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Stabilization and swelling of hagfish slime mucin vesicles

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SUMMARY

When agitated, Atlantic hagfish (*Myxine glutinosa*) produce large quantities of slime that consists of hydrated bundles of protein filaments and membrane-bound mucin vesicles from numerous slime glands. When the slime exudate contacts seawater, the thread bundles unravel and the mucin vesicles swell and rupture. Little is known about the mechanisms of vesicle rupture in seawater and stabilization within the gland, although it is believed that the vesicle membrane is permeable to most ions except polyvalent anions. We hypothesized that the most abundant compounds within the slime gland exudate have a stabilizing effect on the mucin vesicles. To test this hypothesis, we measured the chemical composition of the fluid component of hagfish slime exudate and conducted functional assays with these solutes to test their ability to keep the vesicles in a condensed state. We found K^+ concentrations that were elevated relative to plasma, and Na^+, Cl^- and Ca^{2+} concentrations that were considerably lower. Our analysis also revealed high levels of methylamines such as trimethylamine oxide (TMAO), betaine and dimethylglycine, which had a combined concentration of 388 mmol l^{-1} in the glandular fluid. *In vitro* rupture assays demonstrated that both TMAO and betaine had a significant effect on rupture, but neither was capable of completely abolishing mucin swelling and rupture, even at high concentrations. This suggests that some other mechanism such as the chemical microenvironment within gland mucous cells, or hydrostatic pressure is responsible for stabilization of the vesicles within the gland.

Key words: *Myxine glutinosa*, osmoregulation, ionoregulation, mucus, trimethylamine oxide (TMAO), betaine.

INTRODUCTION

Hagfishes are known for producing large volumes of slime when stressed (Ferry, 1941; Koch et al., 1991). Their slime is believed to act as a defence mechanism against gill-breathing predators, as it has been shown to reduce water flow over the gills of fish (Lim et al., 2006). Hagfish slime is composed of two interacting components, slime thread skeins and mucin vesicles (Fig. 1), which are both released from glands along the ventrolateral length of the animal (Blackstad, 1963). Each slime gland is surrounded by striated muscle and a connective tissue capsule, and contains large numbers of gland thread cells and gland mucous cells (Blackstad, 1963; Spitzer and Koch, 1998). Gland thread cells contain skeins of tightly coiled polymers rich in intermediate filaments, while gland mucous cells produce vesicles containing mucins, a class of glycoproteins (Blackstad, 1963; Koch et al., 1991). Both cell types rupture partially as they pass through the slime gland duct, causing each to lose its plasma membrane, and releasing both thread skeins and mucin vesicles into the external environment (Fernholm, 1981). The mucin vesicles are released by holocrine secretion rather than the more typical mechanism of mucus secretion through fusion of vesicles with the membrane of the mucous cell and release of mucin granules by exocytosis (Spitzer and Koch, 1998; Koch et al., 1991; Luchtel et al., 1991). In this way, the mucin vesicles remain intact until they come into contact with seawater in the external environment (Lutchel et al., 1991).

The mature slime is formed when exudate released from the hagfish contacts convectively mixing seawater (Fudge et al., 2005). Agitation during mixing causes the thread skeins to uncoil to lengths of 10–17 cm, providing a large surface area to which the mucins released from the ruptured vesicles can attach (Koch et al., 1991; Fudge et al., 2005). The fully formed slime is a complex network capable of confining seawater to channels between the slime threads and ruptured mucins like a fine sieve (Fudge et al., 2005). The interaction between the thread skeins and ruptured mucins is critical for the production of the mature slime. In fact, the disruption of the mucin network by reducing agents such as dithiothreitol results in a reduced volume of whole slime and a delayed onset of whole slime formation *in vitro* (Koch et al., 1991; Fudge et al., 2005). However, the mechanism of this interaction is still unknown and further studies are required to better understand this process.

The hagfish mucin vesicle is an ovoid structure with an average length of 7 μm along the major axis. It is surrounded by a membrane composed of a single lipid bilayer, and it is estimated that it contains up to 100 times the number of mucous molecules found in the smaller (~1 μm) mucin vesicles of most vertebrates (Luchtel et al., 1991). Hagfish mucin vesicle rupture occurs almost instantaneously in seawater. Evidence from a previous study suggested that the membrane is permeable to cations of all valencies, but only to univalent anions (Luchtel et al., 1991). Furthermore, the membrane is believed to be impermeable to polyvalent anions such as sulphate, citrate and phosphate, based on the fact that the vesicles are stable in 1 mol l^{-1} solutions of these anions. The same study also revealed that zinc ions at concentrations from 25 to 50 mmol l^{-1} inhibited rupture (Luchtel et al., 1991), which suggests that calcium channels may be important for the rupture of mucin vesicles, as zinc is known to inhibit some calcium channels (Büsellberg et al., 1991; Gore et al., 2004). Furosemide, a $Na^+/K^+/2Cl^-$ symporter inhibitor, has no effect on the stability of hagfish mucin vesicles despite its effectiveness in inhibiting the rupture of mucin vesicles from the land slug *Ariolimax columbianus* (Luchtel et al., 1991).

One hypothesis regarding the rupture of hagfish mucin vesicles is that it is initiated by the influx of permeating ions, which causes a

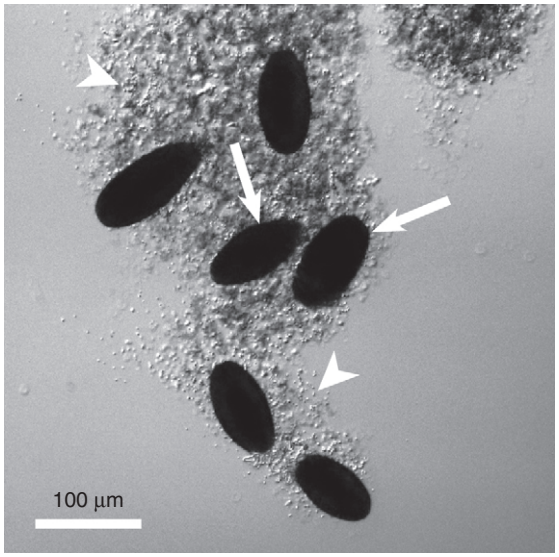

Fig. 1. A small blob of exudate collected from a slime gland and pipetted into a drop of seawater. Hagfish slime gland exudate contains two main solid components – thread skeins (arrows) that consist of a single coiled intermediate filament bundle, and mucin vesicles (arrowheads), which appear as the granular material between the thread skeins. The material shown represents about 5 nl of exudate.

secondary osmotic influx of water (Luchtel et al., 1991). The water then hydrates the mucins, causing them to swell and eventually rupturing the vesicle. Vesicle swelling may be driven further by a 'jack-in-the-box' mechanism involving electrostatic repulsion among negatively charged mucin molecules (Verdugo, 1991). According to this theory, polyvalent cations such as Ca^{2+} shield the charges between adjacent mucin molecules and allow them to be packed at high density within vesicles (Verdugo et al., 1987). When the vesicles are exposed to seawater, Ca^{2+} is exchanged for less effective shielding ions such as Na^{+}, causing repulsion among mucins and rapid swelling.

As osmoconformers, hagfish internal fluids and tissues are isosmotic to seawater (Munz and McFarland, 1964). Rather than actively regulating the internal ion content, which is energetically costly (Kelly and Yancey, 1999), or increasing the salt content of the tissues, which would have adverse effects on the electrostatic properties of enzymes and substrates, the hagfish employs a strategy used by many marine organisms. By using neutral organic compounds to match the osmolarity of the external environment, the hagfish can maintain desirable ion concentrations without compromising protein function (Arakawa and Timasheff, 1985). In the hagfish, this has been found to be true of the serum (Bellamy and Jones, 1961), and if it were also true of the slime gland, the presence of such osmolytes would boost the osmolarity without adding the salts that could cause premature rupture of mucin vesicles (Luchtel et al., 1991).

Of special interest are certain methylamines such as betaine and trimethylamine oxide (TMAO), which, in addition to contributing to total osmolarity, have also been found to stabilize proteins without binding to them (Arakawa and Timasheff, 1985). Both betaine and TMAO are compatible osmolytes that maintain the function of proteins and enzymes without disruptions over a wide range of osmolarities (Yancey, 2001), and are ideal substitutes for inorganic ions that can bind to and destabilize proteins and nucleic acids when present in high concentrations (Yancey, 2005). TMAO is typically found in organisms that accumulate urea in their tissues, where it enhances protein activity and stability and counteracts the detrimental effects that urea has on enzymes (Yancey, 2005). Previous work has revealed that both betaine and TMAO are present in the muscle of *Myxine* at 65 mmol kg^{-1} water and 87 mmol kg^{-1} water, respectively, but neither was reported in the plasma (Robertson, 1976). The purpose of this study was to examine more closely the composition of the fluid component of hagfish slime exudate that surrounds the thread skeins and mucin vesicles, and to identify which inorganic and organic osmolytes are present. Furthermore, we wished to investigate the effects of these osmolytes, most notably TMAO and betaine, on the stability and rupture behaviour of isolated mucin vesicles. Specifically, we wished to test the hypothesis that methylamines such as TMAO and betaine act as impermeable osmolytes that stabilize mucin vesicles. If this is indeed the case then TMAO and betaine should stabilize vesicles at concentrations greater than the osmolarity of the vesicle interior, and should cause rupture in more dilute solutions. In this paper, we report the first detailed investigation of the chemical composition of the fluids in hagfish slime exudate. We also report the first characterization of the swelling behaviour of individual hagfish slime mucin vesicles and provide new insights into the mechanisms of their stabilization within the gland and their deployment in seawater.

MATERIALS AND METHODS

Animals and slime collection

Atlantic hagfish (*Myxine glutinosa* L.) were obtained from the Huntsman Marine Station, St Andrews, New Brunswick, Canada, and were held in the Hagen Aqualab at the University of Guelph in 2000 l tanks at 10°C in artificial seawater with a salinity of 34‰, and were fed once per month. All procedures used were approved by the University of Guelph Animal Care Committee (Protocol 05R154). Prior to exudate collection, the hagfish were anaesthetized in 3.5 l of artificial seawater (33‰) with 3 ml of a 1:9 clove oil to ethanol solution until the animal ceased to respond to touch. Once anaesthetized, the hagfish were electrically stimulated (18 V, 80 Hz) to release exudate from their slime glands. Exudate was then collected using a small scupula for immediate use, or was transferred to a stabilization buffer composed of 0.9 mol l^{-1} sodium citrate and 0.1 mol l^{-1} Pipes buffer [piperazine-*N*,*N*′-bis(ethanesulfonic acid)], pH 6.7 [modified from Downing et al. (Downing et al., 1981)].

Slime supernatant and ruptured slime osmolarity

The osmolarity of the slime supernatant, and artificial seawater (34‰) samples were determined using a Wescor vapor pressure osmometer (VAPRO® 5520; Wescor Inc., Logan, UT, USA). Calibration of the instrument was performed to within ±2 mOsm (±s.e.m.) using Opti-Mole (Wescor, Logan, UT, USA) standards of 100 mOsm, 290 mOsm and 1000 mOsm (Wescor Inc., Logan, UT, USA). Following calibration, 10 μl samples of slime supernatant (N=6), and artificial seawater were tested using the osmometer. The osmometer was tested for accuracy (±2 mOsm) every fourth sample using the 290 mOsm Opti-Mole standard.

Inorganic ion analysis

Five hagfish were collected and anaesthetized as described above. Approximately 0.2 ml of fresh exudate was collected from each hagfish and centrifuged at 16,000 ***g*** for 5 min at 10°C in 1.5 ml tubes. This process separated samples into three distinct layers: the thread skeins, mucin vesicles and a clear fluid, which will hitherto be referred to as the natural supernatant of the slime. After

centrifugation, 50 µl of the natural supernatant from each sample was pipetted off using a micropipette and placed into clean 0.6 ml microcentrifuge tubes. The samples were then covered with 50 µl of mineral oil to minimize evaporation and stored on ice for transport to McMaster University for analysis.

Construction of ion-selective microelectrodes

Ion-selective microelectrodes were used to measure the pH or concentration of Na^+, K^+, Mg^{2+} or Ca^{2+} in hagfish fluid samples (~10 µl) that were placed under paraffin oil to prevent evaporation. Micropipettes were pulled from 1.5 mm o.d. unfilamented borosilicate glass capillary tubing using a vertical micropipette puller (Narishige, Tokyo, Japan), and dried on a hot plate at 200°C for 10 min before silanization. The latter process makes the glass surface hydrophobic and facilitates filling with, and retention of, the hydrophobic ionophore cocktails. A 1 µl drop of dimethyldichlorosilane was pipetted onto the inside of a 150 mm diameter Pyrex Petri dish, which was then inverted over the micropipettes which had been placed on the hot plate. Micropipettes were removed after a minimum of 20 min exposure to the silane vapour, and could be stored over silica gel for up to 2 weeks before filling. This degree of silanization is sufficient to retain the ionophore cocktail but avoids capillary rise of paraffin oil into the pipette tip. Appropriately silanized microelectrodes for use under paraffin oil are characterized by a flat meniscus at the interface between the cocktail and the backfill solution.

The following ionophore cocktails (Fluka, Buchs, Switzerland) and back-fill solutions (in parentheses) were used: Cl^- ionophore I, cocktail A (150 mmol l^{-1} NaCl); K^+ ionophore I, cocktail B (150 mmol l^{-1} KCl); Ca^{2+} ionophore I, cocktail A (100 mmol l^{-1} $CaCl_2$); H^+ ionophore I, cocktail B (100 mmol l^{-1} NaCl + 100 mmol l^{-1} sodium citrate, pH 6.0). The Na^+ ionophore cocktail consisted of 10% Na^+ ionophore X, 89.75% nitrophenyl octyl ether and 0.25% sodium tetraphenylborate (Messerli et al., 2008). The electrode was backfilled with 150 mmol l^{-1} NaCl. The Mg^{2+} ionophore cocktail consisted of 8.8% Mg^{2+} ionophore VI (ETH 5506), 3% potassium tetrakis chlorophenyl borate, 4.4% ETH 500, 71.8% nitrophenyl octyl ether and 12% polyvinylchloride. These components were dissolved in approximately twice their own volume of a 1:1 mixture of tetrahydofuran:cyclohexanone.

Each cocktail (except Mg^{2+}) was taken up in the tip of a plastic tuberculin syringe pulled out over a low flame to a fine tip (Thomas, 1978), then injected into the shank of the micropipette. The cocktail ran to the tip of the micropipette by capillarity. The micropipette was then backfilled with a solution injected through a second tuberculin syringe pulled to a fine tip. Mg^{2+} electrodes were backfilled with a solution of 135 mmol l^{-1} NaCl, 15 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgCl_2$, then tipfilled with a short (100 µm) column of cocktail and left with their tips in air for 1–12 h to allow solvent evaporation. Reference electrodes were fabricated from 1.5 mm o.d. filamented glass tubing and were filled with 500 mmol l^{-1} KCl. Electrodes were connected through chlorided silver wires to an electrometer of high input impedance ($>10^{13}$ Ω) and signals were recorded using a computer-based data acquisition and analysis system (PowerLab, ADInstruments, Bella Vista NSW, Australia) running Chart software.

Calibration solutions were based on NaCl for Cl^-, NaCl–KCl mixtures for Na^+ and K^+ and NaCl–$CaCl_2$ mixtures for Ca^{2+}. In each case the solutions were selected to encompass the range of ion concentrations in the hagfish slime samples. H^+ microelectrodes were calibrated in 150 mmol l^{-1} NaCl buffered with 10 mmol l^{-1} Hepes.

Ion concentrations were calculated from the equation:

$$[\mathrm{Ion}]_{\mathrm{sample}} = [\mathrm{Ion}]_{\mathrm{c}}\, 10^{(\Delta V/S)},$$

where $[\mathrm{Ion}]_{\mathrm{sample}}$ is the ion concentration of the experimental drop, $[\mathrm{Ion}]_{\mathrm{c}}$ is the concentration of the calibration drop, ΔV is the change in potential (mV) between the sample and the calibration drop, and S is the slope (mV) for a tenfold change in ion concentration. All experiments were done at room temperature, 23°C.

Although ion-selective electrodes measure ion activity and not concentration, data can be expressed in terms of concentrations if it is assumed that the ion activity coefficient is the same in calibration and experimental solutions. Expression of data in terms of concentrations simplifies comparisons with studies in which ion concentrations are measured by techniques such as atomic absorption spectroscopy.

Organic osmolyte analysis

Hagfish were anesthetized as describe above, and five 0.2 ml samples were collected from five hagfish. The samples were each centrifuged at 16,000 ***g*** for 5 min at 4°C. Following centrifugation, two 20 µl replicate samples of natural supernatant were taken from each original sample and frozen at –80°C prior to shipping to Whitman College on dry ice. The samples were then analyzed for organic solutes using high-performance liquid chromatography (HPLC) following the protocol of Wolff et al. (Wolff et al., 1989), and for trimethylamine oxide (TMAO) using ferrous sulfate and disodium ethylenediaminetetraacetic acid (EDTA) following the protocol of Wekell and Barnett (Wekell and Barnett, 1991).

Mucin vesicle preparation, chamber design and assay solutions

Fresh exudate from six different hagfish was collected as described above, and placed into the citrate and Pipes stabilization buffer. Mucin vesicles were then separated from thread cells using 53 µm nylon mesh so that they could be studied in isolation. In order to achieve a constant mucin vesicle concentration for experimental assays, each sample of isolated mucin vesicles was quantified and standardized visually using a hemocytometer. Each sample was diluted appropriately to a concentration of approximately 40 mucin vesicles per microlitre, resulting in an average of 41±1 (mean ± s.e.m.) stationary mucin vesicles observed per assay (N=138). The chambers constructed for use in these assays were 6 mm× 18 mm×0.1 mm, with an internal volume of 10.8 µl. Chambers were composed of a glass microscope slide and a coverslip separated by two rails of 0.1 mm diameter glass beads in Vaseline petroleum jelly spaced 6 mm apart.

To test the effect of betaine and TMAO, solutions of both compounds were made in 5.0 mmol l^{-1} Tris buffer at pH 8.0 at concentrations of 0 mmol l^{-1}, 400 mmol l^{-1}, 800 mmol l^{-1}, 1000 mmol l^{-1} and 1200 mmol l^{-1}. To measure the effect of betaine and TMAO on mucin vesicle rupture in the presence of seawater salts, additional betaine and TMAO solutions were prepared containing 33‰ artificial sea salts (Coralife, Energy Savers Unlimited, Inc., Carson, CA, USA). Each solution was filtered through a 0.2 µm filter to minimize bacterial growth during storage. In order to determine at which point the assay solution came into contact with the mucin vesicles, 3 µl of blue food colouring was added to a 1 ml aliquot of each solution so that it was possible to visually differentiate the assay solutions from the stabilization buffer solution in which the mucin vesicles were stored.

To determine the effect of the natural supernatant on isolated mucin vesicles, supernatant was collected from six hagfish as

described above. An additional artificial supernatant was prepared using values obtained from the inorganic and organic osmolyte analyses. To create a solution with the same osmolarity as the natural supernatant, betaine and TMAO concentrations were increased by 8% to make up for the presence of unknown solutes, and match the total osmolarity of 888 mOsm determined in this study. Additional trials were conducted with seawater, 5.0 mmol l^{-1} Tris, 1 mol l^{-1} betaine, 1 mol l^{-1} TMAO, and the sodium citrate stabilization buffer.

Assay procedure and image capture

Each chamber was filled with isolated mucin vesicles in stabilization buffer, and was left alone for 2 min in order to allow the vesicles to settle, as vesicles in contact with the glass surface of the bottom of the chamber remained stationary within the field of view. After this period, 30 µl of stabilization buffer was deposited at one end of the chamber, and drawn through the chamber using a strip of filter paper 6 mm×40 mm to absorb the excess solution from the opposite end and remove the non-stationary mucin vesicles from the chamber. The chambers were then placed under the ×40 DIC objective of a Nikon Eclipse 90i epifluorescent microscope, and 15 µl of a given assay solution was added to one end of the chamber. The rupture of mucin vesicles (Fig. 2) was then recorded using the time-lapse function of the NIS Elements (Nikon Instruments, Inc., Melville, NY, USA) software as the assay solution was drawn through the chamber with another strip of filter paper 6 mm×20 mm. Each rupture chamber was used only once.

Preliminary assessment determined that the majority of swelling and rupture activity occurred in the 10–20 s period of the trials. Therefore, a time-lapse macro was used to automatically capture images at 5 f.p.s. (frames per second) for a period of 5 s, followed by a 10-s period at 2 f.p.s., followed by a period of 15 s at 1 f.p.s., and then one more 15-s period at 1 f.p.s. after 60 s had passed since the beginning of the trial. This setup allowed for more detailed data collection within the first 30 s of the trial, and provided data to calculate the percentage of vesicles that had ruptured after 60 s. Preliminary trials demonstrated that the majority of vesicles ruptured within the first 30 s, and the number of ruptured vesicles was unlikely to change after 60 s. For the purposes of analysis, time zero was defined as the moment when the assay solution contacted the mucin vesicles, as indicated by the appearance of the blue food colouring.

Video analysis and statistical analysis

The propensity of the various test solutions to stabilize or rupture mucin vesicles was quantified by counting the number of vesicles ruptured after 60 s. In all analyses, vesicles were counted as ruptured when their diameter doubled in size from time zero, or when they were no longer visible. Because the proportion data were non-normal (P<0.05), they were transformed by taking the arcsine of the square root of the proportion of ruptured vesicles. For the betaine and TMAO concentration series trials, data were analyzed using SPSS v. 16.0 to conduct a two-way ANOVA with *post-hoc* Tukey's HSD tests for the effects of solute concentration and the presence or absence of seawater on the proportion of ruptured vesicles. Differences were considered significant when P<0.05. For natural and artificial supernatant comparison trials, data were analyzed using a one-way ANOVA with *post-hoc* Tukey's HSD tests for multiple comparisons between pairs of solutes.

To characterize mucin vesicle swelling kinetics in seawater, swelling and rupture were measured as a function of vesicle diameter over time. For these six trials, images were captured at a higher rate of 15–20 f.p.s. so that the fastest rupture events could still be characterized. The rate of swelling was measured as the change in the percentage increase in vesicle diameter over time. The initial swelling slopes for each vesicle was measured using the linear trend function in Microsoft Excel 2007.

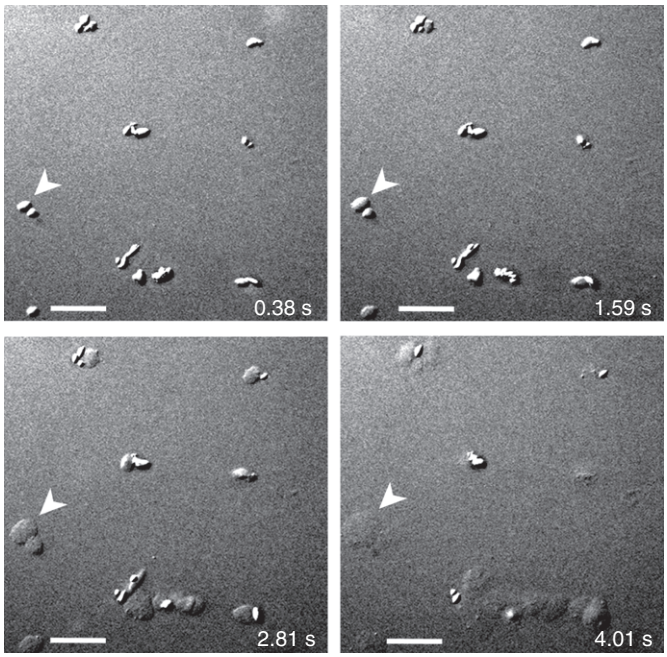

Fig. 2. A composite of stills from a video of isolated mucin vesicles in the slime of *Myxine glutinosa* rupturing on exposure to seawater. Swelling and rupture data in Figs 3–6 were collected from time-lapse video microscopy images such as these. Timestamps in the lower right corner indicate time following contact with seawater. Scale bars, 20 µm. Arrowheads indicate a single mucin vesicle as it changes over time.

RESULTS

Supernatant composition

Following centrifugation, the slime gland exudate yielded three distinct layers. The bottom layer was composed of thread skeins, the middle layer was composed of mucin vesicles, and the top layer was a clear fluid, the natural supernatant. Analysis of this natural supernatant using vapor-pressure osmometry revealed a total osmolarity of 888±3 mOsm (±s.e.m.). Mean concentrations of the inorganic ions measured were: 191 mmol l^{-1} Cl^{-}, 41 mmol l^{-1} Na^{+}, 143 mmol l^{-1} K^{+}, 2.15 mmol l^{-1} Mg^{2+} and 0.45 µmol l^{-1} Ca^{2+} (Table 1). The organic osmolytes detected contributed a total of 490±10 mOsm (s.e.m.), with betaine and TMAO occurring at the highest concentrations of 218 mmol l^{-1} and 101 mmol l^{-1}, respectively (Table 2).

Rupture assays

For rupture trials involving betaine, betaine concentration (P<0.0001) and the presence of seawater salts (P<0.0001) had significant effects on the proportion of mucin vesicles that ruptured after 60 s of exposure to a given solution (Fig. 3). Two-way ANOVA analysis also revealed a significant interactive effect (P<0.0001) between the main effects of betaine and the presence of saltwater salts. Higher concentrations of betaine resulted in a decrease in the proportion of vesicles ruptured, but this was only true in the absence of seawater salts, as revealed by *post-hoc*

Table 1. The concentration of inorganic ions and pH in the supernatant of fresh slime exudate, measured using ion-specific electrodes

Ion	Concentration in supernatant (mmol l^{-1})
Na^+	41.2±2.6
K^+	143.0±3.0
Cl^-	191.5±6.6
Ca^{2+}	0.00045±0.00009
Mg^{2+}	2.15±0.76
Total	379
pH	7.31±0.02

Values are means ± s.e.m., *N*=5.

Table 2. The concentration of organic osmolytes in the supernatant of fresh slime exudate

Osmolyte	Concentration in supernatant (mmol l^{-1})
Glucose	1.23±0.22
Inositol	2.30±0.68
Taurine	2.13±0.42
Betaine	218±7
Dimethyl glycine	68.6±6.0
Glycine	79.9±7.5
Creatine	15.0±1.4
b-Alanine	2.17±0.68
TMAO	101.3±4.8
Total	490±10

TMAO, trimethylamine oxide. Organic solutes were analyzed using HPLC, and TMAO was analyzed using ferrous sulphate and EDTA.
Values are means ± s.e.m., *N*=5.

(Tukey HSD) analysis. The lowest occurrence of rupture was at 1200 mmol l^{-1} betaine, no salts, with 46±6% (±s.e.m.) of vesicles still intact after 60 s. Rupture occurred in 100% of vesicles in 0 mmol l^{-1} betaine (i.e. in 5.0 mmol l^{-1} Tris), and in 96±1% (±s.e.m.) of vesicles exposed to seawater after 60 s.

Rupture trials with TMAO revealed a significant effect of TMAO concentration (P<0.0001) on the proportion of vesicles ruptured after 60 s, but the presence of seawater salts had no effect (P=0.554; Fig. 4). Two-way ANOVA analysis also revealed no interactive effect (P=0.140) between TMAO concentration and the presence of seawater salts. Higher concentrations of TMAO resulted in a decrease in the proportion of vesicles ruptured, and this trend was unaffected by the presence of seawater salts, as revealed by *post-hoc* (Tukey HSD) analysis. The lowest occurrence of rupture was at 1200 mmol l^{-1} TMAO, no salts, with 48±5% of mucin vesicles unruptured after 60 s.

One-way ANOVA analysis of the effect of natural supernatant obtained from fresh slime exudate, artificial supernatant, seawater, stabilization buffer, 5.0 mmol l^{-1} Tris, 1 mol l^{-1} betaine and 1 mol l^{-1} TMAO on the proportion of mucin vesicles that ruptured after 60 s of exposure revealed a significant main effect of solution composition (P<0.000, d.f.=6). *Post-hoc* Tukey's HSD revealed significant differences among comparisons of all treatment groups with the following exceptions: seawater/distilled water, natural supernatant/artificial supernatant/1 mol l^{-1} betaine, and 1 mol l^{-1} betaine/1 mol l^{-1} TMAO (Fig. 5). After 60 s of exposure, 0% of mucin vesicles ruptured when they were exposed to the sodium citrate stabilization buffer, with no variation in this result. 100% of all vesicles ruptured when they were exposed to distilled water, with no variation, and 96±1% of vesicles ruptured when they were exposed to seawater.

Swelling and rupture kinetics

The mean rate of mucin vesicle swelling in seawater, measured as the relative increase in the original diameter per millisecond was 0.75±0.04% ms^{-1}. The graph representing the distribution of swelling rates in seawater for 203 vesicles in six samples shows two distinct groupings of mucin vesicle swelling rates, with the steeper slopes representing vesicles with fast rupture rates, and the more gradual slopes representing vesicles with slower rupture rates (Fig. 6).

DISCUSSION

Our results indicate that the natural supernatant of the hagfish slime exudate contains a higher concentration of K^+ than is present in the plasma, and concentrations of Na^+, Cl^- and Ca^{2+} that were lower. Additionally, we found high levels of the methylamines TMAO, betaine and dimethylglycine at a combined concentration of 388 mmol l^{-1}. However, a large fraction of mucin vesicles ruptured when exposed to a variety of concentrations of TMAO and betaine. These results do not support the hypothesis that these two methylamines are responsible for mucin vesicle stabilization within the slime gland. Our analysis of the rate of mucin vesicle swelling in seawater indicates that there may be two distinct populations of vesicles.

It is not surprising that the osmolarity of the natural supernatant of Atlantic hagfish slime is approximately isosmotic to seawater, since it has been found in other studies that the osmotic concentration of the serum and other tissues of hagfishes match that of the external environment (McFarland and Munz, 1958; Bellamy and Jones, 1961). The supernatant osmolarity of 888 mOsm we measured is highly consistent with the previously estimated value of 897 mOsm vesicle osmolarity (Luchtel et al., 1991). We found that inorganic ions such as Na^+, K^+, Cl^-, Mg^{2+}

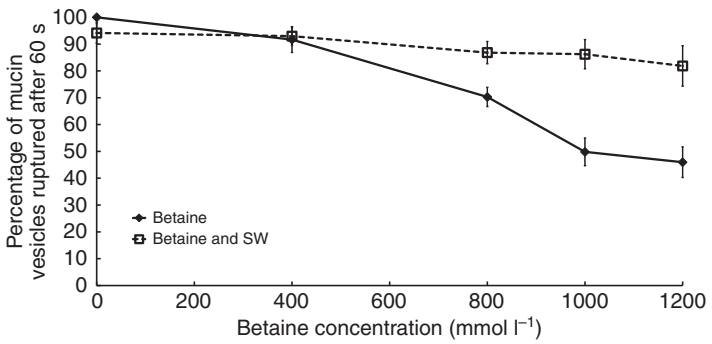

Fig. 3. The percentage of isolated mucin vesicles from the slime of *Myxine glutinosa* that ruptured after 60 s of exposure to varying concentrations of betaine in 5.0 mmol l^{-1} Tris, pH 8.0 (solid line). The dotted line represents the effect of changing betaine concentration in the presence of sea salts (33‰). Values are means ± s.e.m. (*N*=6 in each treatment). Two-way ANOVA showed significant effects of betaine concentration and the presence or absence of sea salts (P<0.05).

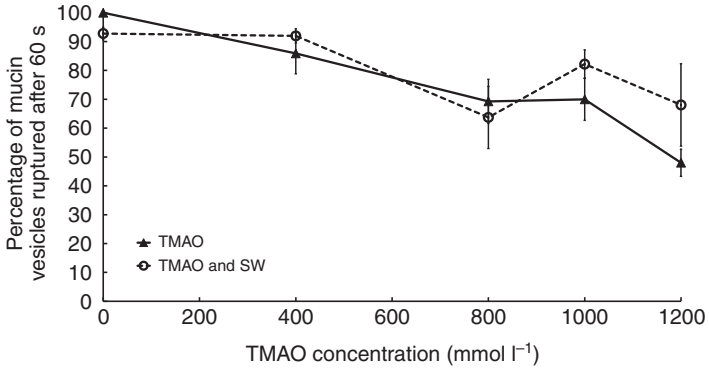

Fig. 4. The percentage of mucin vesicles that ruptured after 60 s of exposure to varying concentrations of TMAO in 5.0 mmol l^{-1} Tris, pH 8.0. Dotted line represents the effect of changing TMAO concentration in the presence of sea salts (33‰). Values are means ± s.e.m. (N=6 in each treatment). Two-way ANOVA showed a significant effect of increasing TMAO concentration (P<0.05) but not for the presence or absence of sea salts.

and especially Ca^{2+} were found in relatively low concentrations in the natural supernatant (Table 1). An analysis of these same ions in whole slime exudate (which included threads, mucin vesicles and additional fluid) found them to be present at much higher concentrations (Munz and McFarland, 1964). The differences in concentration probably arise from higher concentrations of inorganic ions contributed by the thread cells and/or the mucin vesicles after they rupture. Higher concentrations of divalent cations such as Mg^{2+} and Ca^{2+} within the vesicle would be consistent with the jack-in-the-box mechanism of vesicle swelling described by Verdugo (Verdugo, 1991).

Hagfish slime is rich in methylamines

We found that nearly half of the total osmolarity of the natural supernatant was contributed by a variety of organic osmolytes, and that of those organic osmolytes, betaine and TMAO were present at the highest concentrations. These results contrast starkly with the plasma composition in hagfishes, in which approximately 97% of the total osmolarity is contributed by inorganic ions (Robertson, 1976). Although betaine and TMAO were not reported in Robertson's study, they could not have contributed more than 3% of the total osmolarity. Methylamines such TMAO and betaine are thought to be used by marine osmoconformers primarily to counteract the perturbing effect of urea on protein structure and enzyme activity. It has been found that TMAO and other methylamine compounds and amino acids are most effective as protein structure stabilizers at a 1:2 molar concentration ratio of these counteracting osmolytes to urea (Yancey and Somero, 1979). It has also been found that methylamines such as TMAO and betaine can counteract functional inhibition and structural destabilization of proteins by inorganic salts (Yancey, 1994). As counteracting osmolytes, methylamines are often interchangeable (Bedford et al., 1998), and the relative concentrations of betaine and TMAO may not be significant. Bedford et al. (Bedford et al., 1998) speculated that different methylamines serving the same apparent role within different animals and within different tissues of the same animals may represent the most metabolically efficient means of regulating intracellular osmoregulation in external environments of high osmotic strengths. In the case of the hagfish, however, the urea content is extremely low both in plasma (3 mmol kg^{-1} water), and in muscle tissue (1.5 mmol kg^{-1} water), when compared with similar values for the rabbit-fish (*Chimaera monstrosa*) of 332 mmol kg^{-1} water and 335 mmol kg^{-1} water in plasma and muscle, respectively (Robertson, 1976). Our analysis of the natural supernatant in the current study did not reveal the presence of urea; however, methylamines in the natural supernatant may play a role in counteracting the presence of high concentrations of salts within the slime gland.

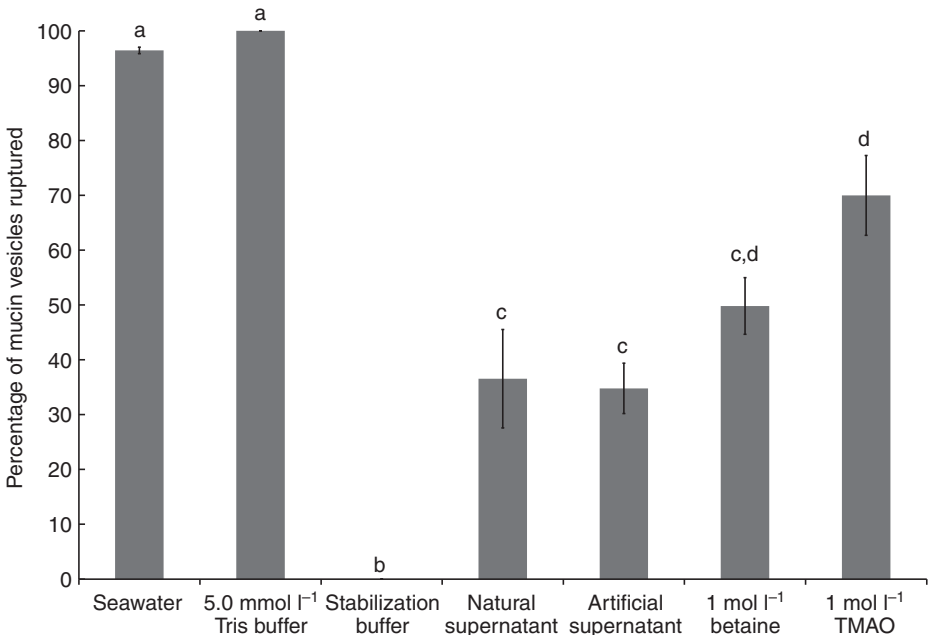

Fig. 5. The percentage of mucin vesicles that ruptured after 60 s of exposure to different solutions. Values are means ± s.e.m. (N=6 in each treatment). Different lower-case letters denote significant differences between means (P<0.05).

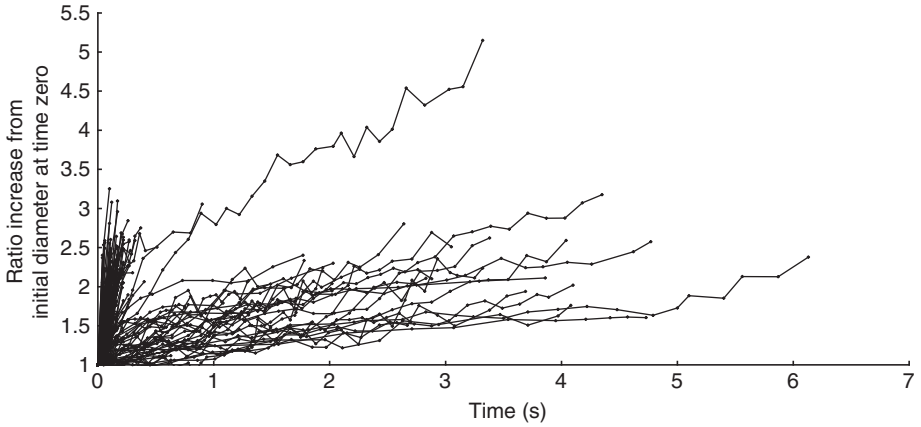

Fig. 6. The swelling of isolated mucin vesicles from the slime of *Myxine glutinosa* over time with exposure to seawater salts. Swelling is represented as the ratio of vesicle diameter to the original diameter at time zero. The slope of each line represents the swelling rate for a single mucin vesicle. *N*=203 vesicles from six samples. The data indicate that there may be two distinct populations of mucin vesicles.

TMAO and betaine alone do not stabilize vesicles

As mucin vesicles from the slime of the Pacific hagfish are thought to be permeable to most ions except polyvalent anions (Luchtel et al., 1991), it is perhaps not surprising that we found high concentrations of organic compounds that could act as osmolytes in slime exudate, given that most inorganic ions would cause rupture. The decrease in the proportion of ruptured vesicles with increasing betaine and TMAO concentrations (Figs 3, 4) is consistent with the idea that these compounds act as osmolytes in the gland, however, the story is more complicated than that. Figs 3 and 4 reveal that even at the highest concentrations of these compounds (1200 mmol l^{-1}), about half of the vesicles still ruptured after a 60 s exposure. This result is in stark contrast to the results for 1 mol l^{-1} sodium citrate, in which none of the vesicles ruptured (Fig. 5). The betaine and TMAO data suggest that the vesicles are partially permeable to these compounds, or there is a substantial fraction of vesicles that are permeable.

If the vesicles are permeable to most inorganic ions (Luchtel et al., 1991) and to the most abundant organic compounds in the gland, this raises the question of how the vesicles are stabilized in the gland at all. One possibility is that compounds such as betaine and TMAO interfere with the permeability of ion channels in the vesicle membrane, thereby making ions such as Na^+ and Cl^- agents of stabilization rather than rupture. We tested this idea by replicating the betaine and TMAO concentration trials in the presence of 33‰ of seawater salts. If these methylamines inhibit ion channels, then we should have seen decreased levels of vesicle rupture when they were present in combination with seawater salts. In the case of betaine, we saw the opposite result, with seawater salts causing an increase in the percentage of vesicles ruptured, which rules out betaine as an inhibitor of ion channels in the vesicle membrane. The results for TMAO were different, in that the presence of seawater salts did not overpower the stabilizing effects of TMAO, as they did in the betaine trials. However, we found no evidence that the presence of seawater salts actually increased the stabilizing effects of TMAO, which also rules out TMAO as an inhibitor of ion channels in hagfish slime mucin vesicle membranes.

The inability of even high concentrations of betaine and TMAO to stabilize all vesicles within a sample led us to investigate the ability of the natural supernatant to stabilize the vesicles. Although we had initially assumed at the start of this study that the supernatant would be 100% effective at stabilizing the vesicles, in fact we found that only ~64% of the vesicles remained intact after 60 s of exposure to the natural supernatant. The artificial supernatant was similar, stabilizing ~65% of mucin vesicles (Fig. 5). In fact, the similar behaviour of the vesicles in natural and artificial supernatant suggests that our compositional analysis did not omit any significant components. Both natural and artificial supernatants were more effective at preventing mucin vesicle rupture than was the 1 mol l^{-1} solution of TMAO, but both were as effective as 1 mol l^{-1} betaine. This suggests that betaine may be an important ingredient for mucin vesicle stabilization, but some other osmolyte within the supernatant (Tables 1, 2), or some combination of osmolytes may be necessary for total stabilization to occur. It is important to note that when samples were exposed to the 1 mol l^{-1} sodium citrate stabilization buffer, no vesicles ruptured in any of the trials. This indicates that in solutions of impermeable ions at concentrations higher than the internal osmolarity of the vesicle, it is possible to stabilize 100% of the isolated vesicles, as must occur in the slime gland. The citrate results also act as negative control and demonstrate that rupture in all other treatments was truly a function of the chemical composition of the solutions in question, and not a result of physical flow of the test solutions in the chamber.

The data presented here suggest that it should be impossible to stabilize mucin vesicles within the hagfish slime gland given the solute composition of the fluids within it and their effects on mucin stability *in vitro*. And yet, of course, the vesicles must be effectively stabilized within the gland. How then does this occur? Consideration of the origin of the slime exudate and the structures within the slime gland that contribute to it may shed some light on this paradox. Mucin vesicles reside within mucous cells, which in turn are found within the slime glands (Downing et al., 1981). The cytosol of the mucous cell may have a different composition from the extracellular fluid surrounding the mucous cells in the gland. In the slime gland, the mucin vesicles are exposed only to the cytosol of the gland mucus cell, which is regulated by the plasma membrane. There is also the cytosol of the thread cells to consider, which is released when the plasma membrane is stripped from the thread cells as they are expelled from the slime gland (Spitzer and Koch, 1998). As a consequence, there are three potentially different sources of fluids that contribute to the natural supernatant that we analyzed: the cytosol of the mucous cell, the cytosol of the thread cell, and the extracellular glandular fluid. Given this analysis, it is possible that the chemical environment within the intact mucous gland cells is considerably different from the composition of the supernatant that we report here. If it is not, then there must be some other mechanism of vesicle stabilization in the slime glands, such as hydrostatic pressure.

Two distinct populations of mucin vesicles?

Analysis of the swelling rates of individual mucin vesicles exposed to seawater (Fig. 6) indicates that there may be two populations of vesicles with distinct swelling behaviours, one with very fast

rupture kinetics, and another with slower kinetics. Although differences in flow rate among trials surely contributed some variability to the swelling behaviours observed, within each trial there were vesicles that showed both types of rupture, and there were vesicles immediately adjacent to one another that exhibited markedly divergent swelling rates. No areas within the chamber appeared to be characterized by a single rupture rate, which eliminates the possibility that the differences were a result of microenvironments within the chamber. One possible explanation for this apparent bimodal distribution of swelling rates could be variability in the number and kind of ion channels in the vesicle membranes. The mucin vesicles are a product of the Golgi apparatus (Luchtel et al., 1991), and as such, the incorporation of ion channels is dependent on their distribution in the organelle. It is possible that a certain fraction of vesicles fail to have ion channels incorporated into their structure. If vesicle swelling is initiated by the influx of water with permeating ions (Luchtel et al., 1991), then the number of channels present would determine the rate at which ions and water enter the vesicle. The vesicles that lack channels completely may therefore represent the vesicles that exhibited slow swelling kinetics. Likewise, variations in the number of aquaporins in the mucin vesicle membrane would also result in differences in the rate of osmotic water influx. It is also possible that there is some adaptive value to having two types of vesicles with different swelling kinetics, although at this time it is unclear what that advantage might be.

Conclusions

We have determined the composition of the fluid component of Atlantic hagfish slime exudate in terms of both inorganic ions and organic compounds. Furthermore, we have established that neither of the most abundant organic osomolytes, the methylamines betaine and TMAO, are capable of fully stabilizing the mucin vesicles on their own, even at high concentrations, suggesting that the vesicle membranes are somewhat permeable to these compounds. We also provide evidence that neither betaine nor TMAO are capable of stabilizing the mucin vesicles by altering the permeability of ion channels in the vesicle membrane. In light of these results, the mechanism by which mucin vesicles are stabilized within the gland is still unclear, but probably involves a stabilizing chemical microenvironment within the gland mucous cells in which the vesicles are produced and stored, or a physical stabilization in the form of hydrostatic pressure. The preliminary analysis of mucin vesicle rupture kinetics in seawater suggests two distinct swelling behaviours of the vesicles, and raises the possibility of multiple vesicle types in the slime. Future work will focus on the nature of the ion channels within the slime as well as other possible mechanisms of vesicle stabilization in the gland and deployment in seawater.

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