Fractionation of the Gulf toadfish intestinal precipitate organic matrix reveals potential functions of individual proteins

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The regulatory mechanisms behind the production of CaCO3 in the marine teleost intestine are poorly studied despite being essential for osmoregulation and responsible for a conservatively estimated 3–15% of annual oceanic CaCO3 production. It has recently been reported that the intestinally derived precipitates produced by fish as a byproduct of their osmoregulatory strategy form in conjunction with a proteinaceous matrix containing nearly 150 unique proteins. The individual functions of these proteins have not been the subject of investigation until now. Here, organic matrix was extracted from precipitates produced by Gulf toadfish (Opsanus beta) and the matrix proteins were fractionated by their charge using strong anion exchange chromatography. The precipitation regulatory abilities of the individual fractions were then analyzed using a recently developed in vitro calcification assay, and the protein constituents of each fraction were determined by mass spectrometry. The different fractions were found to have differing effects on both the rate of carbonate mineral production, as well as the morphology of the crystals that form. Using data collected from the calcification assay as well as the mass spectrometry experiments, individual calcification promotional indices were calculated for each protein, giving the first insight into the functions each of these matrix proteins may play in regulating precipitation.

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1. Introduction

The production of inorganic minerals by living organisms, termed biomineralization, is common from unicellular algae such as coccolithophores to vertebrate skeletons (Weiner and Dove, 2003). These biominerals serve numerous functions ranging from predator protection to body support and food breakdown. Mineral production, in particular carbonate minerals such as calcium carbonate (CaCO3), by marine organisms is particularly abundant and plays a significant role in the oceanic carbon cycle (Cusack and Freer, 2008; Millero, 2007). Although the impacts of well recognized calcifiers such as coccolithophores and foraminiferans on oceanic carbon cycling are well understood, the role of marine fish has not been realized until recently (Wilson et al., 2009).

Marine fish produce carbonate minerals (namely CaCO3 and MgCO3) in their intestine as part of their osmoregulatory process (Perry et al., 2011; Walsh et al., 1991; Wilson et al., 2002). As fish must drink seawater to avoid dehydration, they have evolved numerous adaptations in which to process the imbibed water, which has a nearly three-fold higher osmolality then their extracellular body fluids (Marshall and Grosell, 2006). One such adaptation is the precipitation of carbonate minerals (typically high-magnesium calcite) in the intestinal lumen made possible by the concentration of calcium in the luminal fluid, as well luminal alkalization and bicarbonate concentration by the SLC26a6 anion exchanger (Grosell, 2011). This precipitation reaction lowers the osmotic pressure of the luminal fluid by 70–100 mOsm via the removal of calcium and bicarbonate from solution (Grosell et al., 2009; Wilson et al., 2002), allowing for increased water absorption across the epithelium. It is believed that without this precipitation reaction, fish would be unable to extract sufficient water from imbibed seawater to counteract passive dehydration to their hyperosmotic surroundings, and would therefore be unable to osmoregulate and survive in marine environments (Grosell, 2013).

Intestinally derived fish carbonates are eventually excreted into the water column as a waste product. Due to high production rates, along with the large biomass of marine fish, the annual production of carbonates via this fashion is substantial: conservative estimates suggest 40–150 million metric tons of carbonate is precipitated per year, or 3–15% of total oceanic CaCO3 production (Wilson et al., 2009). Additionally, due at least in part to their high magnesium content, these precipitates are highly soluble, which is believed to account for
the increased total alkalinity above the aragonite saturation horizon that puzzled oceanographers for decades (Wilson et al., 2009; Woosley et al., 2012). Further, the high solubility of these precipitates means that deposits of fish derived mineral in the sediments are likely to serve as “first responders” to decreasing saturation states of seawater resulting from increasing atmospheric CO2 concentrations due to anthropogenic activities, namely the burning of fossil fuels (Morse et al., 2006). Such a response could lessen the effects of the predicted future acidification of oceanic surface waters, which could have far reaching impacts on numerous marine ecosystems (Doney et al., 2009).

As is the case with most biominerals, it has recently been discovered that precipitates formed in the Gulf toadfish (Opsanus beta) intestine are not entirely inorganic, but instead contain an at least partially proteinaceous organic matrix (Schauer et al., 2016). Approximately 150 proteins were identified in this matrix, and the matrix as a whole was found to regulate carbonate mineral precipitation, at least in vitro, in a highly dose-dependent manner as well as regulate the incorporation of magnesium into the forming mineral (Schauer et al., 2016). Although these previous experiments suggest that the proteins associated with the forming mineral can regulate the precipitation reaction, it has not yet been established which proteins are responsible for the observed effects. To begin to address this question, toadfish intestinal precipitate matrix proteins were fractionated here by their net charge and the individual fractions were tested for their ability to modulate carbonate mineral precipitation. The proteins in each fraction were then identified by mass spectrometry (MS) which allowed for the regulatory abilities of individual proteins to be approximated.

2. Materials and methods

2.1. Experimental animals

Gulf toadfish (Opsanus beta) were collected as bycatch from bait-shrimp fisherman operating out of Dinner Key, Miami, FL. After collection, fish were transported to the Rosenstiel School of Marine and Atmospheric Science where they were ectoparasite treated via a three-minute freshwater bath and subsequent malachite green immersion. Prior to experimentation, fish were held in aerated 60 L aquaria on flow-through, sand-filtered seawater from Biscayne Bay (32–37 ppt, 21–26 °C) for at least two weeks. Fish were fed a diet of chopped squid once weekly to satiation except when described otherwise. All experiments were conducted in accordance with the relevant guidelines and regulations, and were approved by the University of Miami Animal Care and Use Committee (protocol no. 13-225), which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

2.2. Intestinal precipitate collection and matrix extraction

Toadfish (37.25 ± 9.14 g; mean ± SD) were randomly selected and placed ten individuals per tank into six, aerated 30 L aquaria filled with 1 μm filtered, UV-sterilized seawater sourced from Biscayne Bay (34–37 ppt, 23–26 °C). All fish were fasted for one week prior to transfer to the experimental tanks and were not fed during the collection period, which lasted no more than two weeks for a single animal. At least 70% of the water was siphoned out of each tank daily, and excreted precipitates which lasted no more than two weeks for a single animal. At least 70% of the water was siphoned out of each tank daily, and excreted precipitates which lasted no more than two weeks for a single animal. At least 70% of the water was siphoned out of each tank daily, and excreted precipitates which lasted no more than two weeks for a single animal. At least 70% of the water was siphoned out of each tank daily, and excreted precipitates which lasted no more than two weeks for a single animal.

Collected precipitates were purified via two rinses with ultrapure water, followed by a 24 h incubation at 4 °C in a NaOCl solution containing 5% available chlorine. Purified precipitates were then collected by a 3 min, 2000 g spin, and the resulting pellet was rinsed twice in ultrapure water to remove residual NaOCl. Inorganic mineral was removed by incubation in excess 0.5 M EDTA (pH 8.0) plus 1 × HALT protease inhibitor cocktail (Thermo Scientific) at 4 °C for a minimum of 3 h. This solution was then centrifuged at 2000g for 10 min and the resulting supernatant consisted of an aqueous solution containing the organic matrix, which was stored at −80 °C until further processing. Upon completion of the collection period, all matrix samples were combined and EDTA was removed by ultrafiltration and buffer exchange into tris buffered saline (TBS; 150 mM NaCl, 10 mM tris, pH 7.5) using Amicon spin concentrators (EMD Millipore) with a 3 kDa molecular weight cutoff. Protein concentration was determined via the bicinchoninic acid (BCA; Thermo Scientific) method per the manufacturer instructions.

2.3. Strong anion exchange fractionation

Strong anion exchange (SAX) was used to fractionate the purified intestinal precipitate matrix by protein isoelectric point (pI). An aliquot of matrix containing 550 μg of protein was first transferred into 20 mM tris (pH 8.0) via ultrafiltration using Amicon spin concentrators with a 3 kDa molecular weight cutoff and then filtered through a 0.45 μm hydrophilic polypropylene (GHP) syringe filter (Pall Corp.). Filtered sample was loaded onto a HiTrap Capto Q ImpRes (GE Life Sciences) SAX column and slowly eluted via 5 ml ‘bumps’ of increasing NaCl concentration in 20 mM tris (pH 8.0). The first bump consisted of 20 mM tris with no NaCl, while subsequent bumps increased in NaCl concentration by intervals of 50 mM up to 500 mM. The final elution contained 1000 mM NaCl to elute highly acidic proteins, resulting in a total of 12 fractions. Individual fractions were again exchanged into TBS via ultrafiltration and concentrated to a final volume of 100 μl. Proteins were also extracted from cryogenically ground toadfish intestinal tissue by sonication in TBS and SAX fractionated for comparison.

2.4. In vitro calcification assay

To determine if the fractionated matrix effects the rate of carbonate mineral production, a previously described in vitro calcification assay was used (Schauer et al., 2016). Briefly, fractionated matrix proteins were added to a 96-well plate at two different concentrations (0.25 μg/ml and 1.0 μg/ml) along with two thymol blue-containing solutions that when combined, mimic the ionic composition of the toadfish intestinal fluid. Six technical replicates were completed at the low concentration (0.25 μg/ml), and three replicates at the high concentration (1.0 μg/ml). The 594 nm absorbance of each of the wells was measured over a period of 20 h, allowing for the pH to be determined due to the presence of thymol blue. The drop in pH (or increase in H+ concentration) was used as a proxy for carbonate mineral formation, as an H+ ion is released as part of the precipitation process according to the following reaction:

$$\text{Ca}^{2+} + \text{HCO}_3^- \rightarrow \text{CaCO}_3 + \text{H}^+$$

From this, two metrics were calculated to compare across samples: nucleation time and calcification rate. Nucleation time was defined as the time at which the H+ concentration increased by 10% of the total increase observed throughout the duration of the experiment. Calcification rate was the slope of a linear regression fit to the calculated H+ concentration from the time at which the H+ concentration increased by 20% of the total increase over the 20-hour experiment, to the time it reached 60% of the total increase. This period was chosen as it accounts for the portion of the experiment with maximum calcification. A more detailed description of the calculations used can be found in Schauer et al. (2016). Nucleation times and calcification rates were compared to the respective values for the no protein control using pairwise permutation tests with an FDR-based (Benjamini-Hochberg) multiple comparison correction.
2.5. Mass spectrometry analysis

With the exception of a 1 μl aliquot retained for the calcification assay and other experiments, the entirety of the matrix fractions were reduced, alkylated and trypsin digested for MS analysis as described previously (Schauer et al., 2013). The total amount of protein digested varied from ~2 to 22 μg depending on the fraction. MS analysis was completed at the Colorado State University Proteomics and Metabolomics Facility (Fort Collins, CO). Complete methodology and instrument parameters are described elsewhere (Adewole et al., 2016). Briefly, approximately 1 μg of digested peptides were loaded onto a 100 mm C18 column and eluted via a 30-minute linear gradient of increasing acetonitrile. Peptides were eluted directly into a Orbitrap Velos Pro (Thermo Scientific) operating in data-dependent acquisition mode where MS scans were collected in the orbitrap (resolution of 60,000), ions were activated using collision induced dissociation (CID), and MS/MS scans were completed in the ion trap. Raw MS/MS data was searched using Mascot (v2.2, Matrix Science) against the Gulf toadfish transcriptome (NCBI BioProject PRJNA313355) that was translated into all six possible reading frames, as described in Schauer et al. (2016). The search database also included sequences for common contaminant proteins from the common Repository of Adventitious Proteins (cRAP; http://www.thegpm.org/crap/) to minimize the likelihood of false positives due to contamination, and was reverse concatenated to allow for the calculation of false discovery rates. Data was searched using trypsin as the digestion enzyme, as well as no enzyme to allow for the detection of non-tryptic peptides. Tryptsin and no enzyme search data were combined, loaded into Scaffold (v4.4, Proteome Software), and filtered using minimum two peptides per protein as well as 95% peptide probability and 99.0% protein probability cutoffs. Probable identifications for protein products were determined via a combination of the Blast2GO pipeline (Conesa et al., 2005) and manual BLAST searching against the NCBI non-redundant database. Calcium binding domains in the proteins were identified using the NCBI conserved domain database (Marchler-Bauer et al., 2015) and isoelectric points were estimated using the isoelectric point calculator web service (Kozlowski, 2016).

2.6. Calculation of protein promotional indices

To determine which proteins were likely responsible for the observed changes in the calcification assay, promotion indices were calculated for each protein (p) identified in the mass spectrometry analysis. Exclusive spectral counts (s; a measure of relative protein abundance) were first normalized to the mass of digested peptides (m) injected into the MS for each fraction (f) by the following equation:

\[ a_{p,f} = \frac{s_{p,f}}{m_f/M} \]

These adjusted spectral count values \( a_{p,f} \) were used as a measure of the relative abundance of each identified protein in the fractions. Control normalized nucleation times for each fraction (\( n_f \)) at the 0.25 μg/ml concentration were then sign-corrected so that values less than one were presented as the inverse, while values greater than one were given as a negative value, resulting in negative values being indicative of inhibition of calcification, and positive values representing promotion. Finally, a promotion factor (\( r_p \)) was calculated for each protein using the equation:

\[ r_p = \sum_{f=1}^{n} a_{p,f} \times n_f \]

The same statistic was also calculated using control normalized calcification rates in place of nucleation time. For these calculations, the calcification rates were corrected using the same method used for the nucleation time (discussed above) except that the signs were reversed such that positive values were again indicative of the promotion of calcification.

2.7. Scanning electron microscopy

Sample preparation was completed using the methods described in Schauer et al. (2016). Fractionated matrix samples were added at a final concentration of 0.25 μg/ml to the calcification assay buffers (lacking thymol blue), and immediately transferred to aluminum specimen mounts. After a 20 h incubation at 26 °C in a humid chamber, the liquid was removed leaving the precipitated mineral bound to the mounts. Carbonates were rinsed 3 × with ultrapure water, dried at 37 °C for 1 h, and palladium coated. Images were acquired on an FEI XL-30 field emission ESEM/SEM.

3. Results

3.1. Fractionation of toadfish intestinal precipitate matrix

A total of 640 μg of matrix protein was isolated from the intestinal precipitates collected throughout the duration of the collection period. When 550 μg of this samples was fractionated by SAX, a large portion of the proteins (nearly 20%) only eluted from the column when the highest (1000 mM NaCl) salt concentration was used (Fig. 1). This suggests that the matrix is enriched in highly acidic proteins, as very little (<2%) of the intestinal proteins required such high salt concentrations to elute.

3.2. In vitro calcification assay

The in vitro calcification assay revealed that different matrix fractions had significant effects on the rate of carbonate mineral formation (Fig. 2). Some fractions promoted calcification (decreased nucleation time, increased calcification rate) while others had an inhibitory effect. Somewhat surprisingly, most of the fractions that showed an increased nucleation time also showed an increased calcification rate, suggesting that although the start of crystal formation was inhibited, the rate of mineral production was faster once seed crystals had formed. Overall, the protein concentration did not influence the general trends (inhibition/promotion) of the fractions, but the higher concentration typically showed a more pronounced effect (Fig. 2). Interestingly, four of the fractions (300 mM–450 mM NaCl eluted) showed inhibition of calcification at the lower concentration, but completely inhibited the process at higher concentrations, with less than a 0.1 drop in pH observed throughout the duration of the 20 h assay.

3.3. Mass spectrometry analysis of fractionated matrix

Between all the fractions, protein products from 67 unique toadfish loci, as well as 11 contaminants proteins from the cRAP database, were identified in the mass spectrometry analysis (Table S1). Protein products from 30 (45%) of the same loci have been previously identified in the toadfish intestinal precipitates using another, less stringent purification procedure, while another 12 proteins (63% in total) mapped to different loci, but upon BLAST searching showed similarity to previously identified matrix proteins isolated via the same method (Schauer et al., 2016). Eleven additional proteins identified here have been shown to associate with fish intestinal precipitates using another, less stringent purification method (Schauer et al., 2016). Therefore, nearly 80% of the proteins identified here have been previously shown to associate with toadfish intestinal precipitates even though previous reports have not included a no enzyme search as was done in this study. When protein identifications using only tryptic peptides are included, the proportion of previously identified matrix proteins increases to over 85%. The distribution of the identified proteins between fractions varied greatly depending on the protein (Fig. 3 and Table S2), with some proteins being present in only a single fraction, while others were found in all twelve.
3.4. **Calculated protein promotional indices**

The promotional indices that were calculated give further insight as to which proteins may be involved in causing the observed effects on calcification, and revealed that slightly more proteins were likely to be involved in inhibiting precipitation rather than promoting it (Fig. 4). Only promotional indices calculated using the nucleation time metric are shown in Fig. 4, as the results shown in Fig. 2 show that this metric is likely more sensitive than calcification rate. Further, only results from the lower tested concentration (0.25 μg/ml) were used to avoid complication of the analysis due to the absence of mineral formation in some of the fractions tested at the higher concentration. Notably, all the proteins containing known calcium binding domains were found to have an inhibitory nature.

When the promotional indices for each protein calculated using the calcification rate metric are compared to those using nucleation time (Fig. 5), the values show a strong, positive correlation when the promotional index is positive (Pearson correlation, \( r = 0.619 \)). In other words, proteins are given a similar promotional index if they were shown to promote calcification, regardless of whether nucleation time in calcification rate is used. This trend breaks down for those proteins given a negative index using the nucleation time calculations. This is not surprising as none of the fractions showed a significant decrease in calcification rate at the lower of the two concentrations tested, therefore negative promotional indices are unlikely when using the calcification rate in the calculations. What is somewhat surprising is that nucleation time calculated protein indices with a negative value were inversely related to the promotional indices calculated using calcification rate (Pearson correlation, \( r = -0.704 \)). This suggests that at least at the lower concentration tested, proteins that cause an increase in nucleation time (delay the start of calcification; negative promotion index) increase calcification rate, which is opposite of what would be expected for a general inhibition of mineral formation. This can also be seen in Fig. 2, as most of the fractions showing increased nucleation time also show an increase in calcification rate when compared to controls.

3.5. **Electron microscopy analysis**

SEM analysis revealed that the presence of different fractions in the calcification solution affected the morphology of the carbonate mineral that was produced during the in vitro calcification assay (Fig. 6). Generally, the specimens were uniformly covered with small crystals upon completion of the assay, yet several of the fractions had fewer, larger crystals (Fig. 6J–K). Distinct changes in morphology were visible in other fractions as well, suggesting that the proteins in the different fractions were capable of influencing crystal morphology.

Further, atomic absorption spectroscopy was used to measure the magnesium to calcium ratio of the mineral formed, as previous work has shown that the matrix is capable of modulating the incorporation of Mg into the mineral (Schauer et al., 2016). Mineral was formed in the presence of the different fractions (0.25 μg/ml) on aluminum specimen mounts using the same method that was used for the SEM analysis. Instead of coating the samples with palladium, the mineral was completely dissolved by the addition of 1 N HCl, and the concentration of Mg\(^{2+}\) and Ca\(^{2+}\) in the resulting solutions was measured. No significant difference in magnesium incorporation was observed between the samples containing the different fractions (data not shown), however sample limitation only allowed for three replicates per fraction resulting in low statistical power.

4. **Discussion**

SAX fractionation of purified toadfish intestinal precipitate organic matrix revealed that the proteinaceous components are enriched with unusually acidic proteins when compared to whole protein extracts from the intestinal epithelium (Fig. 1). This is a common trait among proteins involved in biomineralization, especially those where calcium carbonate is formed (Constantz and Weiner, 1988; Marin and Luquet, 2007). It is believed that acidic domains of matrix proteins interact with the cationic constituents (typically Ca\(^{2+}\)) of the mineralization solution and act as a template for mineral formation, as well as attract further cations to the site of precipitation (Marin and Luquet, 2007; Smeets et al., 2015). It has also been suggested that acidic, hydrophilic peptides or proteins may increase the rate of CaCO\(_3\) formation by decreasing the diffusive barrier around the forming mineral due to perturbation of the structuring of water at the site of mineralization (Elhadj et al., 2006). However, the same proteins that promote mineral formation at low concentrations are often capable of inhibiting precipitation at higher concentrations (Heinemann et al., 2011). Similar trends have been observed in unfractionated organic matrix isolated from toadfish...
precipitates, with low concentrations of matrix promoting mineralization, and high concentrations showing an inhibitory effect (Schauer et al., 2016). The observation that acidic proteins make up a substantial portion of the intestinal precipitate matrix may account for some of these effects.

Results from the in vitro calcification assay testing the unfractionated matrix correspond well with previously collected data (Schauer et al., 2016), with a substantial promotion of mineral production (decreased nucleation time and increase calcification rate) at the lowest concentration (0.25 μg/ml), with a slight reduction of the effect at the 1.0 μg/ml concentration. It is intriguing that two of the fractions (0 mM NaCl, and 1000 mM NaCl) showed effects similar in magnitude to the unfractionated matrix. This suggests that the proteins in these two fractions are the primary drivers of the increased mineral formation. Although it is not surprising that one of these fractions (1000 mM NaCl) contains the most acidic proteins (for the reasons discussed above), it is somewhat unexpected that the fraction which should contain the most basic proteins (0 mM NaCl) showed such an effect. It is possible that the basic nature of these proteins allows them to interact with CO₃²⁻ or HCO₃⁻ in solution, or alternatively, the hydrophilicity of the proteins may be the primary driver of the observed effects, by decreasing the magnitude of the diffusive barrier as described above.

Notably, highly basic proteins are found in the matrix of bivalve mollusk shells, and have been implicated in biomineralization processes in these animals (Hüning et al., 2016; Liang et al., 2016).

The results from the mildly basic fractions (300 mM–500 mM NaCl) are more difficult to interpret due to the increased nucleation time (and therefore inhibition of mineral production), but contrasting increase in calcification rate. We can think of several possible explanations for this effect. First, it is possible that these fractions of matrix contain some

![Figure 2](image-url)

**Figure 2.** Effect of fractionated intestinal precipitate matrix on in vitro carbonate mineral formation. No protein control normalized nucleation times (A) and calcification rates (B) for each of the fractions at two different concentrations (0.25 μg/ml, black bars; and 1.0 μg/ml, gray bars), as determined using the in vitro calcification assay. Values showing significant differences (p < 0.05) from controls, as determined by FDR-corrected pairwise permutation tests, are marked with asterisks. n = 6 for 0.25 μg/ml samples; n = 3 for 1.0 μg/ml.
proteins with an inhibitory nature, while others promote calcification. Should this be the case, one could speculate that early in the precipitation reaction, when few or no crystals are present, the concentration of the inhibitory proteins is great enough to limit the substantial growth of mineral as sufficient protein would be available to block the majority of crystal growth sites. However, as the amount of mineral increases, the concentration of inhibitory proteins may not be sufficient to effectively block further precipitation, allowing for the effects of the proteins which promote calcification to be observed. Alternatively, certain proteins in these fractions may be capable of increasing the rate of mineral formation once seed crystals are formed, but do not alter the nucleation process. Such proteins could be present in these fractions, along with other proteins that inhibit crystal nucleation. Clearly, the mechanisms behind the observed effects on calcification warrant further investigation.

It is also worth considering the changes in crystal morphology when discussing the observed effects on mineral production rates. It is well known that the constituents of organic matrices from different biomineralization systems can change the morphology, and even the polymorph of mineral that forms (Morse et al., 2007; Rahman and Shinjo, 2012). SEM data collected here (Fig. 6) supports that toadfish intestinal precipitate matrix may show a similar effect, as the inclusion of different fractions of the matrix in the precipitation solution affect the morphology of the resulting mineral. Seeing as how most of the fractions with both increased nucleation time and calcification rate also showed altered crystal morphology (Fig. 6J–L) when compared to the mineral produced by other fractions, it is possible that the alterations in mineral formation rate is due to inherent differences in the growth processes of different crystal types. If the matrix proteins in the different fractions are driving the precipitation of different crystal morphologies, some changes in the rate of mineral formation would be expected due to the ability of different crystal structures to accommodate impurities such as Mg$^{2+}$ and SO$_4^{2-}$ into the forming crystal lattice, as well as the different rates of crystal nucleation from solution (Berner, 1975). For example, polymorphs which cannot accommodate Mg$^{2+}$ and SO$_4^{2-}$ would show decreased crystal growth rates due to the presence of these ions in the precipitation solution (Morse et al., 2007), as the presence of these ions would serve to inhibit the precipitation reaction. Alternatively, the changes in crystal morphology may be a result of differing precipitation rates as opposed to a cause, which has also been reported in previous biomineralization investigations (Wheeler and Sikes, 1984). Future work that investigates the physical properties of the forming mineral will help to evaluate these possibilities, but was outside of the scope of this study.

It is difficult to determine what protein concentration is representative of the conditions that would be found in vivo, and therefore, if the concentrations of protein tested here are biologically relevant. The reason for this complication is due to the inherent difficulties with determining the absolute concentrations of numerous individual proteins in a sample, as absolute quantitation currently requires a priori knowledge of the system and targeted analyses (Liebler and Zimmerman, 2013). Total protein concentrations in the intestinal lumen regularly exceeds 500 µg/ml (personal observation), which is several orders of magnitude higher than the concentrations tested here. Of course, the intestinal fluid contains hundreds if not thousands of different proteins (Schauer et al., 2016), most of which are entirely unrelated to mineral precipitation. Adding further complexity to this question is the possibility that the precipitation reaction occurs in some sort of microenvironment such as a modified matrix vesicle or mucus compartment, as has been suggested previously (Humbert et al., 1986; Schauer et al., 2016). Future experiments should aim to determine the absolute concentrations of some of the individual matrix proteins identified here in both the intestinal fluid as well as isolated precipitate matrix to gain further insight as to the conditions which occur in vivo.

MS analysis led to the identification of protein products from 67 unique toadfish loci, 80% of which were identical to or showed high similarity to proteins previously known to associate with precipitates
forming in the toad intestine (Schauer et al., 2016), demonstrating the reproducibility of the purification procedure. The average pI for the identified toad proteins was near neutral (6.75), which is somewhat surprising due to the high amount of protein that required high salt concentrations to elute off the SAX column (Fig. 1). However, it is worth noting the protein pI values were calculated directly from the sequences taken from the translated toad transcriptome, a database which has not been curated to remove untranslated regions, etc. Therefore, the reported pI values may not be indicative of the mature protein, but general trends (highly acidic, highly basic, etc.) can likely be assessed. Interestingly, when the average pI for the identified peptides is calculated, the value is much lower (4.73) than that for the proteins. Seeing as how positive-mode MS analyses conducted under acidic conditions (as was done here) usually favor the identification of more basic peptides (Riley et al., 2015), it is unlikely that the preferential identification of these acidic peptides is an artifact of the MS analysis. This suggests that at least in some instances, acidic protein fragments may associate with the forming mineral as opposed to the whole protein. However, approximately 83% of all peptides identified in the MS analysis were of tryptic origin (C-terminal lysine or arginine, as well as a lysine or arginine immediately N-terminal to the start of the identified peptide), so if only certain portions of the proteins were associating with the forming carbonate mineral, then the portions must have been either quite large (allowing for multiple tryptic digests to be produced), or were derived from in vivo tryptic digestion.

Generally, individual proteins identified in the mass spectrometry analysis were distributed in a single fraction or several adjacent fractions (Fig. 3 and Tables S1 and S2), as would be expected. However, there were several notable exceptions where peptides derived from a single protein were identified across almost all the fractions, or where they were identified in both the highly basic (low NaCl elution concentrations) and highly acidic fractions. Most of the proteins that were identified in all (or nearly all) of the fractions were keratin isoforms. These proteins are likely at least partially human contaminants that were introduced post fractionation. Despite the inclusion of many human keratins in the cRAP database, the list is not comprehensive, and the high homology between the toad and human keratins could lead to the assignment of human derived keratin peptides to toad proteins. This would artificially inflate the number of spectral counts assigned to the toad keratins and would account for the wide distribution of these proteins in the different fractions. Another protein that was identified across all the fractions was ubiquitin-40S ribosomal protein S27a. This protein serves as a precursor to the mature ubiquitin protein that covalently binds to lysine residues of target proteins to form the mature, post-translational modification involved in the regulation of numerous cellular processes (Beaudette et al., 2016; Komander and Rape, 2012). The peptides identified which map to this protein all lie within the mature ubiquitin molecule, suggesting that the identification of this protein in numerous fractions could be due to the presence of ubiquitinated proteins in those fractions. To test this
The calculation of individual protein promotional indices revealed several interesting protein products that may be involved in the regulation of carbonate mineral precipitation in the teleost intestine. One such protein was NADPH-cytochrome P450 reductase, which had the second highest calculated promotional index (Fig. 4A). This enzyme is typically located in the endoplasmic reticulum and is involved in oxidative metabolism of numerous compounds (Wang et al., 1997). This same protein was the second most abundant matrix constituent identified in the previous characterization of toadfish derived intestinal precipitate matrix, yet was not identified elsewhere in the intestinal fluid, suggesting strong enrichment of this enzyme in the matrix (Schauer et al., 2016). Further, although the protein itself has a nearly neutral isoelectric point (7.44), portions of the protein are highly acidic, as the mean pI for the peptides identified in the MS analysis was 4.72, with seven of the eight unique identified peptides having a pI under five. This protein is clearly of particular interest and warrants further investigation in the future.

Fig. 5. Correlation of promotional indices calculated using nucleation time versus calcification rate. Promotional indices were calculated using either nucleation time or calcification rate. Each black dot represents a single protein and its corresponding indices calculated using the two different methods. A perfect 1:1 correlation is shown by the black dashed line, where an exact inverse relationship (1:−1) is illustrated in gray.

hypothesis, MS data was searched again, this time including a variable diglycine modification to determine if ubiquitin modification sites could be determined (Xu and Jaffrey, 2013). None were identified, but this is not necessarily surprising due to the likely low abundance of these modifications at a specific site, and the lack of inclusion of an enrichment step for these experiments. Nonetheless, future investigations may wish to examine the ubiquitination of proteins found in the precipitate organic matrix.

For cases where the same protein was identified in both acidic and basic fractions, it is possible that the proteins were degraded prior to the fractionation procedure, and therefore the more basic portions of the protein would elute off the SAX column at lower salt concentrations than the more acidic portions. Such degradation would not be surprising due to the high numbers of proteases present in the intestinal fluid where the precipitates form, in conjunction with the numerous steps involved in the purification and fractionation process. However, as discussed previously, approximately 83% of all peptides identified in the MS analysis were of tryptic origin, suggesting that degradation was not substantial, as many non-tryptic peptides would be expected in the case of rampant degradation.

Other interesting proteins assigned a positive promotional index include two heat shock proteins (heat shock cognate 70 kDa protein and heat shock protein 90-alpha) as well an unknown protein (Muscle_L_10615_T_1/1) that does not show significant similarity to any proteins in the NCBI non-redundant database. Both heat shock proteins were identified only the fractions that showed the highest degrees of promotion (0 and 1000 mM NaCl) which attributes to their high calculated promotional index, despite having relatively low abundance. Similarly, the unknown protein (Muscle_L_10615_T_1/1) was only identified in the most acidic fractions. Purification of, and investigation of these proteins individually would aid in understanding their roles in the regulation of the precipitation reaction.

Over half of the proteins identified in the matrix had calculated promotional indices that were negative, suggesting they serve to limit mineral formation. All proteins found to contain known calcium binding domains fell in this category. This could be the result of the proteins sequestering free calcium in the solution, limiting that which is available for mineral precipitation. This seems somewhat unlikely, however, due to the low concentration of protein present in the calcification assay (0.25 µg/ml), and the large amount of calcium in solution (20 mM). Such a mechanism would therefore only be effective should the proteins be concentrated around the forming mineral, allowing for modification of the micro-environment at the site of precipitation. More likely, the calcium binding domains of these proteins are interacting with calcium that has already been incorporated into the mineral, as has been shown for the molluskan acidic matrix protein, Pif (Suzuki et al., 2009). Acidic proteins involved in biomineralization have been previously shown to interact with formed mineral, and inhibit further precipitation by binding to crystal steps, and therefore preventing further crystal growth (Mann et al., 2007). Such a mechanism is likely at play here.

The observation that mucin-2 has the lowest promotional index (most inhibitory) is particularly intriguing. Precipitates formed in the intestine are typically covered in a thick mucus layer (Humbert et al., 1986; Schauer et al., 2016; Walsh et al., 1991). It is possible that the formation of this mucus layer around the precipitates signals the end of mineral formation, and prevents the production of
precipitates that are too large, which could cause intestinal blockage. This idea is in contrast to early electron microscopy studies that suggested that the mucus may serve to nucleate mineral production, or produce a microenvironment for calcium concentration allowing for precipitation to occur (Humbert et al., 1986). These speculations were drawn from visual observation only, so it is possible (if not likely), that the mechanisms at work could not be fully captured via the microscopy analysis.

Phospholipase A2 was the protein with the second lowest promotional index, and is another protein that was previously shown to be highly enriched in the precipitate organic matrix compared to the surrounding intestinal fluid (Schauer et al., 2016). This protein is part of a superfamily of enzymes involved in the hydrolysis of fatty acids which can be either secreted or found intracellularly, and are perhaps best known for their presence in many snake venoms (Burke and Dennis, 2009). These proteins are known to bind calcium ions, which in most cases serve as a cofactor for the catalysis of lipid breakdown. Just behind phospholipase A2, with the third lowest promotional index, is the well-known calcium binding protein calmodulin. The low calculated promotional indices of these two proteins, along with the presence of all the other proteins containing known calcium binding domains in the inhibitory list, suggests that calcium binding by proteins is likely involved in the regulation of precipitation in the teleost intestine via the mechanisms discussed earlier.

Fig. 6. Changes in mineral crystal morphology in the presence fractionated matrix. SEM images showing the resulting mineral produced in the absence of protein (A), versus 0.25 μg/ml of unfractionated intestinal precipitate matrix protein (B), or fractionated matrix eluted from SAX columns via 0 mM to 500 mM NaCl (in 50 mM increments; C–M) or 1000 mM NaCl (N). Scale bar = 20 μm.
Also of note are the three unknown proteins (Muscle_L_6293_T_1/4, Muscle_L_12311_T_1/1, and Neural_L_6714_T_1/1) with positive promotion indices which showed no substantial homology to any proteins in the NCBI database. The function of these proteins in the matrix is entirely unknown and therefore provides an interesting subject for future study. For one of these proteins, Neural_L_6714_T_1/1, the peptides identified were all long stretches of glutamic acid repeats. Due to the acidic nature of glutamic acid, these peptides would be highly acidic and would therefore likely interact with the forming mineral. It is difficult to determine the source of the glutamic acid repeats – whether they were derived from a larger protein product, or were synthesized in some alternative fashion – providing an intriguing question for future studies.

The observation that promotion indices that are calculated using nucleation time or calcification rate are directly correlated when relative nucleation time values are positive, but inversely correlated when the values are negative is intriguing (Fig. 5). This effect is clearly related to the observation discussed above that fractions showing increased nucleation time generally showed increased calcification rate as well (contrary to the expectation for general precipitation inhibition), but is also affected by the abundance of each protein in the different fractions. We hypothesize that these observed effects are at least partially due to the low protein concentration (0.25 μg/ml) used in the calcification assay from which these values were derived, due to previously discussed mechanisms of precipitation regulation. If we were to take the case of a calcium binding protein in the precipitation medium as an example, one could imagine that even at low concentrations, this protein would bind nearly all small crystals that precipitate out of solution early in the assay and block further precipitation by blocking the growth sites. As time goes on and the number of precipitated crystals increases, a point would be reached at which the protein concentration is no longer high enough to block all crystal growth sites. At this point, mineral would begin forming more rapidly and the calcium binding ability of the protein may further the precipitation reaction by concentrating calcium ions near the site of precipitation. This is only one of many possible mechanisms that would cause such an effect, and is an intriguing avenue for future study.

The observed changes in crystal morphology suggest that matrix proteins may be involved in determining the crystal structure or polymorphism of the mineral that forms in the fish intestine. Toadfish precipitates produced in vivo consist of many small (<0.05 μm) fairly round crystals (Woosley et al., 2012), which are distinct from the crystals shown in Fig. 6. However, this is not necessarily surprising due to the in vitro nature of these experiments. Unfortunately, limited sample availability prohibited further investigation of crystal structure by more sensitive methods such as X-ray diffraction (XRD). Limited sample availability may also be responsible for the lack of observed changes in mineral magnesium incorporation, due to the low sample sizes used for the atomic absorption spectroscopy analysis. However, previous work only observed changes in magnesium incorporation at levels higher than those tested here (2.0 μg/ml versus the 0.25 μg/ml tested here), which could also be responsible for the lack of any observed effect (Schafer et al., 2016). Further, it should be emphasized that these analyses are bulk analyses that measure the incorporation of magnesium but do not address finer scale changes at the individual crystal level, which could certainly occur.

The data presented here provides further confirmation that proteins associated with the forming mineral are involved in regulating the precipitation of carbonates in the marine teleost intestine, and provides the first evidence as to what the individual functions of these matrix proteins may be. Analysis of fractionated matrix on the effects of precipitation allowed for individual protein promotional indices to be calculated, giving the first insights into the function of individual matrix proteins. Using the data presented here, future studies can isolate and study individual proteins to further test the predictions proposed here. By increasing our knowledge of the role proteins play in regulating teleost intestinal precipitation, we can better understand how the largest group of vertebrates on the planet, marine fish, are able to maintain homeostasis, as well as how fish may respond and contribute to future changes in the marine environment. An increased understanding of the molecular mechanisms that control not only the rate of teleost intestinal precipitation, but also the physical characteristics of the forming mineral will aid in future investigations that aim to quantify the large-scale impacts of teleost derived precipitate production and dissolution, and the effects of these processes on oceanic carbon cycling.

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References


