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RESEARCH ARTICLE

Spontaneous unraveling of hagfish slime thread skeins is mediated by a seawater-soluble protein adhesive

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ABSTRACT

Hagfishes are known for their ability to rapidly produce vast quantities of slime when provoked. The slime is formed via the interaction between seawater and two components released by the slime glands: mucin vesicles from gland mucous cells, which swell and rupture in seawater to form a network of mucus strands, and intermediate filament-rich threads, which are produced within gland thread cells as tightly coiled bundles called skeins. A previous study showed that the unraveling of skeins from Atlantic hagfish (*Myxine glutinosa*) requires both the presence of mucins and hydrodynamic mixing. In contrast, skeins from Pacific hagfish (*Eptatretus stoutii*) unravel in the absence of both mucins and mixing. We tested the hypothesis that spontaneous unraveling of *E. stoutii* skeins is triggered by the dissolution of a seawater-soluble protein adhesive and the release of stored strain energy within the coiled thread. Here we show that, as predicted by this hypothesis, unraveling can be initiated by a protease under conditions in which unraveling does not normally occur. We also demonstrate, using high resolution scanning electron microscopy, that the treatment of skeins with solutions that cause unraveling also leads to the disappearance of surface and inter-thread features that remain when skeins are washed with stabilizing solutions. Our study provides a mechanism for the deployment of thread skeins in Pacific hagfish slime, and raises the possibility of producing novel biomimetic protein adhesives that are salt, temperature and kosmotrope sensitive.

KEY WORDS: Hagfish, *Eptatretus stoutii*, Slime, Mucus, Skein, Unraveling, Adhesive

INTRODUCTION

Hagfishes are bottom-dwelling craniates known for their ability to produce copious amounts of slime very rapidly when they are provoked (Ferry, 1941; Newby, 1946). The slime is used as a defense mechanism against gill-breathing predators (Lim et al., 2006), and may be used for other functions as well (Fernholm, 1981; Zintzen et al., 2011). Its components are produced in and released from glands situated ventrolaterally along the length of the animal on both sides (Newby, 1946). The glands primarily contain two different types of cells: gland mucous cells (GMCs) and gland thread cells (GTCs) (Downing et al., 1981; Newby, 1946; Koch et al., 1991). Each GMC produces hundreds of tiny (3–7 µm in diameter) mucin vesicles containing condensed mucous (Luchtel et al., 1991), while each GTC produces a single, ~15 cm-long intermediate filament-rich thread that is tightly coiled and packed in a precisely organized manner into a 120–150 µm by 50–60 µm ovoid bundle called a skein (Newby, 1946; Fernholm, 1981; Downing et al., 1981; Fudge et al., 2003). When the muscle layer surrounding a gland contracts, mature GMCs and GTCs are forced through the narrow gland pore, which shears off their plasma membranes and releases their contents into the environment (Fernholm, 1981). Each gland only releases a fraction of a milliliter of slime exudate at a time.

Once in the surrounding seawater, the mucin vesicles rupture and the skeins unravel (Koch et al., 1991; Fudge et al., 2005). The two components then interact, in ways that are not yet fully understood, to create a vast network of threads and mucous that entrains large volumes of bulk seawater (Fudge et al., 2005). This unique process allows the tiny volume of exudate released from the glands to transform into about a liter of slime in a fraction of a second. Although the mechanisms underlying mucin vesicle rupture have been investigated to some extent (Luchtel et al., 1991; Herr et al., 2010), little is known about the mechanisms involved in skein unraveling. Winegard and Fudge (Winegard and Fudge, 2010) demonstrated that the presence of mucin vesicles as well as mixing forces are required for skein unraveling in the slime of the Atlantic hagfish (*Myxine glutinosa*). Pacific hagfish [*Eptatretus stoutii* (Lockington 1878)] skeins, however, will undergo spontaneous unraveling upon exposure to seawater (Koch et al., 1991), even in the absence of mucin vesicles and turbulence. In this study, we investigated the mechanisms underlying spontaneous skein unraveling in *E. stoutii*.

Preliminary observations of spontaneous skein unraveling led us to explore two possible hypotheses. One hypothesis is that unraveling is driven by swelling of the slime thread itself in seawater, which forces the curved loops of thread to separate and straighten out. This hypothesis predicts that thread dimensions should increase in unraveling solutions compared with stabilizing solutions. The second hypothesis is that strain energy is added to the thread as it is coiled within the developing GTC, and this energy is released when an adhesive that holds the loops of thread together is dissolved away in seawater. This hypothesis predicts that skeins should unravel under stabilizing conditions if the adhesive is disrupted, and that there should be visual evidence of adhesive on or within intact skeins, and less or none on unraveled skeins. Here, we present results from several experiments that strongly support the adhesive/stored strain energy hypothesis and also provide information on the effects of temperature and ionic strength on spontaneous unraveling.

RESULTS

Spontaneous unraveling

We confirmed the finding by Koch et al. (Koch et al., 1991) that *E. stoutii* skeins unravel spontaneously upon exposure to seawater and solutions similar to seawater (e.g. 1 mol l^{-1} NaCl) (Fig. 1; supplementary material Movie 1).

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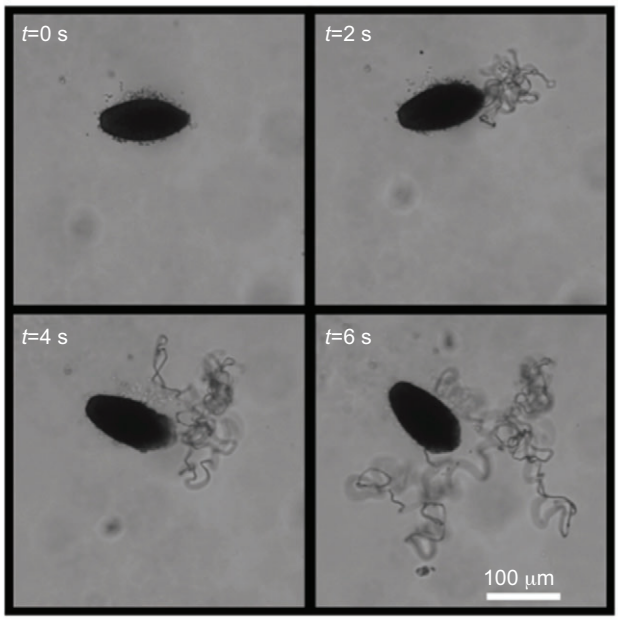

Fig. 1. Time-lapse images of the initial interaction between a slime thread skein from *Eptatretus stoutii* and artificial seawater (ASW). Loops of thread spring from the bundle following ASW exposure. Note how unraveling proceeds from the apical tip of the skein. See supplementary material Movie 1 for a video of spontaneous unraveling.

Temperature and salt concentration

Temperature had a significant effect ($F_{3,132}$=86.09, P<0.0001) on unraveling, with the highest unraveling efficiencies occurring at 5 and 15°C (Fig. 2). NaCl concentration also had a strong effect ($F_{10,132}$=74.03, P<0.0001) on unraveling, with the optimal concentration occurring between 1 and 2 mol l^{-1} and dropping off to zero, or close to zero, at higher and lower concentrations (Fig. 2). Two-way ANOVA analysis revealed a significant interactive effect ($F_{30,132}$=9.49, P<0.0001) between temperature and [NaCl] on unraveling efficiency, suggesting that the effects of salt and temperature are not simply additive. More specifically, higher temperatures tended to decrease the [NaCl] at which optimal unraveling occurred.

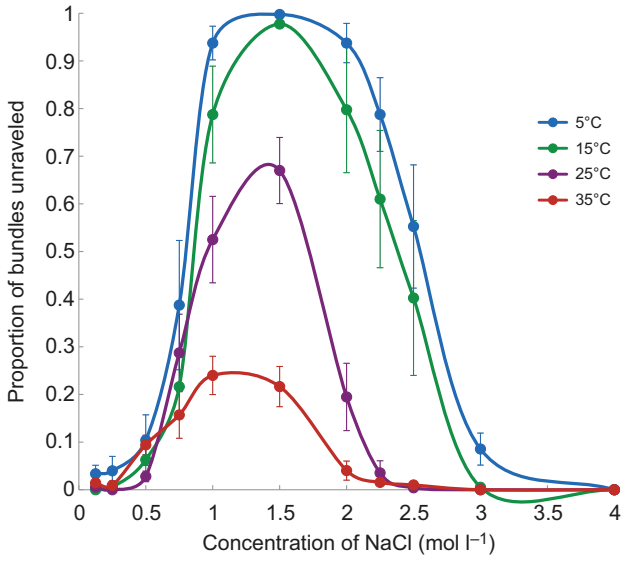

Fig. 2. Proportion of slime thread bundles unraveled after 30 s in various NaCl solutions at various temperatures. Temperature (P<0.0001) and NaCl concentration (P<0.0001) both had significant effects on unraveling, as did the interaction between these two variables (P<0.0001). Error bars are ±s.e.m.

Disruption of the adhesive via trypsin

As predicted by the adhesive/stored strain energy hypothesis, the protease trypsin was able to trigger unraveling under conditions in which unraveling does not normally occur (0.25 mol l^{-1} NaCl, 35°C) ($F_{6,21}$=26.46, P<0.0001) (Fig. 3). Unraveling occurred more slowly in 0.25 mol l^{-1} NaCl + 0.5 mg ml^{-1} trypsin at 35°C than in unraveling conditions such as 1 mol l^{-1} NaCl at 5°C. However, unraveling proceeded in the same manner – beginning at the apical tip, with thread loops springing apart from each other and the rest of the skein (Fig. 3; supplementary material Movie 2).

Visualization of the adhesive

High-power scanning electron microscopy (SEM) images (Figs 4, 5) revealed dramatic differences in the appearance of slime threads that were fixed in solutions that promote unraveling [i.e. artificial seawater (ASW)] compared with those that inhibit it (i.e. stabilization buffer, SB). ASW-exposed threads had significantly less material covering their surface than skeins exposed to SB (t_{38}=14.79, P<0.0001). ASW-exposed threads also had significantly fewer connections between adjacent threads compared with threads fixed in stabilizing conditions (t_{38}=11.23, P<0.0001) (Fig. 6).

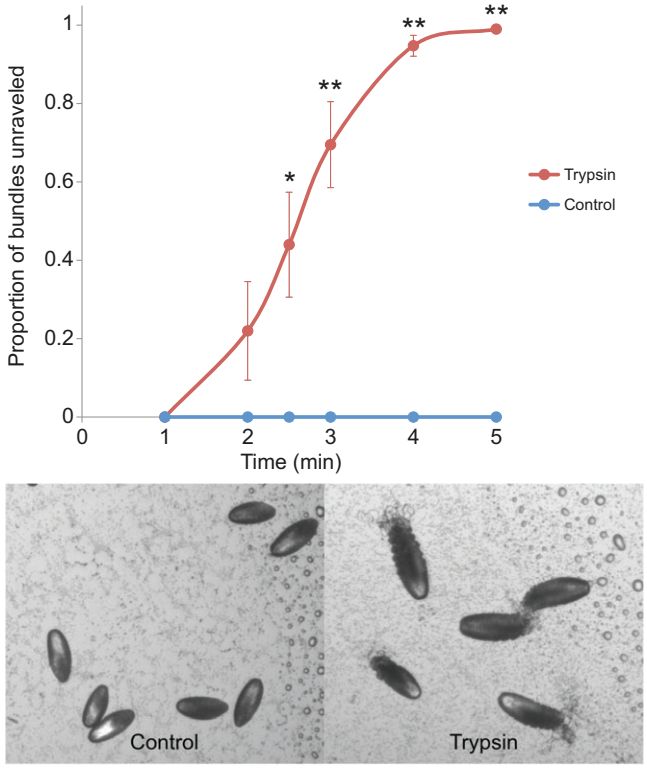

Fig. 3. The effect of protease on skein unraveling. Skeins were observed in 0.25 mol l^{-1} NaCl with and without a protease (trypsin) at 35°C over a period of 5 min. Asterisks denote a statistically significant difference between trypsin and control groups (*P<0.01 and **P<0.0001). Error bars are ±s.e.m. Note how unraveling proceeds from the apical tip in the trypsin treatment, much like spontaneous unraveling in 1 mol l^{-1} NaCl (Fig. 1), but does not occur at all in the control. See supplementary material Movies 2 and 3 for videos of trypsin-aided unraveling and stabilized skeins.

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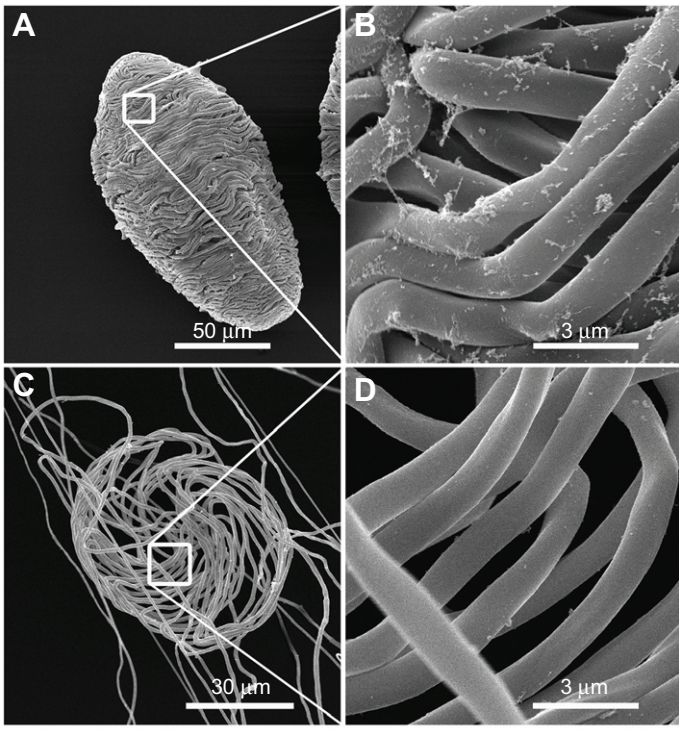

Fig. 4. Scannning electron microscopy (SEM) of *E. stoutii* slime thread skeins. Skeins were exposed to stabilization buffer (SB) (A,B) and artificial seawater (ASW) (C,D). Samples were washed with SB prior to treatment, and fixed in 3% paraformaldehyde in PBS, dehydrated in ethanol, critical point dried and Au/Pd sputter coated prior to imaging.

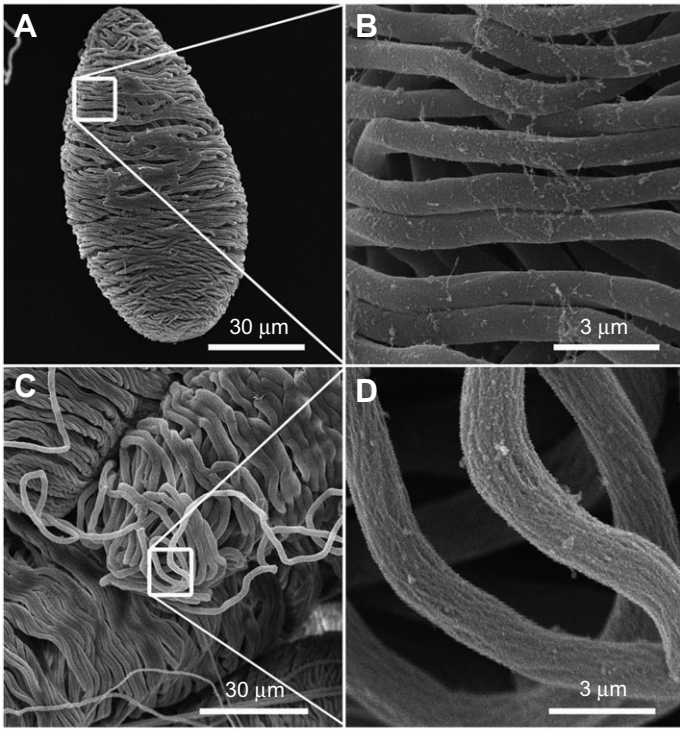

Fig. 5. SEM of *E. stoutii* slime thread skeins exposed to 0.25 mol l^{-1} NaCl (A,B) and 0.25 mol l^{-1} NaCl + 0.5 mg ml^{-1} trypsin (C,D). Experiments were carried out at 35°C. All samples were washed with SB prior to treatment, and fixed in 3% paraformaldehyde in PBS, dehydrated in ethanol, critical point dried and Au/Pd sputter coated prior to imaging. Note the etched appearance of thread exposed to trypsin compared with the smoother surface of control threads.

Trypsin also had a significant effect on the amount of surface material (t_{38}=2.04, P=0.024), as well as the number of connections between adjacent threads (t_{38}=6.91, P<0.0001) (Fig. 7). However, the difference in surface material between the trypsin and control treatments was less significant than the difference between ASW- and SB-treated skeins, and there was significantly more surface material on the trypsin-exposed threads compared with the ASW-treated threads (t_{38}=15.61, P<0.0001). Trypsin also appeared to digest the proteins making up the threads themselves, causing etching of the surface, breaks in the thread, and even completely digesting the skeins at longer exposure times.

DISCUSSION

Our results are consistent with the hypothesis that spontaneous unraveling in *E. stoutii* skeins is governed by the dissolution of a protein adhesive and the release of stored strain energy in seawater. We found that the optimal unraveling conditions (1–2 mol l^{-1} NaCl, 5–15°C) for *E. stoutii* skeins correspond closely with the natural environmental conditions for this species (and most hagfishes, in fact), suggesting that the dissolution properties of the adhesive are adaptive. High temperatures inhibit skein unraveling, perhaps because the adhesive proteins denature and aggregate at high temperatures, making them less soluble, similar to the way in which egg white proteins can be rendered insoluble by cooking. The strong sensitivity of the unraveling response to [NaCl] is interesting, considering the fact that hagfishes are osmoconformers and most of their tissues contain levels of sodium and chloride ions similar to those in seawater (Munz and McFarland, 1964; Currie and Edwards, 2010). However, previous research demonstrated that slime gland exudate contains relatively low concentrations of sodium and chloride ions, and high levels of methylamines and other organic osmolytes (Herr et al., 2010). This finding was initially interpreted in light of the stabilization of mucin vesicles within the slime gland, but no evidence was found to support this hypothesis (i.e. many vesicles ruptured even in high concentrations of methylamines) (Herr et al., 2010). The salt sensitivity of skein unraveling in *E. stoutii* provides another explanation for the low salt content of fluids in the slime gland, which is that it stabilizes the protein adhesive that keeps coiled skeins in the condensed state within the gland. Furthermore, the high concentration of zwitterionic methylamines such as betaine, trimethylamine oxide (TMAO) and dimethyl glycine (DMG), which are known to have kosmotropic (i.e. stabilizing) effects on proteins, may also contribute to the prevention of premature skein unraveling in the gland.

The fact that trypsin can initiate unraveling under conditions that are normally stabilizing provides compelling evidence that a protein glue is responsible for holding the skeins together, and furthermore that its dissolution triggers unraveling via the release of stored strain energy. Trypsin-aided unraveling is very slow compared with unraveling in ASW or other suitable unraveling conditions; however, the pattern of unraveling is identical, suggesting that normal unraveling also proceeds via the disruption of a protein glue. The slower rate of unraveling in the trypsin solution likely has two origins. One is that proteolytic digestion of the glue via trypsin is almost certainly slower than the dissolution rate in ASW. Furthermore, trypsin appears to digest both adhesive proteins and thread proteins, and proteolysis of the latter is likely to dissipate the stored strain energy that drives unraveling. These results also demonstrate that unraveling does not require thread

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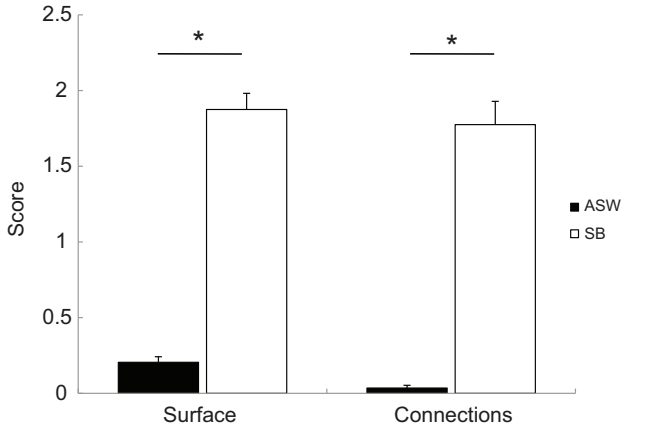

Fig. 6. A comparison of the amount of material on and between threads of stabilized (SB) and unraveled (ASW) skeins. Scores were assigned using a scale of 0–3, based on the amount of surface material and number of inter-thread connections. See Materials and methods for a detailed explanation of scores. ASW treatment significantly decreased (*P<0.0001) both the amount of surface material on the threads and the connections between them. Error bars are s.e.m.

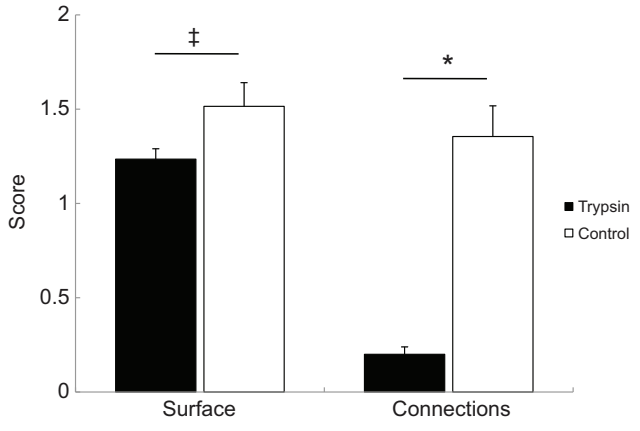

Fig. 7. The effect of trypsin on the amount of material on and between slime threads. Stabilized 'control' skeins were stabilized in 0.25 mol l^{-1} NaCl; and unraveled 'trypsin' skeins were treated with 0.25 mol l^{-1} NaCl + trypsin. Scores were assigned using a scale of 0–3, based on the amount of surface material and number of inter-thread connections. See Materials and methods for a detailed explanation of scores. Trypsin treatment significantly decreased (*P<0.0001) the number of inter-thread connections, but had a less significant effect on the amount of surface material on the threads (‡P=0.024). Error bars are s.e.m.

swelling, because trypsin alone was sufficient to trigger unraveling, and it should have no swelling effects on the threads. Moreover, we were unable to detect any changes in thread dimensions in stabilizing versus unraveling solutions (data not shown).

Using SEM, Fernholm (Fernholm, 1981) observed fibrous structures bridging adjacent threads of intact skeins, but did not speculate on their significance. Our SEM analysis revealed that the same structures observed by Fernholm are present in stabilizing solutions, but not in unraveling treatments. These results suggest that these structures are load-bearing portions of the seawater-soluble adhesive that we hypothesized is involved in skein stabilization and unraveling. The ASW-treated skeins were generally very clean, with little or no surface aggregation and few or no inter-thread connections, which suggests that the adhesive is completely dissolved by seawater. While there were few to no inter-thread connections observed in the trypsin-treated skeins, there was still significantly more surface material on them compared with ASW-treated skeins. This suggests that, while the trypsin may fully digest the adhesive structures in some cases, it likely digests the inter-thread connections faster than the surface aggregations, perhaps because of their higher surface area to volume ratio. Another possibility is that the inter-thread connections are under tension (which they in fact must be if they are what prevents release of stored strain energy in stabilized skeins) and therefore only a small amount of digestion is enough for these connections to fail catastrophically and disappear from view.

Our results raise interesting questions about how exactly the thread is coiled in the developing GTC and the nature of the stored strain energy that is released during unraveling. What kinds of forces are required to bend and coil the thread in the skein? Is it possible that thread bending can be achieved by thermal agitation of a flexible slime thread? Answering this last question requires knowing something about the persistence length (L_p) of the thread, which is proportional to the flexural stiffness and gives an indication of the length scale over which different parts of a polymer remain correlated in space as it gyrates in response to thermal collisions. L_p can be calculated at a given temperature from the Young's modulus of the thread and its second moment of area using the equation $L_p=EI/kT$, where E is Young's modulus, I is the second moment of area and kT is a measure of the thermal energy available. A previous study reports a Young's modulus for slime threads of about 5 MPa (Fudge et al., 2003), and assuming a circular cross-section for a thread of diameter 2 μm, I can be calculated as $\pi r^4/4$ or 7.85×10^{-25} m^4. This gives a L_p of about a kilometer, which means that it is not possible that thermal energy provides the driving force for thread bending in the developing thread cell and suggests that the energy of bending must be supplied by another source, although at this time it is unclear what that source is.

Although entropic elasticity does not appear to be relevant to the bending of the entire thread, it is likely to be important for the rubber-like behavior of the intermediate filament proteins that make up the thread. Fudge et al. (Fudge et al., 2003) demonstrated that slime threads in water can stretch reversibly up to strains as high as 35% and return to their original dimensions in a rubber-like tensile behavior. The utility of this rubber-like elasticity has not been clear up to this point, but the current study suggests that the proposed entropic elasticity of the thread may be important for the storage of strain energy as the thread is coiled and the release of that energy when skeins encounter seawater and the thread relaxes to a straighter conformation.

How can we be sure that the inter-thread structures we have identified are adhesive protein and not just remnants of ruptured mucin vesicles that have coated the skein? There are several reasons why we believe these structures are not ruptured mucin vesicles. The first is that SB is very effective at stabilizing vesicles, which allows us to separate them from the skeins with great efficiency. Second, ruptured mucin vesicles were rare, and in those cases where they were present, the material was much thicker and more coherent than the fine strands we observed between the threads in stabilized skeins. Third, if the surface features are ruptured mucin vesicles, then we should see a lot more surface material on skeins exposed to solutions like ASW, which ruptures the vesicles, and in fact we see far less on unraveled skeins. Further confirmation will involve isolation and biochemical characterization of both the mucins and the adhesive proteins.

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Eptatretus versus *Myxine* skeins

Our data raise the question of why and how the unraveling process differs between the skeins of *M. glutinosa* and *E. stoutii*. One possibility is that the difference is related to the contrasting habitats and lifestyles of these two species. *Myxine glutinosa* spends most of its time in burrows that it forms within soft sediment, whereas *E. stoutii* lives on rockier bottoms and generally finds refuge in crevices. *Eptatretus stoutii* may therefore be more exposed and more vulnerable to predators than *M. glutinosa*. If this is true, then perhaps selection has favored a sliming defense in *E. stoutii* that can deploy faster and this has come about via spontaneous skein unraveling. If this scenario is correct, then it raises the question of why *M. glutinosa* has not also evolved a faster sliming response, as this would seem to have obvious benefits for both species. Perhaps there are drawbacks to having a slime defense that deploys too rapidly, especially for animals that spend most of their time in burrows. These observations raise the issue of whether spontaneous unraveling of *E. stoutii* skeins does in fact increase the speed of slime deployment.

There are two plausible mechanisms that could account for the differences in skein deployment between the *E. stoutii* and *M. glutinosa*. One is that the protein adhesive in *M. glutinosa* is less soluble in seawater than the adhesive in *E. stoutii* skeins, and another is that *E. stoutii* skeins contain more stored strain energy. The latter possibility seems unlikely, however, given that the strain energy that is released during unraveling is most likely added as the thread is coiled within the developing GTC, and the coiling patterns between the two species are remarkably similar.

Future directions

Future work will focus on identifying and characterizing the adhesive protein(s) involved in skein stabilization and deployment. The study of these proteins could inform the development of new bio-inspired adhesives that are stable at low ionic strengths, but soluble in seawater. We will also look for the energy source that is involved in coiling the thread in developing thread cells. We have examined skein deployment in two species of hagfishes and have found substantial differences between them. We strongly suspect that further interesting variability exists among the sliming defenses of the approximately 80 species of hagfishes that we have not yet examined.

MATERIALS AND METHODS

Experimental animals

Pacific hagfish were collected from Bamfield Marine Station in Bamfield, BC, Canada, and housed in a 2000 l tank filled with chilled artificial seawater (ASW; 34‰, 10°C) at the Hagen Aqualab at the University of Guelph, Guelph, ON, Canada. The hagfish were fed squid monthly to satiety as per the University of Guelph Animal Care Protocol 09R128.

Slime collection

Hagfish were placed in 3 l of ASW with 3 ml of an anesthetic solution (1:9 clove oil to 95% ethanol) and left until they ceased to respond to touch. Once fully anesthetized, the hagfish were removed from the ASW and placed on a chilled dissection tray with the slime glands on one side of the animal facing up. The skin surrounding the glands was washed with ddH_2O, blotted dry, and the glands stimulated to release slime (60 Hz, 18 V) using a Grass SD9 Stimulator (Grass Instruments, Quincy, MA, USA) with a custom-made stimulation wand. Several adjacent glands were stimulated, each releasing a small pool of exudate, which were then collected using a small spatula and placed either into mineral oil for whole-slime observations or 0.9 mol l^{-1} sodium citrate, 0.1 mol l^{-1} Pipes [piperazine-*N*,*N*′-bis(ethanesulfonic acid)] slime SB (pH 7.55) for observation of isolated skeins (mucin vesicles removed). Slime collected in mineral oil could be used throughout the day that it was collected, but slime collected in SB had to be used for experimentation immediately, as skeins cease to unravel if exposed to SB for too long.

Unraveling assay

The effects of temperature, [NaCl] and trypsin were assessed using a custom-designed unraveling assay. A 25 µl drop of test solution was placed on a coverslip on a temperature-controlled microscope stage (Nikon Eclipse Ti Inverted Microscope System, Nikon Instruments Inc., Melville, NY, USA) and allowed to acclimate to the stage's temperature. A 1 µl drop of slime exudate was then placed on the coverslip and the acclimated drop of test solution was picked up and pipetted across the drop of slime. After 30 s, an image was captured and analyzed to determine the proportion of bundles that had begun to unravel. Temperature and NaCl effects were measured simultaneously using four slime samples from each of four different hagfish that were exposed to every combination of 11 NaCl concentrations (0.125, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.25, 2.5, 3 and 4 mol l^{-1}) and four temperatures (5, 15, 25 and 35°C). Trypsin effects were measured by comparing skein behavior in a treatment shown to completely inhibit unraveling (0.25 mol l^{-1} NaCl, 35°C) with behavior under the same conditions plus 0.5 mg ml^{-1} trypsin.

SEM and image analysis

Slime was collected in SB to isolate the skeins from the vesicles. Samples underwent three SB-washing cycles before being exposed to one of four treatments (ASW, SB, 0.25 mol l^{-1} NaCl + 0.5 mg ml^{-1} trypsin at 35°C, or 0.25 mol l^{-1} NaCl at 35°C). The samples were then fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) and dehydrated in an ethanol series. Dehydrated samples were critical point dried, and then sputter coated using an Emitech K550 Sputter Coater (Emitech Ltd, Ashford, Kent, UK) to produce an ~12 nm Au/Pd coating (20 mA for 2 min). The prepared skein samples were then observed using a Hitachi S-570 scanning electron microscope (Hitachi High Technologies, Tokyo, Japan) and images were captured using Quartz PCI Image Management System v. 8.5. Image analysis was carried out by five volunteers who were blinded to the identity of the treatments for each image. Volunteers were asked to assign two scores (a 'surface' score and a 'connections' score) to each image on a scale of 0–3. The surface scores were based on the amount of material on the surface of the threads: a score of 0 was assigned if the threads were clean (i.e. very little or no material) and a score of 3 was assigned if a large proportion of the thread surface was covered with material. The connections scores were based on the amount of non-thread material bridging between adjacent threads: a score of 0 was assigned if there were no connections, and a score of 3 was assigned if all adjacent thread segments were linked by one or more connections. The number of adjacent threads was also taken into account when assigning connections scores, so they are a reflection of the number of inter-thread connections relative to the number of adjacent threads, rather than simply the total number of connections.

Statistical analysis

Main effects of temperature and [NaCl] on unraveling efficiency as well as interactive effects were calculated using two-way ANOVA analysis. Effects of trypsin on unraveling efficiency were calculated using ANOVA analysis. Comparisons of surface and connections scores for ASW- and SB-treated skeins as well as trypsin versus control treatments were carried out using two-tailed *t*-tests. All statistical tests were executed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.A.B. designed and conducted experiments and wrote the manuscript. I.O. designed and conducted experiments. A.H. designed and conducted experiments. D.S.F. designed experiments and wrote the manuscript.

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Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.096909/-/DC1

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