (0.1–0.6 mg ml$^{-1}$), only two of five trials showed a clear effect. At higher concentrations (near 5 mg ml$^{-1}$) there was no clear effect in three trials.

Changing the structure of the glue proteins by breaking their disulfide bonds with DTT correspondingly weakened their effect. Instead of forming a firm gel with a distinct shape, the treated samples formed loose clumps of gel that were only slightly stiffer than the control. In contrast, treatment with DTT had no effect on control gels, which were uniformly loose and barely gelled.

*Identification of glue proteins in other species*

As was true of periwinkles and limpets, there were specific proteins correlated with adhesion in the mucus of the slug *A. subfuscus* (Fig. 3A). There were six major protein bands ranging from 10 to 200 kDa that were found in both the trail and adhesive mucus. Two proteins of roughly 15 kDa and 61 kDa, however, were consistently associated with adhesion. They were, respectively, 1.8 and 2.2 times as concentrated in the adhesive mucus (paired Student's *t*-test, $N$=6, $P$=0.02 and 0.04, respectively). This difference would probably have been greater but for contamination of trail mucus samples with adhesive mucus during collection. In contrast, none of the other proteins differed significantly between the two types of mucus.

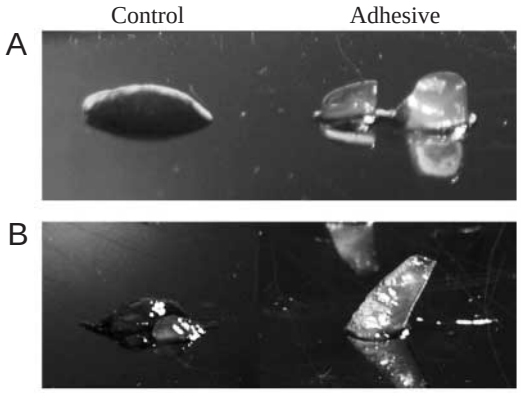


Fig. 2. Examples of the qualitative effect of glue proteins from *L. irrorata* on gel mechanics. Samples were mixed in a microcentrifuge tube and poured or scooped out. Samples with glue proteins (0.5–1 mg ml$^{-1}$) are on the right (Adhesive). Samples with the same concentration of BSA are on the left (Control). (A) 0.6% agar, (B) 2% polygalacturonic acid.

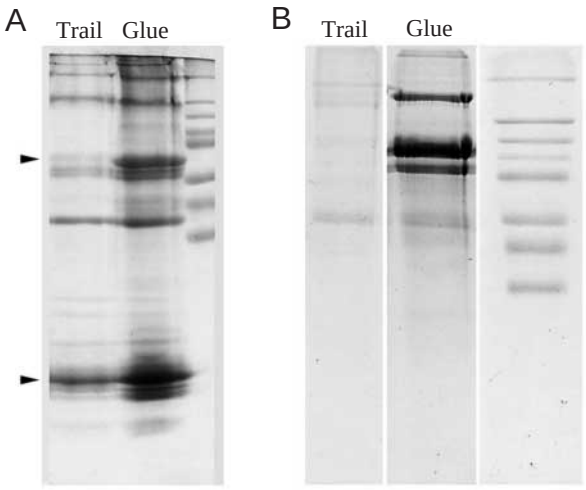


Fig. 3. Identification of glue proteins from slug and land snail glue. (A) SDS-PAGE comparison between equal amounts of trail and adhesive mucus (glue) from the slug *A. subfuscus*. Arrowheads mark the 15 and 61 kDa proteins that are significantly more common in the adhesive mucus. (B) SDS-PAGE comparison between roughly equal amounts of trail and adhesive mucus from the land snail *H. aspersa*. Molecular mass markers are in the right lane, from top to bottom: 205, 116, 97, 84, 66, 55, 45 and 36 kDa.

As was true with periwinkles, the glue proteins made up a substantial fraction of the mucus. In the adhesive mucus, the 15 kDa protein accounted for 47% of the protein on SDS-PAGE, based on staining intensity, while the 61 kDa protein accounted for 8%. Both of these proteins were associated with fainter bands that traveled slightly farther in the gel and whose staining intensity seemed to correspond with the larger proteins.

In addition to the proteins that were small enough to appear on SDS-PAGE, a substantial amount of protein eluted from the gel filtration column with an apparent molecular mass in the MDa range. This fraction was variable in magnitude but roughly equal to half of the organic material in the adhesive mucus. This high molecular mass fraction was primarily proteinaceous with some associated carbohydrates. It should be noted, however, that the carbohydrates seemed to be lost more readily through the procedure and were less readily detected due to the specificity of the staining reaction. Thus, the carbohydrate content may have been underestimated.

The difference between adhesive and trail mucus was even more striking in the land snail *H. aspersa* (Fig. 3B). The trail mucus had few proteins in the size range that could be visualized on SDS-PAGE. In contrast, the adhesive mucus had prominent protein bands at molecular weights of approximately 82, 97 and 175 kDa. As with periwinkles and slugs, the results from the gel filtration column showed that the adhesive also had an MDa-sized component. The amount of this large molecular mass fraction varied considerably, but was often roughly similar to the amount of the glue proteins. It appeared to be primarily proteinaceous, though there were also carbohydrates, as with the slug adhesive. It should be noted that in all three species tested, the MDa-sized polymers constitute roughly half of the organic material of the adhesive mucus. The glue proteins make up the rest in *H. aspersa* and *L. irrorata*, and roughly half of the rest in *A. subfuscus*.

### *Qualitative effects of* H. aspersa *and* A. subfuscus *glue proteins*

As with *L. irrorata* glue proteins, the glue proteins from *A. subfuscus* and *H. aspersa* stiffened gels. The 15 kDa protein from *A. subfuscus* triggered citrus pectin to gel, while other proteins that were equally common to both the trail and adhesive mucus did not appear to have this effect. The purity of the fractions was not sufficient to be certain of this, however. Gel filtration resulted in early fractions enriched in proteins larger than 500 kDa, middle fractions enriched in proteins ranging from 40 to 500 kDa, and later fractions that contained primarily the 15 kDa glue protein. The 15 kDa protein, however, was present to a lesser extent in all earlier fractions as well. In fact, while most of this protein eluted as expected based on its mass, SDS-PAGE analysis of the pooled fractions showed that a significant peak of this protein also eluted in the void volume with the MDa-sized polymers. This suggests that it may bind to the larger polymers, even under the dissociating conditions used here. In any case, the extent to which pooled column fractions with the same overall protein concentration triggered gel formation was roughly proportional to the amount of 15 kDa protein in the fraction. When the overall protein concentration of the pooled fractions was roughly 0.1 mg ml$^{-1}$, only the last fractions containing primarily the 15 kDa protein triggered gelling. In another set of tests with all the fractions concentrated to 0.3 mg ml$^{-1}$, the last fractions again triggered strong gelling. In addition, the fraction containing the MDa-sized polymers, which contained roughly half as much of the 15 kDa protein as the last fractions, also triggered strong gelling. The other fractions, which had less than a third as much of the 15 kDa protein, only caused the formation of softer, looser lumps of gel.

For *H. aspersa*, the primary protein peak contained the 82, 97 and 175 kDa glue proteins and little else. These triggered gel formation in agar, while fractions containing the MDa-sized polymers at the same concentration had no qualitative effect.

### *Quantitative effects of* H. aspersa *glue proteins*

Quantitative measurements confirmed the qualitative results. The land snail glue proteins had a strong effect on gels, and this effect was concentration dependent (Fig. 4). There was a clear linear increase in pectin stiffness as the glue protein concentration increased from 0.1 to 1.3 mg ml$^{-1}$. In this range, the slope was significantly different from zero (linear regression; $P=2\times10^{-5}$, $r^2=0.75$). At higher concentrations, the effect dropped off. Note that at 1.3 mg ml$^{-1}$ the glue proteins constituted 6% of the total organic material.

The results were similar but more variable with agar. At low concentrations of glue proteins ($\leq$0.5 mg ml$^{-1}$), there was no

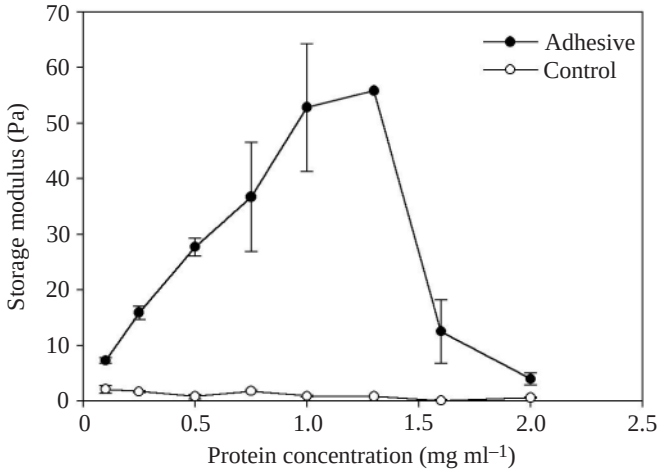


Fig. 4. The effect of different concentrations of *H. aspersa* glue proteins on citrus pectin mechanics. The storage modulus of 2% citrus pectin was measured with a dynamic rheometer. Gels contained different concentrations of glue proteins or BSA as a control. Three comparisons were performed at each concentration between 0.1 and 1 mg ml$^{-1}$, and two comparisons were performed at each concentration above 1 mg ml$^{-1}$ (except at 1.3 mg ml$^{-1}$, which had only one trial). Values are means ± S.E.M. Note that the error bars do not show up on most of the controls because the variability is so small.

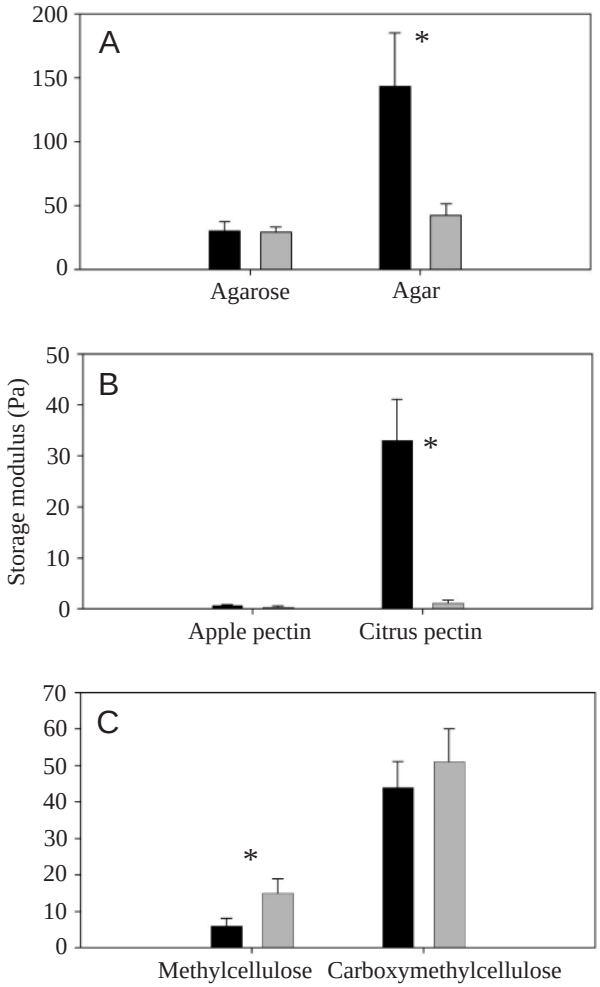


Fig. 5. The effect of *H. aspersa* glue proteins on polymers with different degrees of charge. The storage moduli were measured with a dynamic rheometer. Gels contained 1 mg ml$^{-1}$ glue proteins (dark bars) or 1 mg ml$^{-1}$ BSA as a control (light bars). Agarose (0.2%, *N*=5), apple pectin (2%, *N*=7) and methylcellulose (2%, *N*=6) are neutral or less charged than agar (0.6%, *N*=7), citrus pectin (2%, *N*=7) and carboxymethylcellulose (2%, *N*=7). Values are means ± S.E.M. Asterisks indicate a significant difference (*t*-test or Wilcoxon two-sample test, $P<0.05$).

significant difference from controls (Wilcoxon two-sample test; $P>0.1$, $N=8$). There was a clear effect in some samples, but it was not consistent. At concentrations of 0.75–2 mg ml$^{-1}$, the glue proteins increased the storage modulus of agar by a factor of 4.2 ($P<0.001$, $N=13$). The storage modulus was greatest with glue protein concentrations of 1–1.85 mg ml$^{-1}$ (17–27% of the total organic material) and weakened at 2 mg ml$^{-1}$ where the mean stiffness was only slightly higher than that of the control. The loss modulus (viscosity) followed the same pattern as the storage modulus in this and all other quantitative experiments, though the values were typically much lower and in the range where the rheometer was less accurate.

The glue proteins worked best with negatively charged polymers. While the *H. aspersa* glue proteins clearly stiffened agar, they had no significant effect on agarose (Fig. 5A). The primary difference between agarose and agar is that agarose is neutral, while agar also contains sulfated and acetylated sugars. Similarly, the glue proteins caused a 27-fold increase in the stiffness of citrus pectin, but no change in the stiffness of apple pectin (Fig. 5B). The glue proteins did, however increase the loss modulus of apple pectin from 1.0±0.2 Pa to 1.8±0.1 Pa (*t*-test, $P=0.0005$). The primary structural difference between these two forms of pectin is that apple pectin is more methylated, blocking a larger fraction of its negative charge. Finally, methylcellulose, which is neutral, had lower moduli with the glue proteins, though it went into solution more rapidly. With negatively charged carboxymethylcellulose, the glue proteins had no effect on the measured stiffness (Fig. 5C). In six of the seven comparisons with carboxymethylcellulose, however, the samples with glue proteins appeared lumpier, somewhat stiffer and more gelled than the controls. Thus, the lack of a change in modulus may be due to measurement error resulting from sample inhomogeneity. For example, the presence of clumps could break up the sample and lower the measured modulus. In addition, the glue proteins caused substantial bubble formation during sample mixing and these bubbles were trapped within the high viscosity cellulosic samples. The presence of trapped bubbles would presumably lower the measured moduli. The carboxymethylcellulose did become sticky with the glue proteins, adhering to the spatula used to spread them on the test plates. This was the only material that responded in this way.

In addition to the effect on gel mechanics, the glue proteins from all species created a characteristic increase in the ability

of a solution to wet any surface. Solutions containing the glue proteins were easily recognizable by their ability to wet plastic and acrylic (Plexiglass) surfaces fully, forming a thin film that stayed spread on the surface, while solutions containing other proteins beaded up.

## Discussion

In the three molluscan species studied, the key structural difference between trail mucus and adhesive mucus is the presence of specific proteins. These glue proteins can markedly stiffen different polymer solutions. The effects depend on the concentration of the glue proteins and their structure. The effects are not shared with other proteins or carbohydrates found in the mucus. Finally, the glue proteins work best on charged polymers. These results are consistent with the hypothesis that the glue proteins serve as relatively non-specific cross-linkers of large, ionic polymers. This is what one might expect of an adhesive protein, as Eagland (1990) points out that most polymer adhesives require cross-linking to form effective glues.

### *The mechanism of action of molluscan glue proteins*

The experiments described in this paper effectively rule out several other possible interpretations of the difference in structure between adhesive and trail mucus. It seems unlikely that the proteins serve as enzymes that act on the other polymers in the mucus. If they were, one would not expect them to affect unrelated polymers such as agar and pectin. Furthermore, the changes often occur almost instantly, which would not be expected for enzymes. Given these observations and the fact that the glue proteins make up as much as half of the secretion, it seems likely that they play a direct structural role. It is unlikely, though, that the proteins act independently of the other polymers in the gel. Even at a concentration of 5 mg $ml^{-1}$ (0.5%), a solution of glue proteins has roughly the same viscosity as water. It is only in concert with other gel-forming polymers that an effect is seen. It is still uncertain whether the glue proteins cross-link the trail mucus polymers and thus literally convert the non-adhesive mucus into a glue, or whether they crosslink large polymers in the adhesive mucus that may differ from the trail polymers in an as yet undetermined way. Because the glue proteins are non-specific, they may work with any large polymer in mucus, but some modifications of the large polymer may improve the adhesiveness.

The non-specific action of the glue proteins is intriguing. They appear to be able to cross-link a variety of carbohydrates. They appear to work best with large, negatively charged polymers. This would make sense given that the mechanics of molluscan mucus depend heavily on giant carbohydrate-rich molecules with substantial negative charge due to the presence of sulfated sugars or uronic acids (Denny, 1983). These findings suggest that the glue proteins have charged regions that mediate ionic cross-links. Consistent with this, Smith et al. (1999) and Smith and Morin (2002) found that the glue proteins of limpets and periwinkles have a large number of charged amino acids. Overall, the proteins are acidic, but they may have regions with substantial positive charge, since they contain 15–17% basic amino acid residues. The fact that the glue proteins affected both agar and polygalacturonic acid was interesting. Both of these are capable of gelling, but while polygalacturonic acid normally forms electrovalent cross-links, agar depends on association between helical regions (Williams and Phillips, 2000). Perhaps the glue proteins provide additional crosslinks to stabilize the ones that typically form. It is worth noting that the effect on agar was not as large as the effect on more highly charged citrus pectin or polygalacturonic acid.

Several other characteristics of the glue proteins may give insight into their nature. The fact that they increased the ability of solutions to wet surfaces makes sense since an adhesive must be able to bond to an interface in addition to stiffening the bulk adhesive. Since the proteins also triggered substantial foaming, one or more of them may have some surfactant properties. This may also explain why they caused the neutral polysaccharides to go into solution more rapidly. A structure involving separate non-polar and charged regions would be intriguing. Several other researchers have had success developing gels from synthetic block copolymers with this type of structure (Petka et al., 1998; Nowak et al., 2002).

Another aspect to consider is the possibility that different glue proteins within a secretion play different roles. In the species tested, there was typically one glue protein that was present in large quantities, along with one or two others. It is possible that the others are minor variants of the major glue protein. There is some evidence from amino acid composition and isoelectric points that the two glue proteins in periwinkles are related, and some evidence that the two glue proteins in limpets may be related as well (Smith et al., 1999; Smith and Morin, 2002). In some cases, the glue protein forms a dark band in SDS-PAGE with one or two fainter bands below it. These may be different size variants, possibly differing in degree of glycosylation. As yet, though, there is no indication of whether or not the different proteins have different roles. In this study, no attempt was made to separate the different glue proteins from each other. Hence, for example, we know that together the 41 and 36 kDa proteins from periwinkle glue affect gels, but whether each can work on its own is unknown.

While the effect of the glue proteins depended on their concentration, the effect weakened at concentrations above approximately 1.5 mg $ml^{-1}$. There are several possible explanations for this. As noted in the Materials and methods, it is difficult to achieve much higher concentrations in the initial extract. This may be partly due to a tendency to aggregate, as demonstrated for limpet glue proteins (Smith et al., 1999). Thus, while it may be possible to concentrate the proteins beyond 1.5 mg $ml^{-1}$ after chromatography, they may begin to interact with each other and cease functioning normally. The weakened effect at higher concentrations may also have been an artifact of the experimental system; there may have been problems because of inhomogeneous mixing and the use of commercial gel-forming polymers rather than the native ones.

It should be noted that this type of concentration dependence is not unusual for an adhesive. In commercial adhesives consisting of two components, there is often an optimum ratio of the components. Given this, it is worth noting the ratio of glue proteins to large polymers in the adhesive mucus of the molluscs that have been studied. The glue proteins from the land snail, slug and periwinkle constitute roughly 25–50% by mass of the total organic material. In limpets, the glue proteins make up a somewhat smaller percentage of the whole. With the gel-forming polymers in this study, the glue proteins worked best when they constituted roughly 6–27% of the total material. This suggests that the glue proteins should make up a substantial fraction of the material, but not more than half.

Finally, the use of commercial polymers may lead to an underestimate of the effectiveness of the glue proteins. There are many characteristics of gel-forming polymers that could impact their response to a potential cross-linker. For example, they may have structural features or charge distributions that are not ideally suited for interactions with the glue proteins. The glue proteins may also directly or indirectly change the structure of the gel-forming polymers. For instance, they may cause the polymers to aggregate or to take on a less extended conformation. Thus, they could actually weaken the gel. Given this, it will be important to characterize more precisely the features of the mucus gel-forming polymers that are important for adhesion.

### *Different animals that use glue proteins to modify gel mechanics*

The identification of gel-stiffening glue proteins in *A. subfuscus* and *H. aspersa* broadens the potential scope of this adhesive mechanism. To date, four molluscan species have been tested, all from very different environments. *L. irrorata* lives in intertidal salt marshes attached to blades of marsh grass. Limpets live in the rocky wave-swept intertidal and are subject to strong wave forces. Both alternate between glued and active states with a periodicity of several hours (Smith et al., 1999; Smith and Morin, 2002). In contrast, the slug *A. subfuscus* lives in temperate forests and gardens and rapidly secretes a sticky, orange mucus when disturbed. This suggests that it is a defense mechanism. Finally, the land snail *H. aspersa* also lives in temperate forests, but secretes a sheet of mucus around its aperture to form a dried attachment that can last for months. This sheet is called an epiphragm, and in addition to adhesion, it contributes to desiccation resistance (Campion, 1961; Barnhart, 1983). Though the adhesive mucus is used in different environments for different purposes, in all four cases there were similar changes in mucus structure between non-adhesive mucus and adhesive mucus. In the three species tested, the glue proteins had a similar effect on gel mechanics. Thus, it is likely that this is a commonly used mechanism for controlling gel mechanics among invertebrates.

It is intriguing that there were differences in the size of the glue proteins and the carbohydrate content of the MDa-sized polymers of the adhesive. Such differences may play a role in the mechanics. For example, a 15 kDa protein may spread through the gel more rapidly than a 100 kDa protein, causing the gel to set sooner. Given the differences in environment and functional needs, it will be interesting to compare the effectiveness of each glue protein quantitatively. It will also be interesting to look for similar proteins in other animals. Many marine invertebrates such as molluscs, interstitial worms and echinoderms secrete adhesive gels (Smith, 2002). Echinoderms are particularly interesting because of the strength of their adhesion and their ability to stay attached for extended periods or let go rapidly. Echinoderms are also interesting since they have been shown to stiffen their dermis with a system that may be analogous; holothurians appear to cross-link large collagen fibers with a proteinaceous ‘stiffening factor’ (Trotter and Koob, 1995; Koob et al., 1999; Trotter et al., 2000).

### *Summary and future work*

This research has clear practical implications. The adhesive gels produced by molluscs form strong attachments in wet, irregular environments, using a minimum of organic material. Adhesives used by marine animals are likely to have unusual and useful characteristics because of the demands of adhesion underwater (Waite, 2002). Furthermore, there is substantial interest in the development of gels with unusual properties, particularly for applications such as drug delivery and biomedical adhesives (Petka et al., 1998; Miyata et al., 1999; Wang et al., 1999; Peppas et al., 2000; Nowak et al., 2002), as well as food science (Williams and Phillips, 2000). Despite this potential, there has been little research on the gels produced by animals. The results described in this paper provide the first direct evidence of a mechanism by which a dilute gel can become adhesive. Until recently, almost no work has been done linking changes in biochemical structure to function in these kind of gels (Davies and Hawkins, 1998). Now, specific differences in composition have been shown to control gel mechanics. Further work needs to be done to determine the structure of these glue proteins and to elucidate how they act on the gel-forming polymers. Also, in addition to changes in stiffness, other characteristics of the adhesive mucus should be investigated, such as the ability to bond effectively to the substrate and the ability to dissipate fracture energy. Finally, other animals should be investigated, as the addition of similar proteins to control gel mechanics may be a widespread phenomenon.

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