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In-depth proteomic analysis of a mollusc shell: acid-soluble and acid-insoluble matrix of the limpet *Lottia gigantea*

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Abstract

Background: Invertebrate biominerals are characterized by their extraordinary functionality and physical properties, such as strength, stiffness and toughness that by far exceed those of the pure mineral component of such composites. This is attributed to the organic matrix, secreted by specialized cells, which pervades and envelops the mineral crystals. Despite the obvious importance of the protein fraction of the organic matrix, only few in-depth proteomic studies have been performed due to the lack of comprehensive protein sequence databases. The recent public release of the gastropod *Lottia gigantea* genome sequence and the associated protein sequence database provides for the first time the opportunity to do a state-of-the-art proteomic in-depth analysis of the organic matrix of a mollusc shell.

Results: Using three different sodium hypochlorite washing protocols before shell demineralization, a total of 569 proteins were identified in *Lottia gigantea* shell matrix. Of these, 311 were assembled in a consensus proteome comprising identifications contained in all proteomes irrespective of shell cleaning procedure. Some of these proteins were similar in amino acid sequence, amino acid composition, or domain structure to proteins identified previously in different bivalve or gastropod shells, such as BMSP, dermatopontin, nacrein, perlustrin, perlucin, or Pif. In addition there were dozens of previously uncharacterized proteins, many containing repeated short linear motifs or homorepeats. Such proteins may play a role in shell matrix construction or control of mineralization processes.

Conclusions: The organic matrix of *Lottia gigantea* shells is a complex mixture of proteins comprising possible homologs of some previously characterized mollusc shell proteins, but also many novel proteins with a possible function in biomineralization as framework building blocks or as regulatory components. We hope that this data set, the most comprehensive available at present, will provide a platform for the further exploration of biomineralization processes in molluscs.

Background

Molluscan shells are extraordinarily stable biocomposites of calcium carbonate and an organic matrix consisting of polysaccharides and proteins. The organic matrix, although constituting a very minor fraction of the biocomposite by weight, is thought to be of utmost importance for the construction of the biocomposite and its final properties because it controls crystal nucleation, crystal growth, crystal shape and choice of calcium carbonate polymorph [1,2]. Previously established methods

to identify new mollusc shell matrix proteins, such as isolation by chromatography and biochemical characterization or molecular biology approaches, have been complemented recently by mass spectrometry-based proteomic analysis or combination of proteomic and transcriptomic studies [3-11]. However, proteomic approaches depend on the comparison of experimentally determined spectra with theoretical spectra obtained by *in silico* digestion of proteins and *in silico* fragmentation of resulting peptides [12,13]. Therefore protein sequence databases that are as comprehensive as possible, usually derived from genome sequencing, are presently indispensable for high-throughput proteomics. The need for a comprehensive database is highlighted by previously published proteomic studies of

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shell matrices in various molluscan species [3-11]. These studies relied on translated EST databases contributed by a number of groups [7,11,14-18] and usually less than 15 proteins were identified from isolated organic matrices. Sometimes database searches were combined with *de novo* mass spectrometric sequencing. However, *de novo* sequencing algorithms, which attempt to interpret spectra independently of a sequence database [19], are not compatible with high-throughput analysis at present. Transcriptomics, on the other hand, does not identify matrix proteins directly, making additional techniques, such as immunohistochemical localization, necessary to demonstrate the actual location of potential shell matrix proteins. Thus, although previous studies have identified several very interesting new matrix proteins, these studies may fail to show the actual complexity of the shell matrix proteome indicated by proteomic studies of biomineral matrices of organisms with sequenced genomes, such as chicken [20] or the sea urchin *Strongylocentrotus purpuratus* [21-23].

The first genome sequence of a mollusc, the limpet *Lottia gigantea*, was made public recently (<http://genome.jgi-psf.org/Lotgi1/Lotgi1.download.html>) [24]. In the present report we used a protein sequence database derived from this genome sequence to perform a high-throughput in-depth proteomic analysis of the shell matrix of this marine snail.

The shell of *Lottia* and related limpets consists of five layers [25,26], which are divided into 3 outer layers, M + 1, M + 2 and M + 3 and separated from an inner layer M - 1 by the intermediate myostracum (M layer). The outermost layer, M + 3, is reported to contain calcite as mineral phase. This layer appears eroded and often disappears altogether around the top of the shell. The M + 2 layer consists of flat prismatic crystals made of aragonite, another common calcium carbonate mineral. The M + 1 and M - 1 layers are described to consist of lamellar prisms similarly made of aragonite. Compared to the other layers, the M layer, sandwiched between M + 1 and M - 1, is very thin and has a prismatic structure of aragonite. Organic matrix was visible in M + 3 and M + 2, but was not detected in other layers [25].

Using LTQ Orbitrap Velos high-performance mass spectrometers [27] in combination with the MaxQuant software package designed for analysis of large high-resolution mass spectrometric data sets [28-30] we identified 311 proteins in the organic matrix of the *Lottia* shell with very high stringency. This is the first in-depth proteomic study of a mollusc shell matrix.

Materials and methods

The shells of freshly collected limpets were carefully cleaned manually and treated with sodium hypochlorite solution (Merck, Darmstadt; Germany; 6-14% active chlorine) to remove organic surface contaminants. Shells

were either treated with hypochlorite for 2 h at room temperature (A), for 2 h with two 5 min ultrasonic treatments at the start of each hour (B), or for 24 h with two 5 min ultrasound bursts as before and one after 24 h (C). The shells were then washed with de-ionized water, dried, and crushed into small pieces using a hammer. The pieces were demineralized in 50% acetic acid (20 ml/g of shell) in a cold room overnight, yielding a dark brown suspension. Acid-soluble and acid-insoluble matrix was separated by centrifugation at 14000g_{av} at 5°C for 1 h. The pellet was washed twice by re-suspension in approximately 20 volumes of 50% acetic acid, centrifugation for 30 min at 14000g_{av}, and lyophilized. The supernatant was dialyzed twice against 10 volumes of 10% acetic acid followed by three times 10 volumes of 5% acetic acid at 4-6°C (Spectra/Por 6 dialysis membrane, molecular weight cut-off 2000; Spectrum Europe, Breda, The Netherlands), and lyophilized.

SDS-PAGE was done using pre-cast 4-12% Novex Bis-Tris gels in MES buffer with reagents and protocols supplied by the manufacturer (Invitrogen, Carlsbad, CA). Samples were suspended in 30 µl sample buffer/200 µg of organic matrix and heated to 95°C for 5 min. Sample buffer-insoluble matrix was removed by centrifugation in an Eppendorf bench top centrifuge for 5 min at 13000 rpm. Gels were loaded with 30 µl of matrix sample supernatant per lane and stained with colloidal Coomassie (Invitrogen) after electrophoresis. The protein standard used for molecular weight estimation was Novex Sharp, pre-stained (Invitrogen). Gels were sliced into 12 sections for in-gel digestion with trypsin [31]. The eluted peptides were purified on C18 Stage Tips [32].

Peptide mixtures were analyzed by on-line nanoflow liquid chromatography using the EASY-nLC system (Proxeon Biosystems, Odense, Denmark; now Thermo Fisher) with 15 cm capillary columns of an internal diameter of 75 µm filled with 3 µm Repronil-Pur C18-AQ resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The gradient consisted of 5-30% acetonitrile in 0.5% acetic acid at a flow rate of 250 nl/min for 85 min, 30-60% acetonitrile in 0.5% acetic acid at a flow rate of 250 nl/min and 60-80% acetonitrile in 0.5% acetic acid at a flow rate of 250 nl/min for 7 min. The eluate was electrosprayed into an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) through a Proxeon nanoelectrospray ion source. The Orbitrap Velos was operated in a HCD top 10 mode essentially as described [Olsen et al., 2009] at a resolution of 30,000 for full scans and of 7,500 (both at m/z 400) for MS/MS scans.

Data analysis was performed with MaxQuant (v1.1.1.36) [28,29], a computational proteomics platform based on the Andromeda search engine [30] (<http://www.maxquant.org/>), using the Lotgi1_GeneModels_Filtered Models1_aa.fasta.gz protein sequence database comprising 23,851 gene

models at present (<http://genome.jgi-psf.org/Lotgi1/Lotgi1.download.html>) [24], together with the corresponding reversed database and the sequences of common contaminants, including human keratins from IPIhuman. Carbamidomethylation was set as fixed modification. Variable modifications were set as oxidation (M), N-acetyl (protein) and pyro-Glu/Gln (N-term). Initial peptide mass tolerance was set to 7 ppm and fragment mass tolerance was 20 ppm. Two missed cleavages were allowed and the minimal length required for peptide identification was seven amino acids. The peptide and protein false discovery rates (FDR) were both set to 0.01. The maximal posterior error probability (PEP) for peptides, which is the probability of each peptide to be a false hit considering identification score and peptide length [28,29], was set to 0.01. The Re-quantify and Second Peptide [30] options were enabled. At least two MaxQuant group sequence-unique peptides with a score >100 were required for protein identification. Furthermore, identifications were only accepted if the peptides were identified in at least two replicates within the respective group A, B or C. Identifications with only two unique peptides were manually validated considering the assignment of major peaks, occurrence of uninterrupted γ - or β -ion series of at least 4 consecutive amino acids, preferred cleavages N-terminal to proline bonds, the possible presence of α_2/β_2 ion pairs and immonium ions, and mass accuracy. The ProteinProspector MS-Product program (<http://prospector.ucsf.edu/>) was used to calculate the theoretical masses of fragments of identified peptides for manual validation. BLAST and FASTA searches against non-redundant databases (all organisms) were performed using the programs provided by NCBI (<http://www.ncbi.nlm.nih.gov/blast>) and EBI (<http://www.ebi.ac.uk/Tools/sss/>). Domains were predicted with InterProScan (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) and PROSITE (<http://prosite.expasy.org/>). For sequence alignments we employed Kalign (<http://www.ebi.ac.uk/Tools/msa/kalign/>) and ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Sequence repeats were predicted using RADAR (<http://www.ebi.ac.uk/Tools/Radar/index.html>). The abundance of proteins was estimated by calculating the exponentially modified protein abundance index (emPAI) [33]. Observable peptides were determined and counted with Protein Prospector (<http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest>) using zero miss-cleavages, a peptide mass of 700–2800, and a minimal peptide length of seven amino acids. Observed unique parent ions with a minimal length of seven amino acids and a mass between 700–2800 used for emPAI calculation included ions with up to two miss-cleavages, modifications specified for MaxQuant analysis (see above), different charges, and neutral losses [33]. Proteins with emPAI ≥ 9 were referred to as major proteins in this report.

Results and discussion

Matrix isolation and characterization by SDS-PAGE

The cleaning of invertebrate biominerals usually involves washing in sodium hypochlorite using different incubation lengths. This is supposed to destroy and remove organic material at the biomineral surface, while intracrystalline organic matrix components are thought to be shielded from the destructive action of hypochlorite by the surrounding, densely packed, mineral. Because we wanted to study the effect of different sodium hypochlorite treatment length and the effect of ultrasonic treatment of shells during hypochlorite treatment on matrix composition, shells were either washed in hypochlorite solution for 2 h without (A) or with (B) short ultrasonic treatment, or for 24 h with short ultrasonic treatment (C). Comparison of the protein band pattern of the isolated matrices typically showed some minor, apparently predominantly quantitative rather than qualitative, differences (Figure 1A). However, PAGE comparison of matrices from different shells treated according to the same protocol showed comparable differences (Figure 1B). This suggests that not only experimental variables in the extraction protocol played a role, but possibly also individual biological factors, such as shell size, preservation and thickness of the outer calcitic shell layer, or environmental factors. The yields of organic matrix were between 2.2–5.3 mg/g of shell for the acid-soluble matrix, and between 2.1–4.6 mg/g for the acid-insoluble matrix (total of nine shells). The acid-insoluble matrix formed approximately half of the total organic matrix and the PAGE protein band patterns of soluble and insoluble matrices were very different (Figure 2). Therefore the proteomes of both fractions were analyzed separately. Several sets of data from different shells were evaluated together to establish a representative shell proteome. For A and B, four data sets (replicates) of matrices isolated from three different shells (8.8, 5.6, and 3.8 g of weight and 11.5, 9.1 and 4.1 g of weight, respectively) were analyzed. For C, two data sets were from a single large shell (8.6 g) and two data sets were from the pooled matrices of two small shells (2.9 and 1.5 g). Each data set was obtained from the analysis of tryptic peptides extracted from three gel lanes cut into 12 slices (Figure 2).

Proteomic analysis of matrix fractions

Proteomic analysis of all fractions (Figure 3; Additional file 1 and Additional file 2) clearly showed the effect of ultrasound treatment. Approximately 28% of the proteins of the acid-soluble matrix and 21% of the acid-insoluble matrix of shells not treated with ultrasound during hypochlorite cleaning (A) were identified only in these matrices but not in B or C (Figure 3). Differences between B (2 h hypochlorite) and C (24 h hypochlorite) were less clear. Surprisingly the number of protein and

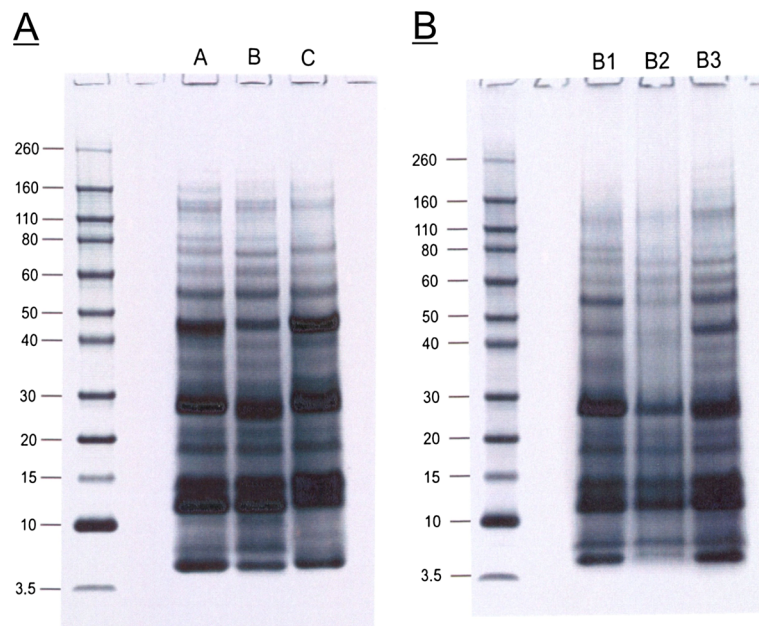
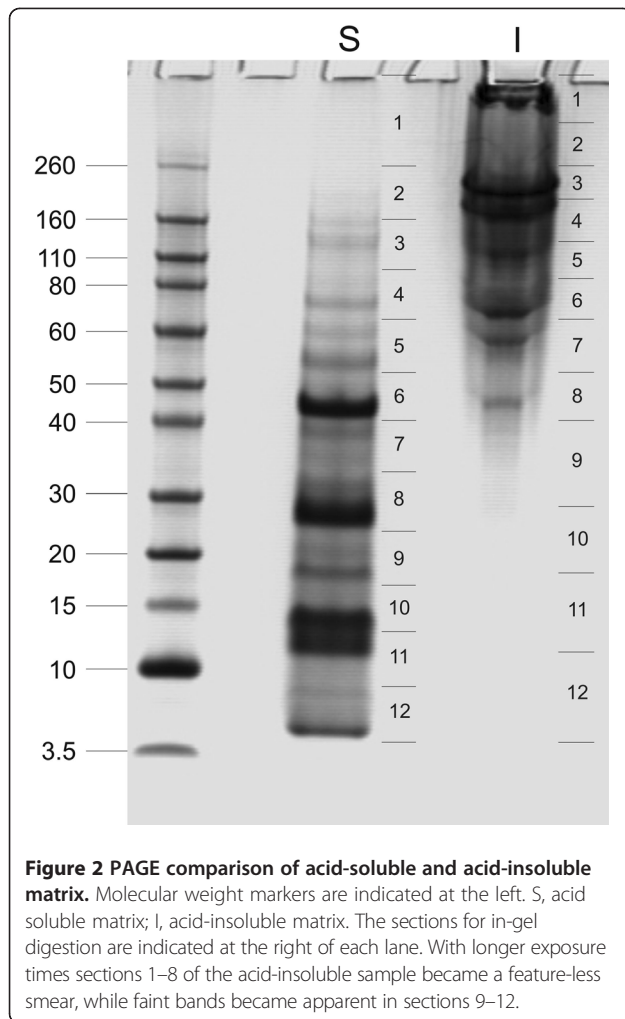


Figure 1 PAGE comparison of acid-soluble matrices from shells. Molecular weight markers are indicated at the left. Each lane was loaded with 200 μg of matrix in a volume of 30 μl . **A**, matrices of shells cleaned with different sodium hypochlorite protocols. Lane A, 2 h hypochlorite at room temperature; lane B, 2 h hypochlorite with 2 x 5 min ultrasound treatment at the start of each hour; lane C, cleaned with hypochlorite for 24 h with 2 x 5 min ultrasound bursts as before and one after 24 h. **B**, matrices of different shells, all cleaned with hypochlorite according to protocol B (2 h hypochlorite, 2 x 5 min ultrasound).

peptide identifications in the soluble fraction of C was greater than that of B (Additional file 1). Most of the proteins distinct between the two preparations were not unique but also occurred in A. This was difficult to explain, because all four replicates showed the same effect although they were prepared and analyzed at different times, sometimes on different mass spectrometers and often in sequence with replicates from other preparations. However, the qualitative differences between B and C were minor and focused almost exclusively on low abundance proteins. This may indicate that ultrasound treatment during cleaning with hypochlorite may have helped to solubilize and destroy proteins that stuck tenaciously to the biomineral surface. The length of hypochlorite treatment, however, apparently did not play a dominant role, at least after two hours of treatment. This aspect of hypochlorite treatment may become more important with nacreous shell layers, as our experience with *Haliotis laevis* has shown that lengthy treatments start to degrade the matrix surrounding nacre plates, leading to a partial loss of the outermost nacre layers.

Altogether 569 proteins were identified in matrices obtained after different hypochlorite treatments. To obtain a representative, high-confidence, shell matrix proteome of *Lottia gigantea*, we assembled a consensus proteome

comprising all database entries identified in all three types of samples (Figure 3). The consensus proteome of the acid-soluble fraction included 204 proteins and the consensus proteome of the acid-insoluble fraction contained 242 proteins. Given an overlap of 135, this summed up to a total of 311 *Lottia* database entries containing shell matrix protein sequences. However, these numbers should not be regarded as final because some database entries may eventually turn out to contain the sequence of more than one protein and some protein sequences may be divided among several database entries. Furthermore, the identifications not comprised in the consensus proteome are by no means to be considered as false positives but may be true shell matrix components. In most cases these were minor proteins and their absence or presence in different fractions may be due to experimental variability or the still limited dynamic range of mass spectrometers. Additional files 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 contain protein and peptide details, such as accession numbers of proteins sharing group-unique peptides, scores, masses, peptide sequences, and distribution in gel slices (Additional files 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14). Unlike Additional file 1 and Additional file 2 (Additional file 1 and Additional file 2), Additional files 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 contain data of all peptides and proteins identified within the set thresholds



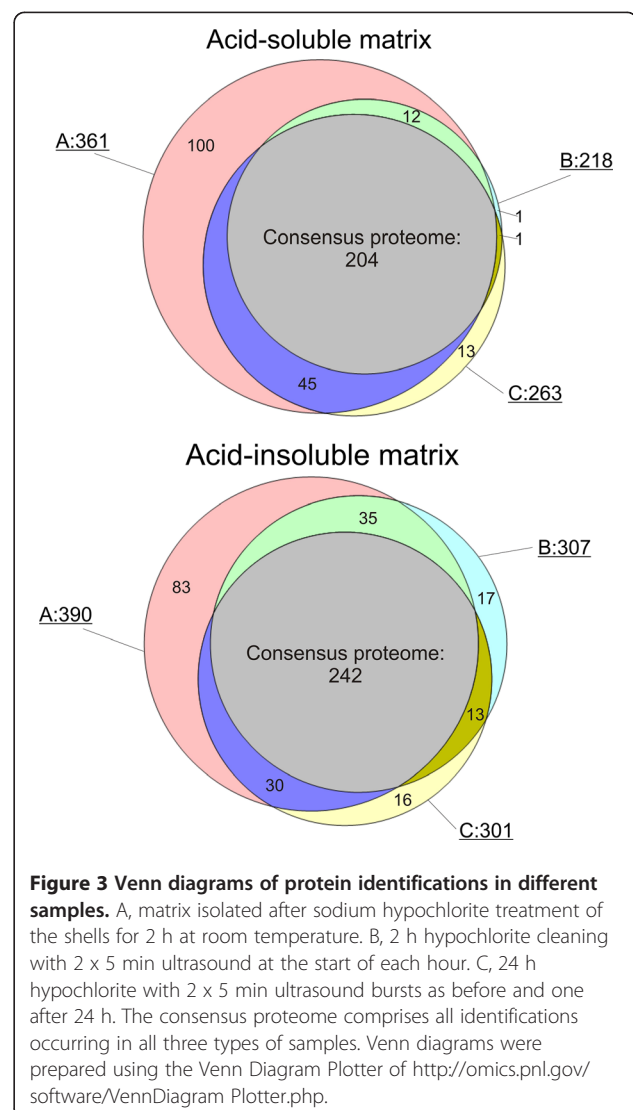
for MaxQuant searches (including identifications with one sequence-unique peptide), irrespective of whether they were accepted after manual inspection or not.

Both consensus proteomes contained intracellular proteins. In the soluble proteome these amounted to approximately 15% (Additional file 1). The acid-insoluble fraction contained approximately 36% (Additional file 2). Many of these proteins, such as the endoplasmatic reticulum and Golgi apparatus residents, may be by-products of secretion processes. Others may be releases into the extrapallial fluid by damaged or decaying cells of the epithelium lining the mantle cavity. Once in the extrapallial fluid, they have free access to the growing shell surface, may bind there, and may eventually be overgrown by further calcium carbonate deposition in shell growth periods. As true intra-crystalline components, although probably without any function, they may not be removed even by rigorous hypochlorite cleaning. Because the acid-insoluble consensus proteome contained more of these intracellular components, one may conclude that many of them were already structurally modified and

aggregated before incorporation into the growing shell. Proteins of previously known intracellular location were also found in other invertebrate skeletal matrices analyzed in depth using similar proteomic technology [22-24]. However, it is rather unlikely that matrix components with a well-defined intracellular location have any function in the shell. However, specific functional shell matrix proteins may be found among the major matrix proteins and those with recognized or predicted extracellular location.

Uncharacterized *Lottia* matrix proteins with unusual amino acid composition and short sequence repeats

The matrix of the *Lottia gigantea* shell contained many previously uncharacterized proteins (i.e. proteins without obvious sequence homology to known mollusc shell proteins) with unusual amino acid composition, short tandem repeats, and blocks of identical or similar amino acids (homorepeats). Often these characteristic primary



sequence features are found in terminal regions of shell proteins that have been proposed to be structurally unstable, unfolded domains able to adopt a specific structure only upon binding to a ligand, such as a crystal surface [34]. This proposition was based on experiments with synthetic polypeptides confirming the intrinsically disordered conformation of such shell protein domains and the *in vitro* interaction with calcium carbonate [35-39]. However, most known features of such short linear motifs and homorepeats come from intracellular examples [40,41]. Apart from occurring predominantly in natively disordered structures, such motifs mediate protein-protein interactions with low affinity, which is usually compensated by frequent repetition of the motif. Examples of major (average emPAI ≥ 9) *Lottia* matrix proteins with peculiar primary sequence features are shown in Table 1. Many of these proteins either do not contain cysteines, which usually are disulfide-bonded in extracellular proteins and stabilize structured domains (except in the predicted signal peptide), or have cysteine-containing domains apart from the presumed intrinsically disordered sequence motifs. However, there are exceptions. Thus, in Lotgi1|173200, one of the most abundant proteins of the acid-soluble matrix (Additional file 1), 30% of the sequence consists of Asn, Pro and Ser, but the sequence also contains 20 Cys, indicating a well-ordered structure stabilized by disulfide bonds. Database searches indicated some similarity to the *Pinctada fucata* shell mpn88 protein B7X6R9_PINFU (unpublished; submitted to EMBL by Nogawa et al., 2007). The proteins showed 27% sequence identity, but none of the 20 cysteines of Lotgi1|173200 was preserved in mpn88, which contains no cysteine at all in the predicted mature sequence. Therefore we prefer to accredit the similarity in database searches to regions of similar amino acid composition, but not to sequence homology. The same may be true for Lotgi1|231186 (Table 1).

Selected sequences and spectra of this group are shown in Figures 4, 5, 6. Several of these proteins shared their sequence features with recently discovered shell proteins. Thus, the very acidic protein in Lotgi1|233420, which is one of the most abundant proteins in *Lottia* shell matrix (Additional file 1 and Additional file 2), shows 36% sequence identity to aspein [42], but this is based almost exclusively on alignment of aspartic acids. Extended Asp-rich sequences also occur in other shell proteins, such as MSP-1 [43] and asprich [44]. A very similar acidic domain was also contained in the C-terminal third of Lotgi1|239188, while the N-terminal domain was similar to nacrein (Table 2). Glycine-rich proteins may be relatives of shematrins [45]. However, in the absence of significant sequence similarity in non-repetitive sequence regions a possible homology is difficult to prove. The *Lottia gigantea* shell matrix also contained several proteins with sequence similarity

to previously identified mollusc shell proteins (Table 2) discussed below.

Proteins with possible homologs in other shells

Dermatopontin, ependymin-like and gigasin-2-like proteins

The first mollusc shell dermatopontin was isolated from the freshwater snail *Biomphalaria glabrata* shell matrix [49]. Since then several molluscan dermatopontin-encoding genes have been identified and some of them were transcribed in mantle cells, implying the shell matrix as final destination [17,54,55]. A protein very similar to dermatopontin, Lotgi1|133595 (Figure 7), was identified at moderate abundance in the acid-insoluble matrix consensus proteome and in the soluble fraction of A and C (Additional file 1 and Additional file 2). The function of this protein remains unknown at present [55].

A protein similar to the ependymin-related proteins recently discovered in *Haliotis asinina* shells [6] was found in Lotgi1|233583, a minor protein of the acid-insoluble consensus proteome (Additional file 1 and Additional file 2). It was also similar to an unpublished *Haliotis discus* protein submitted to databases by Kang et al. (2006) under the name X-box binding protein with the accession number B6RB39 (Additional file 15: Figure SA). The function of ependymin and related proteins is unknown at present.

Entry Lotgi1|235548 contained a protein sequence partially (~aa170-540) similar to the recently discovered *Crassostrea gigas* shell protein gigasin-2 (*Cgigas*-IMSP-2) [9] and the related proteins EGF-like domain containing protein-1 and -2 from *Pinctada maxima* [Jackson et al., 2009] (Additional file 15: Figure SB). Lotgi1|235548 was a minor protein in both, acid-soluble and acid-insoluble, consensus proteomes (Additional file 1 and Additional file 2).

Nacrein-like proteins

One of the most important enzymes in biomineralization events is carbonic anhydrase, which catalyzes the formation of hydrogen carbonate from CO₂ and water. The first carbonic anhydrase isolated from a mollusc shell and characterized at the molecular level was nacrein [46]. This protein, which was isolated from the nacreous layer of *Pinctada fucata* shells, contained two carbonic anhydrase domains separated by a Gly-X-Asn repeat domain. The same protein was also identified in the prismatic layer [56]. Since then nacrein-like proteins or nacrein-encoding genes have been identified in several other molluscs [4,7,10,57,58].

The *Lottia* shell matrix contained three entries that showed some similarity to nacreins (Table 2). Of these Lotgi1|238082 belonged to the most abundant proteins in the shell matrix (Additional file 1 and Additional file 2) and its sequence was 25% identical to that of *Mytilus*

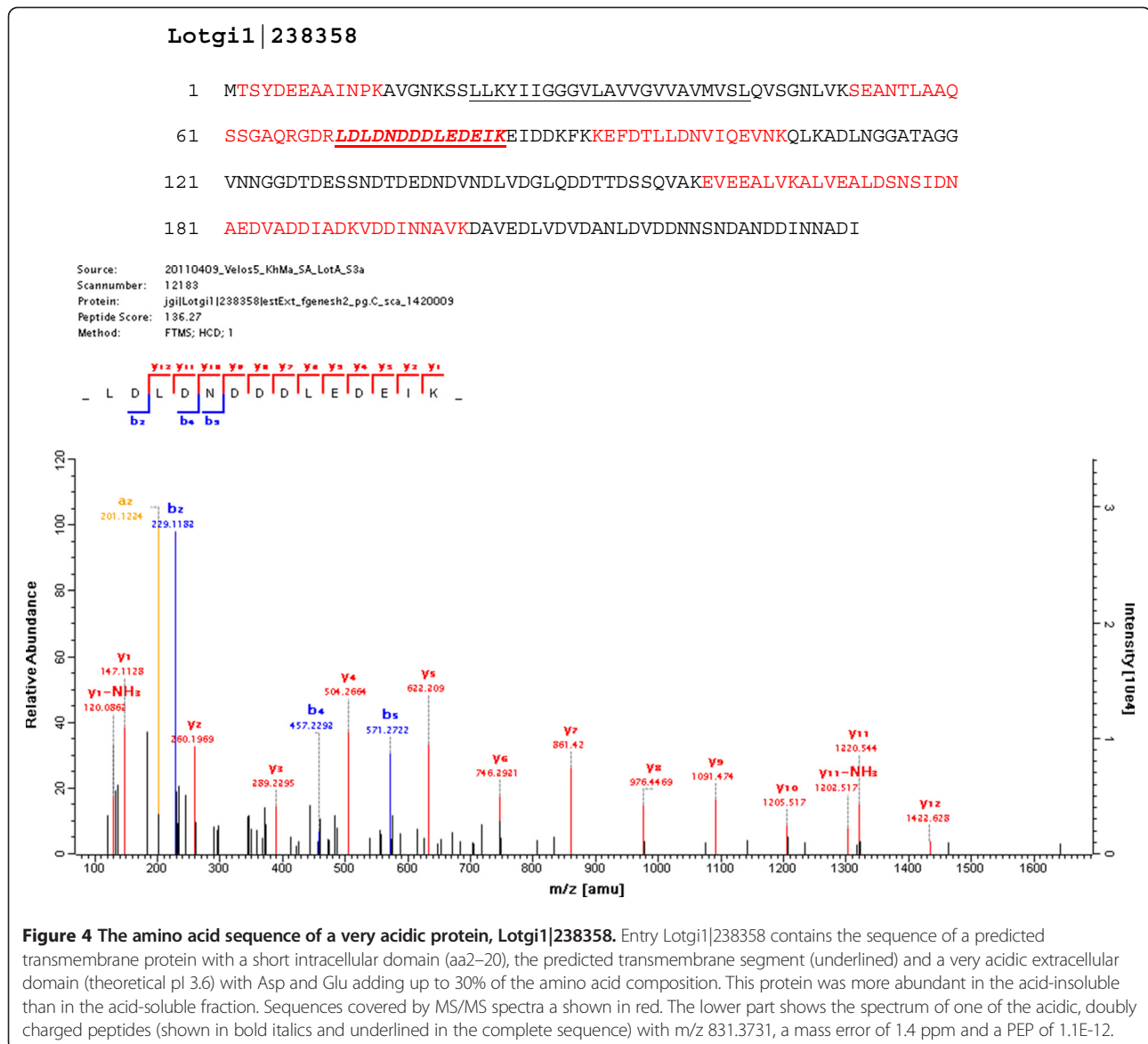
Table 1 Previously uncharacterized major *Lottia* shell matrix proteins with unusual primary sequence features

| Accession | Feature |
|----------------------|--|
| Lotgi1 115147 | 14% P, 11% T, 6 repeats of ~30aa, starting with MITPE; pl: 4.7; 319aa |
| Lotgi1 142790 | 25% Q, 10% E, 17% P, 12% V, 10% N 10% L; 6 short repeats: k/qQQPxVELNKQQP; pl 5.2; 182aa |
| Lotgi1 142814 | 38% Q, 11% L, 10% P; 5 ~70aa repeats containing shorter repeat motifs like NQQQ and KQQQ; pl: 10.5; 322aa |
| Lotgi1 152688 | 20% G, 12% P; pl: 9.7; 137aa |
| Lotgi1 158113 | 11% P; Q-rich C-term (aa210–240); pl: 9.7; 258aa |
| Lotgi1 159331 | 26% E, 13% L, 12% T; pl: 4; starting with aa156 8x SNLLQQPDa/tTQqLa/tTNeQQQ; (Figure 6) |
| Lotgi1 163637 | 17% D, 16% A; EFh, pl: 3.8; 643aa; 12 ca30aa repeats similar to AxVDNxxMADMIDTxQDxxEDAADNMADNIDTAQDAQ between aa32–453 |
| Lotgi1 171084 | 13% S; frequent doublets (SS, QQ, TT, YY, NN); G/E block aa322–337; pl: 4.4; 357aa |
| Lotgi1 172698 | 23% Q, 13% N, 13% S; aa130–702: 31 x 14aa repeats similar to QSNQQFNxxQSNQQF; pl: 7.1; 1184aa |
| Lotgi1 173200 | ~10% of P, N and G; in aa107–170 10x GAMP/GSMP; pl: 9.6; 563aa |
| Lotgi1 174003 | 19% P in aa50–400 and 35% P in aa778–882; pl: 9.5; 882aa |
| Lotgi1 227783 | aa17–126: 17% R + K, 12% P, 11% L; pl: 11; 126aa |
| Lotgi1 228385 | 16% R, 11% S; pl: 11.7; 160aa; R/H-rich from aa103–150 |
| Lotgi1 231186 | 19% G, 12% P; aa433–481: 27% M; pl: 4.6; 481aa; R/H-rich C-term half |
| Lotgi1 231509 | aa26–230: 18% P; pl: 4.2; 230aa; acidic blocks in N-term half |
| Lotgi1 233397 | A/P-rich motif aa150–170; H-rich motif aa171–185; pl: 8.8; 219aa |
| Lotgi1 233420 | 31% D, 10% E; pl: 3.6; similar to aspein? |
| Lotgi1 234884 | 42% Q in aa281–630; G/L/A-rich region aa631–928; pl: 9.2; 928aa |
| Lotgi1 235497 | aa120–247: 20% P, 16% A, 10% Q; pl: 9.7; 247aa |
| Lotgi1 235610 | 15% P, 15% T; pl: 5.7; 557aa |
| Lotgi1 235621 | aa171–270: 33% G, 25% T, 15% P, 14% Q; 16 x GGQPs/tT; pl: 5.4; 303aa |
| Lotgi1 235812 | 24% P, 18% Q, 10% N; pl: 8.9; 729aa; aa57–376: 17 repeats of 16aa, similar to NNxa/vQPPxxQxxYQPt/p |
| Lotgi1 236689 | 19% P, 10% A, 10% V, 10% R; pl: 10; 317aa |
| Lotgi1 236690 | 21% Q, 18% P; aa268–356: 4 xAQPGAYQQP(x) ₂₋₄ GAYxQQP repeats; pl: 8.4; 440aa |
| Lotgi1 236691 | 22% P, 13% Q, 10% A; Q-rich regions: ~aa61–160 and ~aa721–990; P-rich: ~aa280–600 and ~780–970; pl: 8.8; 1035aa |
| Lotgi1 238358 | aa61–232: 32% D + E, 12% N; pl: 3.7; 323aa; (Figure 4) |
| Lotgi1 238831 | 13% A, 11% R, 11% L; K/R/A-rich C-terminus (aa185–219); pl: 10.3; 219aa |
| Lotgi1 239170 | 16% G, 12% M, 10% Q; G blocks in N-term half; pl: 9.9; 145aa |
| Lotgi1 239174 | 20% G, 18% M, 12% A, 10% L; pl: 11.2; 186aa; some similarity to shematrins |
| Lotgi1 239339 | 13% T, 12% S, 10% P; blocks of T from aa185–240; pl: 9.7; 609aa |
| Lotgi1 239447 | 22% G, 12% N; pl: 9.5; 191aa; some similarity to GAAP_HALAI (Figure 5) |
| Lotgi1 77105 | 19% P, 15% S; 12% G; 9 x g/dSQPGIYP and 4 x imperfect; pl: 4.5; 173aa |
| Lotgi1 84059 | 23% N, 15% P, 15% T, 11% S; 7 repeats similar to TPxxxNNVNPGETPxTxNNVNPGE and 2 incomplete; pl: 3.8; 234aa |

For complete lists of matrix proteins see Additional file 1 and Additional file 2. Accessions in bold belong to the 26 most abundant proteins with average emPAI >1000 (Additional file 1 and Additional file 2).

californianus nacrein-like protein [10] (Additional file 15: Figure SC). It is comprised of a single α -CA₂ domain preceded by a predicted secretion signal sequence. The peak of protein distribution along gels was in slice 6. This was in agreement with the predicted protein mass (44.7 kDa) and coincided with a major band in the PAGE pattern (Figure 2). A less abundant but still major protein was Lotgi1|239188. The sequence contained a predicted secretion signal sequence and a single α -CA₂

domain (aa87–411). This was followed by a region containing 26% Asp, 23% Gly, 22% Arg and 13% Asn that aligned with 32–37% identity to the GN- and GXN-rich domains of nacreins. The CA domain was 33% identical to the sequence of an unpublished *Haliotis tuberculata* protein (accession G0YY03 of UniProt, submitted as carbonic anhydrase by LeRoy et al., 2011) and only 23% to the sequence of *Mytilus californianus* nacrein-like protein [10]. Lotgi1|233461 contained neither a secretion



signal sequence nor a predicted CA domain, but showed 36–38% sequence identity to nacrein regions preceding and comprising the GN- and GXN-rich domains. Therefore its relation to nacrein remains inconclusive. In addition to nacrein-like proteins the *Lottia* shell matrix contained two other predicted carbonic anhydrases apparently completely unrelated to nacreins (see below and Table 3).

Proteins with CLECT, IGFBP and WAP domains

The C-type lectin perlocin was first identified and isolated as a major protein of the nacreous layer of *Haliotis laevigata* shells [61,62]. C-type lectin-like (CLECT) domains were detected in several *Lottia* matrix proteins (Additional file 1 and Additional file 2), two of which were reasonably similar to perlocin to be considered as homologs (Lotgi1|229175 and Lotgi1|235529; Figure 8). However, in

both entries the perlocin-like domain was joined to a ZP (zona_pellucida)_2 domain. This resulted in a predicted mass of approximately 57,000 for the presumed products. The peptides of both domains were found predominantly in gel slices four and five (Figure 2). This was in good agreement with the predicted M_r of the complete protein, indicating that the domains occurred in the same protein. Therefore it remains questionable whether the *Lottia* shell matrix contained a true perlocin homolog. While Lotgi1|229175 was an abundant protein in the consensus proteomes of acid-soluble and acid-insoluble fractions, Lotgi1|235529 was a minor protein only identified in the acid-soluble fraction of preparation A (Additional file 1 and Additional file 2). Lotgi1|235549 was a minor consensus proteome component with a chain of 11 predicted CLECT domains preceded by two predicted EGF and one ZP_2

Lotg1 | 239447

1 MARFLPK**EPTNQQLPTLTIATS**NPVITKGNII IADPTT**GGGGNGGGNGGS**NGGGGNNG
 61 **GGGNGGWGNGG**INGGSGNNGGGGNGGWGNNGGNNVGPWPPFTNNPIFSIVDTMARKTVLR
 121 RLK**KTVSQVYGF**GKFLSP**YDG**PMTHN**IPIDPRRR****SIGY****CPYPVTEEWK**IKTML
 181 SRYGTFSEMMSS

Source: 20110414_Velos2_KhMa_SA_LotA_S11b
 Scannumber: 12969
 Protein: jgilLotg1|239447|estExt_fgensch2_pg.C_sca_2510014
 Peptide Score: 104.17
 Method: FTMS; HCD; 1

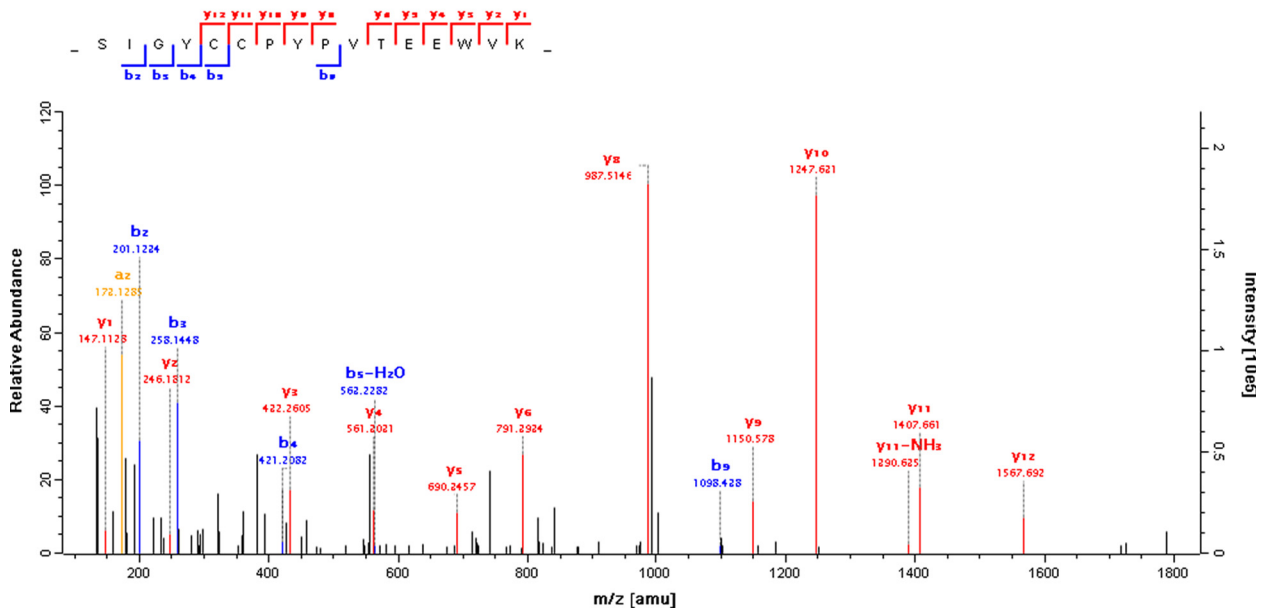


Figure 5 The amino acid sequence of the Gly/Asn-rich protein in Lotg1|239447. This was one of the most abundant proteins in the acid-soluble matrix. The sequence contained a Gly/Asn-rich domain (aa41–105; shaded yellow) consisting of 55% Gly and 28% Asn. This is followed by a cysteine-containing domain (cysteines shaded green) that can be presumed to have a more rigid structure stabilized by disulfide bonds. The Gly/Asn-rich domain did not yield a peptide because of the lack of tryptic cleavage sites. However, it is framed by MS/MS-sequenced peptides. A very similar G/N-rich sequence region was found in the otherwise unrelated shell protein GAAP_HALAI, identified in *Haliotis asinina* [6] and in nacrein-like proteins [7,46]. Sequences covered by MS/MS are in red, the peptide giving rise to the spectrum is in bold italics and underlined. The doubly charged peptide with m/z 994.4501 and a deviation from the calculated value of 0.1 ppm had a PEP of 4.7E-13. Very typically, the most intense fragments, y8 and y10, were produced by preferential fragmentation N-terminal to Pro and in the +1 position of Pro.

domains. Finally, in the predicted minor transmembrane protein Lotg1|156525 a single CLECT domain with limited similarity to mollusc perlucins was joined by several CUB; Sushi and EGF domains. Perlucin was recently also detected in the shell of a *Mytilus* species [10].

Compared to perlucin, the EGF- and insulin-binding protein perlustrin was a minor component of the *Haliotis laevigata* shell nacre matrix [50,61]. However, its predicted homolog (Figure 9) Lotg1|174065 was one of the most abundant proteins in the *Lottia* matrix (Additional file 1 and Additional file 2). A second perlustrin-like protein (Figure 9), Lotg1|238970, was less abundant, but still a major protein. To our knowledge no perlustrin-like protein

has been found in shells other than *Haliotis laevigata* and *Lottia gigantea*.

Another major protein of *Haliotis laevigata* nacre matrix is perlwapin [51], which derives its name from three whey acidic protein (WAP), also called four-disulfide core domains. WAP domains are widespread among vertebrates and invertebrates [63] and proteins very similar to *Haliotis laevigata* perlwapin were recently identified in *Haliotis asinina* [6] and *Mytilus galloprovincialis* [10]. The *Lottia* shell matrix contained three proteins with WAP domains (Figure 10). Lotg1|143247 and Lotg1|201804 were minor proteins of the acid-soluble consensus proteome, while Lotg1|239125 was a major constituent of both, acid-soluble

Lotgi1 | 159331

1 MKILSLIVLPLMAIHSTSGQEDIWLLCLLR**NCYQTPTQQTSY****SPSTYSYNEAFR**PQQQY
 61 QQYTS¹QQQPY²QQPTATQQE³LDTIQ⁴QEPNI⁵IQ⁶QEPNI⁷IQ⁸QEPNI⁹IQ¹⁰QEPNI¹¹IQ¹²QEPNI¹³IQ¹⁴QEPNI¹⁵IQ¹⁶QEPNI¹⁷IQ¹⁸QEPNI¹⁹IQ²⁰QEPNI²¹IQ²²QEPNI²³IQ²⁴QEPNI²⁵IQ²⁶QEPNI²⁷IQ²⁸QEPNI²⁹IQ³⁰QEPNI³¹IQ³²QEPNI³³IQ³⁴QEPNI³⁵IQ³⁶QEPNI³⁷IQ³⁸QEPNI³⁹IQ⁴⁰QEPNI⁴¹IQ⁴²QEPNI⁴³IQ⁴⁴QEPNI⁴⁵IQ⁴⁶QEPNI⁴⁷IQ⁴⁸QEPNI⁴⁹IQ⁵⁰QEPNI⁵¹IQ⁵²QEPNI⁵³IQ⁵⁴QEPNI⁵⁵IQ⁵⁶QEPNI⁵⁷IQ⁵⁸QEPNI⁵⁹IQ⁶⁰QEPNI⁶¹IQ⁶²QEPNI⁶³IQ⁶⁴QEPNI⁶⁵IQ⁶⁶QEPNI⁶⁷IQ⁶⁸QEPNI⁶⁹IQ⁷⁰QEPNI⁷¹IQ⁷²QEPNI⁷³IQ⁷⁴QEPNI⁷⁵IQ⁷⁶QEPNI⁷⁷IQ⁷⁸QEPNI⁷⁹IQ⁸⁰QEPNI⁸¹IQ⁸²QEPNI⁸³IQ⁸⁴QEPNI⁸⁵IQ⁸⁶QEPNI⁸⁷IQ⁸⁸QEPNI⁸⁹IQ⁹⁰QEPNI⁹¹IQ⁹²QEPNI⁹³IQ⁹⁴QEPNI⁹⁵IQ⁹⁶QEPNI⁹⁷IQ⁹⁸QEPNI⁹⁹IQ¹⁰⁰QEPNI¹⁰¹IQ¹⁰²QEPNI¹⁰³IQ¹⁰⁴QEPNI¹⁰⁵IQ¹⁰⁶QEPNI¹⁰⁷IQ¹⁰⁸QEPNI¹⁰⁹IQ¹¹⁰QEPNI¹¹¹IQ¹¹²QEPNI¹¹³IQ¹¹⁴QEPNI¹¹⁵IQ¹¹⁶QEPNI¹¹⁷IQ¹¹⁸QEPNI¹¹⁹IQ¹²⁰QEPNI¹²¹IQ¹²²QEPNI¹²³IQ¹²⁴QEPNI¹²⁵IQ¹²⁶QEPNI¹²⁷IQ¹²⁸QEPNI¹²⁹IQ¹³⁰QEPNI¹³¹IQ¹³²QEPNI¹³³IQ¹³⁴QEPNI¹³⁵IQ¹³⁶QEPNI¹³⁷IQ¹³⁸QEPNI¹³⁹IQ¹⁴⁰QEPNI¹⁴¹IQ¹⁴²QEPNI¹⁴³IQ¹⁴⁴QEPNI¹⁴⁵IQ¹⁴⁶QEPNI¹⁴⁷IQ¹⁴⁸QEPNI¹⁴⁹IQ¹⁵⁰QEPNI¹⁵¹IQ¹⁵²QEPNI¹⁵³IQ¹⁵⁴QEPNI¹⁵⁵IQ¹⁵⁶QEPNI¹⁵⁷IQ¹⁵⁸QEPNI¹⁵⁹IQ¹⁶⁰QEPNI¹⁶¹IQ¹⁶²QEPNI¹⁶³IQ¹⁶⁴QEPNI¹⁶⁵IQ¹⁶⁶QEPNI¹⁶⁷IQ¹⁶⁸QEPNI¹⁶⁹IQ¹⁷⁰QEPNI¹⁷¹IQ¹⁷²QEPNI¹⁷³IQ¹⁷⁴QEPNI¹⁷⁵IQ¹⁷⁶QEPNI¹⁷⁷IQ¹⁷⁸QEPNI¹⁷⁹IQ¹⁸⁰QEPNI¹⁸¹IQ¹⁸²QEPNI¹⁸³IQ¹⁸⁴QEPNI¹⁸⁵IQ¹⁸⁶QEPNI¹⁸⁷IQ¹⁸⁸QEPNI¹⁸⁹IQ¹⁹⁰QEPNI¹⁹¹IQ¹⁹²QEPNI¹⁹³IQ¹⁹⁴QEPNI¹⁹⁵IQ¹⁹⁶QEPNI¹⁹⁷IQ¹⁹⁸QEPNI¹⁹⁹IQ²⁰⁰QEPNI²⁰¹IQ²⁰²QEPNI²⁰³IQ²⁰⁴QEPNI²⁰⁵IQ²⁰⁶QEPNI²⁰⁷IQ²⁰⁸QEPNI²⁰⁹IQ²¹⁰QEPNI²¹¹IQ²¹²QEPNI²¹³IQ²¹⁴QEPNI²¹⁵IQ²¹⁶QEPNI²¹⁷IQ²¹⁸QEPNI²¹⁹IQ²²⁰QEPNI²²¹IQ²²²QEPNI²²³IQ²²⁴QEPNI²²⁵IQ²²⁶QEPNI²²⁷IQ²²⁸QEPNI²²⁹IQ²³⁰QEPNI²³¹IQ²³²QEPNI²³³IQ²³⁴QEPNI²³⁵IQ²³⁶QEPNI²³⁷IQ²³⁸QEPNI²³⁹IQ²⁴⁰QEPNI²⁴¹IQ²⁴²QEPNI²⁴³IQ²⁴⁴QEPNI²⁴⁵IQ²⁴⁶QEPNI²⁴⁷IQ²⁴⁸QEPNI²⁴⁹IQ²⁵⁰QEPNI²⁵¹IQ²⁵²QEPNI²⁵³IQ²⁵⁴QEPNI²⁵⁵IQ²⁵⁶QEPNI²⁵⁷IQ²⁵⁸QEPNI²⁵⁹IQ²⁶⁰QEPNI²⁶¹IQ²⁶²QEPNI²⁶³IQ²⁶⁴QEPNI²⁶⁵IQ²⁶⁶QEPNI²⁶⁷IQ²⁶⁸QEPNI²⁶⁹IQ²⁷⁰QEPNI²⁷¹IQ²⁷²QEPNI²⁷³IQ²⁷⁴QEPNI²⁷⁵IQ²⁷⁶QEPNI²⁷⁷IQ²⁷⁸QEPNI²⁷⁹IQ²⁸⁰QEPNI²⁸¹IQ²⁸²QEPNI²⁸³IQ²⁸⁴QEPNI²⁸⁵IQ²⁸⁶QEPNI²⁸⁷IQ²⁸⁸QEPNI²⁸⁹IQ²⁹⁰QEPNI²⁹¹IQ²⁹²QEPNI²⁹³IQ²⁹⁴QEPNI²⁹⁵IQ²⁹⁶QEPNI²⁹⁷IQ²⁹⁸QEPNI²⁹⁹IQ³⁰⁰QEPNI³⁰¹IQ³⁰²QEPNI³⁰³IQ³⁰⁴QEPNI³⁰⁵IQ³⁰⁶QEPNI³⁰⁷IQ³⁰⁸QEPNI³⁰⁹IQ³¹⁰QEPNI³¹¹IQ³¹²QEPNI³¹³IQ³¹⁴QEPNI³¹⁵IQ³¹⁶QEPNI³¹⁷IQ³¹⁸QEPNI³¹⁹IQ³²⁰QEPNI³²¹IQ³²²QEPNI³²³IQ³²⁴QEPNI³²⁵IQ³²⁶QEPNI³²⁷IQ³²⁸QEPNI³²⁹IQ³³⁰QEPNI³³¹IQ³³²QEPNI³³³IQ³³⁴QEPNI³³⁵IQ³³⁶QEPNI³³⁷IQ³³⁸QEPNI³³⁹IQ³⁴⁰QEPNI³⁴¹IQ³⁴²QEPNI³⁴³IQ³⁴⁴QEPNI³⁴⁵IQ³⁴⁶QEPNI³⁴⁷IQ³⁴⁸QEPNI³⁴⁹IQ³⁵⁰QEPNI³⁵¹IQ³⁵²QEPNI³⁵³IQ³⁵⁴QEPNI³⁵⁵IQ³⁵⁶QEPNI³⁵⁷IQ³⁵⁸QEPNI³⁵⁹IQ³⁶⁰QEPNI³⁶¹IQ³⁶²QEPNI³⁶³IQ³⁶⁴QEPNI³⁶⁵IQ³⁶⁶QEPNI³⁶⁷IQ³⁶⁸QEPNI³⁶⁹IQ³⁷⁰QEPNI³⁷¹IQ³⁷²QEPNI³⁷³IQ³⁷⁴QEPNI³⁷⁵IQ³⁷⁶QEPNI³⁷⁷IQ³⁷⁸QEPNI³⁷⁹IQ³⁸⁰QEPNI³⁸¹IQ³⁸²QEPNI³⁸³IQ³⁸⁴QEPNI³⁸⁵IQ³⁸⁶QEPNI³⁸⁷IQ³⁸⁸QEPNI³⁸⁹IQ³⁹⁰QEPNI³⁹¹IQ³⁹²QEPNI³⁹³IQ³⁹⁴QEPNI³⁹⁵IQ³⁹⁶QEPNI³⁹⁷IQ³⁹⁸QEPNI³⁹⁹IQ⁴⁰⁰QEPNI⁴⁰¹IQ⁴⁰²QEPNI⁴⁰³IQ⁴⁰⁴QEPNI⁴⁰⁵IQ⁴⁰⁶QEPNI⁴⁰⁷IQ⁴⁰⁸QEPNI⁴⁰⁹IQ⁴¹⁰QEPNI⁴¹¹IQ⁴¹²QEPNI⁴¹³IQ⁴¹⁴QEPNI⁴¹⁵IQ⁴¹⁶QEPNI⁴¹⁷IQ⁴¹⁸QEPNI⁴¹⁹IQ⁴²⁰QEPNI⁴²¹IQ⁴²²QEPNI⁴²³IQ⁴²⁴QEPNI⁴²⁵IQ⁴²⁶QEPNI⁴²⁷IQ⁴²⁸QEPNI⁴²⁹IQ⁴³⁰QEPNI⁴³¹IQ⁴³²QEPNI⁴³³IQ⁴³⁴QEPNI⁴³⁵IQ⁴³⁶QEPNI⁴³⁷IQ⁴³⁸QEPNI⁴³⁹IQ⁴⁴⁰QEPNI⁴⁴¹IQ⁴⁴²QEPNI⁴⁴³IQ⁴⁴⁴QEPNI⁴⁴⁵IQ⁴⁴⁶QEPNI⁴⁴⁷IQ⁴⁴⁸QEPNI⁴⁴⁹IQ⁴⁵⁰QEPNI⁴⁵¹IQ⁴⁵²QEPNI⁴⁵³IQ⁴⁵⁴QEPNI⁴⁵⁵IQ⁴⁵⁶QEPNI⁴⁵⁷IQ⁴⁵⁸QEPNI⁴⁵⁹IQ⁴⁶⁰QEPNI⁴⁶¹IQ⁴⁶²QEPNI⁴⁶³IQ⁴⁶⁴QEPNI⁴⁶⁵IQ⁴⁶⁶QEPNI⁴⁶⁷IQ⁴⁶⁸QEPNI⁴⁶⁹IQ⁴⁷⁰QEPNI⁴⁷¹IQ⁴⁷²QEPNI⁴⁷³IQ⁴⁷⁴QEPNI⁴⁷⁵IQ⁴⁷⁶QEPNI⁴⁷⁷IQ⁴⁷⁸QEPNI⁴⁷⁹IQ⁴⁸⁰QEPNI⁴⁸¹IQ⁴⁸²QEPNI⁴⁸³IQ⁴⁸⁴QEPNI⁴⁸⁵IQ⁴⁸⁶QEPNI⁴⁸⁷IQ⁴⁸⁸QEPNI⁴⁸⁹IQ⁴⁹⁰QEPNI⁴⁹¹IQ⁴⁹²QEPNI⁴⁹³IQ⁴⁹⁴QEPNI⁴⁹⁵IQ⁴⁹⁶QEPNI⁴⁹⁷IQ⁴⁹⁸QEPNI⁴⁹⁹IQ⁵⁰⁰QEPNI⁵⁰¹IQ⁵⁰²QEPNI⁵⁰³IQ⁵⁰⁴QEPNI⁵⁰⁵IQ⁵⁰⁶QEPNI⁵⁰⁷IQ⁵⁰⁸QEPNI⁵⁰⁹IQ⁵¹⁰QEPNI⁵¹¹IQ⁵¹²QEPNI⁵¹³IQ⁵¹⁴QEPNI⁵¹⁵IQ⁵¹⁶QEPNI⁵¹⁷IQ⁵¹⁸QEPNI⁵¹⁹IQ⁵²⁰QEPNI⁵²¹IQ⁵²²QEPNI⁵²³IQ⁵²⁴QEPNI⁵²⁵IQ⁵²⁶QEPNI⁵²⁷IQ⁵²⁸QEPNI⁵²⁹IQ⁵³⁰QEPNI⁵³¹IQ⁵³²QEPNI⁵³³IQ⁵³⁴QEPNI⁵³⁵IQ⁵³⁶QEPNI⁵³⁷IQ⁵³⁸QEPNI⁵³⁹IQ⁵⁴⁰QEPNI⁵⁴¹IQ⁵⁴²QEPNI⁵⁴³IQ⁵⁴⁴QEPNI⁵⁴⁵IQ⁵⁴⁶QEPNI⁵⁴⁷IQ⁵⁴⁸QEPNI⁵⁴⁹IQ⁵⁵⁰QEPNI⁵⁵¹IQ⁵⁵²QEPNI⁵⁵³IQ⁵⁵⁴QEPNI⁵⁵⁵IQ⁵⁵⁶QEPNI⁵⁵⁷IQ⁵⁵⁸QEPNI⁵⁵⁹IQ⁵⁶⁰QEPNI⁵⁶¹IQ⁵⁶²QEPNI⁵⁶³IQ⁵⁶⁴QEPNI⁵⁶⁵IQ⁵⁶⁶QEPNI⁵⁶⁷IQ⁵⁶⁸QEPNI⁵⁶⁹IQ⁵⁷⁰QEPNI⁵⁷¹IQ⁵⁷²QEPNI⁵⁷³IQ⁵⁷⁴QEPNI⁵⁷⁵IQ⁵⁷⁶QEPNI⁵⁷⁷IQ⁵⁷⁸QEPNI⁵⁷⁹IQ⁵⁸⁰QEPNI⁵⁸¹IQ⁵⁸²QEPNI⁵⁸³IQ⁵⁸⁴QEPNI⁵⁸⁵IQ⁵⁸⁶QEPNI⁵⁸⁷IQ⁵⁸⁸QEPNI⁵⁸⁹IQ⁵⁹⁰QEPNI⁵⁹¹IQ⁵⁹²QEPNI⁵⁹³IQ⁵⁹⁴QEPNI⁵⁹⁵IQ⁵⁹⁶QEPNI⁵⁹⁷IQ⁵⁹⁸QEPNI⁵⁹⁹IQ⁶⁰⁰QEPNI⁶⁰¹IQ⁶⁰²QEPNI⁶⁰³IQ⁶⁰⁴QEPNI⁶⁰⁵IQ⁶⁰⁶QEPNI⁶⁰⁷IQ⁶⁰⁸QEPNI⁶⁰⁹IQ⁶¹⁰QEPNI⁶¹¹IQ⁶¹²QEPNI⁶¹³IQ⁶¹⁴QEPNI⁶¹⁵IQ⁶¹⁶QEPNI⁶¹⁷IQ⁶¹⁸QEPNI⁶¹⁹IQ⁶²⁰QEPNI⁶²¹IQ⁶²²QEPNI⁶²³IQ⁶²⁴QEPNI⁶²⁵IQ⁶²⁶QEPNI⁶²⁷IQ⁶²⁸QEPNI⁶²⁹IQ⁶³⁰QEPNI⁶³¹IQ⁶³²QEPNI⁶³³IQ⁶³⁴QEPNI⁶³⁵IQ⁶³⁶QEPNI⁶³⁷IQ⁶³⁸QEPNI⁶³⁹IQ⁶⁴⁰QEPNI⁶⁴¹IQ⁶⁴²QEPNI⁶⁴³IQ⁶⁴⁴QEPNI⁶⁴⁵IQ⁶⁴⁶QEPNI⁶⁴⁷IQ⁶⁴⁸QEPNI⁶⁴⁹IQ⁶⁵⁰QEPNI⁶⁵¹IQ⁶⁵²QEPNI⁶⁵³IQ⁶⁵⁴QEPNI⁶⁵⁵IQ⁶⁵⁶QEPNI⁶⁵⁷IQ⁶⁵⁸QEPNI⁶⁵⁹IQ⁶⁶⁰QEPNI⁶⁶¹IQ⁶⁶²QEPNI⁶⁶³IQ⁶⁶⁴QEPNI⁶⁶⁵IQ⁶⁶⁶QEPNI⁶⁶⁷IQ⁶⁶⁸QEPNI⁶⁶⁹IQ⁶⁷⁰QEPNI⁶⁷¹IQ⁶⁷²QEPNI⁶⁷³IQ⁶⁷⁴QEPNI⁶⁷⁵IQ⁶⁷⁶QEPNI⁶⁷⁷IQ⁶⁷⁸QEPNI⁶⁷⁹IQ⁶⁸⁰QEPNI⁶⁸¹IQ⁶⁸²QEPNI⁶⁸³IQ⁶⁸⁴QEPNI⁶⁸⁵IQ⁶⁸⁶QEPNI⁶⁸⁷IQ⁶⁸⁸QEPNI⁶⁸⁹IQ⁶⁹⁰QEPNI⁶⁹¹IQ⁶⁹²QEPNI⁶⁹³IQ⁶⁹⁴QEPNI⁶⁹⁵IQ⁶⁹⁶QEPNI⁶⁹⁷IQ⁶⁹⁸QEPNI⁶⁹⁹IQ⁷⁰⁰QEPNI⁷⁰¹IQ⁷⁰²QEPNI⁷⁰³IQ⁷⁰⁴QEPNI⁷⁰⁵IQ⁷⁰⁶QEPNI⁷⁰⁷IQ⁷⁰⁸QEPNI⁷⁰⁹IQ⁷¹⁰QEPNI⁷¹¹IQ⁷¹²QEPNI⁷¹³IQ⁷¹⁴QEPNI⁷¹⁵IQ⁷¹⁶QEPNI⁷¹⁷IQ⁷¹⁸QEPNI⁷¹⁹IQ⁷²⁰QEPNI⁷²¹IQ⁷²²QEPNI⁷²³IQ⁷²⁴QEPNI⁷²⁵IQ⁷²⁶QEPNI⁷²⁷IQ⁷²⁸QEPNI⁷²⁹IQ⁷³⁰QEPNI⁷³¹IQ⁷³²QEPNI⁷³³IQ⁷³⁴QEPNI⁷³⁵IQ⁷³⁶QEPNI⁷³⁷IQ⁷³⁸QEPNI⁷³⁹IQ⁷⁴⁰QEPNI⁷⁴¹IQ⁷⁴²QEPNI⁷⁴³IQ⁷⁴⁴QEPNI⁷⁴⁵IQ⁷⁴⁶QEPNI⁷⁴⁷IQ⁷⁴⁸QEPNI⁷⁴⁹IQ⁷⁵⁰QEPNI⁷⁵¹IQ⁷⁵²QEPNI⁷⁵³IQ⁷⁵⁴QEPNI⁷⁵⁵IQ⁷⁵⁶QEPNI⁷⁵⁷IQ⁷⁵⁸QEPNI⁷⁵⁹IQ⁷⁶⁰QEPNI⁷⁶¹IQ⁷⁶²QEPNI⁷⁶³IQ⁷⁶⁴QEPNI⁷⁶⁵IQ⁷⁶⁶QEPNI⁷⁶⁷IQ⁷⁶⁸QEPNI⁷⁶⁹IQ⁷⁷⁰QEPNI⁷⁷¹IQ⁷⁷²QEPNI⁷⁷³IQ⁷⁷⁴QEPNI⁷⁷⁵IQ⁷⁷⁶QEPNI⁷⁷⁷IQ⁷⁷⁸QEPNI⁷⁷⁹IQ⁷⁸⁰QEPNI⁷⁸¹IQ⁷⁸²QEPNI⁷⁸³IQ⁷⁸⁴QEPNI⁷⁸⁵IQ⁷⁸⁶QEPNI⁷⁸⁷IQ⁷⁸⁸QEPNI⁷⁸⁹IQ⁷⁹⁰QEPNI⁷⁹¹IQ⁷⁹²QEPNI⁷⁹³IQ⁷⁹⁴QEPNI⁷⁹⁵IQ⁷⁹⁶QEPNI⁷⁹⁷IQ⁷⁹⁸QEPNI⁷⁹⁹IQ⁸⁰⁰QEPNI⁸⁰¹IQ⁸⁰²QEPNI⁸⁰³IQ⁸⁰⁴QEPNI⁸⁰⁵IQ⁸⁰⁶QEPNI⁸⁰⁷IQ⁸⁰⁸QEPNI⁸⁰⁹IQ⁸¹⁰QEPNI⁸¹¹IQ⁸¹²QEPNI⁸¹³IQ⁸¹⁴QEPNI⁸¹⁵IQ⁸¹⁶QEPNI⁸¹⁷IQ⁸¹⁸QEPNI⁸¹⁹IQ⁸²⁰QEPNI⁸²¹IQ⁸²²QEPNI⁸²³IQ⁸²⁴QEPNI⁸²⁵IQ⁸²⁶QEPNI⁸²⁷IQ⁸²⁸QEPNI⁸²⁹IQ⁸³⁰QEPNI⁸³¹IQ⁸³²QEPNI⁸³³IQ⁸³⁴QEPNI⁸³⁵IQ⁸³⁶QEPNI⁸³⁷IQ⁸³⁸QEPNI⁸³⁹IQ⁸⁴⁰QEPNI⁸⁴¹IQ⁸⁴²QEPNI⁸⁴³IQ⁸⁴⁴QEPNI⁸⁴⁵IQ⁸⁴⁶QEPNI⁸⁴⁷IQ⁸⁴⁸QEPNI⁸⁴⁹IQ⁸⁵⁰QEPNI⁸⁵¹IQ⁸⁵²QEPNI⁸⁵³IQ⁸⁵⁴QEPNI⁸⁵⁵IQ⁸⁵⁶QEPNI⁸⁵⁷IQ⁸⁵⁸QEPNI⁸⁵⁹IQ⁸⁶⁰QEPNI⁸⁶¹IQ⁸⁶²QEPNI⁸⁶³IQ⁸⁶⁴QEPNI⁸⁶⁵IQ⁸⁶⁶QEPNI⁸⁶⁷IQ⁸⁶⁸QEPNI⁸⁶⁹IQ⁸⁷⁰QEPNI⁸⁷¹IQ⁸⁷²QEPNI⁸⁷³IQ⁸⁷⁴QEPNI⁸⁷⁵IQ⁸⁷⁶QEPNI⁸⁷⁷IQ⁸⁷⁸QEPNI⁸⁷⁹IQ⁸⁸⁰QEPNI⁸⁸¹IQ⁸⁸²QEPNI⁸⁸³IQ⁸⁸⁴QEPNI⁸⁸⁵IQ⁸⁸⁶QEPNI⁸⁸⁷IQ⁸⁸⁸QEPNI⁸⁸⁹IQ⁸⁹⁰QEPNI⁸⁹¹IQ⁸⁹²QEPNI⁸⁹³IQ⁸⁹⁴QEPNI⁸⁹⁵IQ⁸⁹⁶QEPNI⁸⁹⁷IQ⁸⁹⁸QEPNI⁸⁹⁹IQ⁹⁰⁰QEPNI⁹⁰¹IQ⁹⁰²QEPNI⁹⁰³IQ⁹⁰⁴QEPNI⁹⁰⁵IQ⁹⁰⁶QEPNI⁹⁰⁷IQ⁹⁰⁸QEPNI⁹⁰⁹IQ⁹¹⁰QEPNI⁹¹¹IQ⁹¹²QEPNI⁹¹³IQ⁹¹⁴QEPNI⁹¹⁵IQ⁹¹⁶QEPNI⁹¹⁷IQ⁹¹⁸QEPNI⁹¹⁹IQ⁹²⁰QEPNI⁹²¹IQ⁹²²QEPNI⁹²³IQ⁹²⁴QEPNI⁹²⁵IQ⁹²⁶QEPNI⁹²⁷IQ⁹²⁸QEPNI⁹²⁹IQ⁹³⁰QEPNI⁹³¹IQ⁹³²QEPNI⁹³³IQ⁹³⁴QEPNI⁹³⁵IQ⁹³⁶QEPNI⁹³⁷IQ⁹³⁸QEPNI⁹³⁹IQ⁹⁴⁰QEPNI⁹⁴¹IQ⁹⁴²QEPNI⁹⁴³IQ⁹⁴⁴QEPNI⁹⁴⁵IQ⁹⁴⁶QEPNI⁹⁴⁷IQ⁹⁴⁸QEPNI⁹⁴⁹IQ⁹⁵⁰QEPNI⁹⁵¹IQ⁹⁵²QEPNI⁹⁵³IQ⁹⁵⁴QEPNI⁹⁵⁵IQ⁹⁵⁶QEPNI⁹⁵⁷IQ⁹⁵⁸QEPNI⁹⁵⁹IQ⁹⁶⁰QEPNI⁹⁶¹IQ⁹⁶²QEPNI⁹⁶³IQ⁹⁶⁴QEPNI⁹⁶⁵IQ⁹⁶⁶QEPNI⁹⁶⁷IQ⁹⁶⁸QEPNI⁹⁶⁹IQ⁹⁷⁰QEPNI⁹⁷¹IQ⁹⁷²QEPNI⁹⁷³IQ⁹⁷⁴QEPNI⁹⁷⁵IQ⁹⁷⁶QEPNI⁹⁷⁷IQ⁹⁷⁸QEPNI⁹⁷⁹IQ⁹⁸⁰QEPNI⁹⁸¹IQ⁹⁸²QEPNI⁹⁸³IQ⁹⁸⁴QEPNI⁹⁸⁵IQ⁹⁸⁶QEPNI⁹⁸⁷IQ⁹⁸⁸QEPNI⁹⁸⁹IQ⁹⁹⁰QEPNI⁹⁹¹IQ⁹⁹²QEPNI⁹⁹³IQ⁹⁹⁴QEPNI⁹⁹⁵IQ⁹⁹⁶QEPNI⁹⁹⁷IQ⁹⁹⁸QEPNI⁹⁹⁹IQ¹⁰⁰⁰QEPNI¹⁰⁰¹IQ¹⁰⁰²QEPNI¹⁰⁰³IQ¹⁰⁰⁴QEPNI¹⁰⁰⁵IQ¹⁰⁰⁶QEPNI¹⁰⁰⁷IQ¹⁰⁰⁸QEPNI¹⁰⁰⁹IQ¹⁰¹⁰QEPNI¹⁰¹¹IQ¹⁰¹²QEPNI¹⁰¹³IQ¹⁰¹⁴QEPNI¹⁰¹⁵IQ¹⁰¹⁶QEPNI¹⁰¹⁷IQ¹⁰¹⁸QEPNI¹⁰¹⁹IQ¹⁰²⁰QEPNI¹⁰²¹IQ¹⁰²²QEPNI¹⁰²³IQ¹⁰²⁴QEPNI¹⁰²⁵IQ¹⁰²⁶QEPNI¹⁰²⁷IQ¹⁰²⁸QEPNI¹⁰²⁹IQ¹⁰³⁰QEPNI¹⁰³¹IQ¹⁰³²QEPNI¹⁰³³IQ¹⁰³⁴QEPNI¹⁰³⁵IQ¹⁰³⁶QEPNI¹⁰³⁷IQ¹⁰³⁸QEPNI¹⁰³⁹IQ¹⁰⁴⁰QEPNI¹⁰⁴¹IQ¹⁰⁴²QEPNI¹⁰⁴³IQ¹⁰⁴⁴QEPNI¹⁰⁴⁵IQ¹⁰⁴⁶QEPNI¹⁰⁴⁷IQ¹⁰⁴⁸QEPNI¹⁰⁴⁹IQ¹⁰⁵⁰QEPNI¹⁰⁵¹IQ¹⁰⁵²QEPNI¹⁰⁵³IQ¹⁰⁵⁴QEPNI¹⁰⁵⁵IQ¹⁰⁵⁶QEPNI¹⁰⁵⁷IQ¹⁰⁵⁸QEPNI¹⁰⁵⁹IQ¹⁰⁶⁰QEPNI¹⁰⁶¹IQ¹⁰⁶²QEPNI¹⁰⁶³IQ¹⁰⁶⁴QEPNI¹⁰⁶⁵IQ¹⁰⁶⁶QEPNI¹⁰⁶⁷IQ¹⁰⁶⁸QEPNI¹⁰⁶⁹IQ¹⁰⁷⁰QEPNI¹⁰⁷¹IQ¹⁰⁷²QEPNI¹⁰⁷³IQ¹⁰⁷⁴QEPNI¹⁰⁷⁵IQ¹⁰⁷⁶QEPNI¹⁰⁷⁷IQ¹⁰⁷⁸QEPNI¹⁰⁷⁹IQ¹⁰⁸⁰QEPNI¹⁰⁸¹IQ¹⁰⁸²QEPNI¹⁰⁸³IQ¹⁰⁸⁴QEPNI¹⁰⁸⁵IQ¹⁰⁸⁶QEPNI¹⁰⁸⁷IQ¹⁰⁸⁸QEPNI¹⁰⁸⁹IQ¹⁰⁹⁰QEPNI¹⁰⁹¹IQ¹⁰⁹²QEPNI¹⁰⁹³IQ¹⁰⁹⁴QEPNI¹⁰⁹⁵IQ¹⁰⁹⁶QEPNI¹⁰⁹⁷IQ¹⁰⁹⁸QEPNI¹⁰⁹⁹IQ¹¹⁰⁰Q

Table 2 *Lottia* matrix proteins with possible sequence homologs in other shells

| Accession | Suggested homolog ¹ | Organism | Reference | Sequence identity ² | Alignment |
|---------------------------|--|--|-----------|--------------------------------|-------------------------------|
| Lotgi1 140660 | BMSP (fragment)Pif (fragment) | <i>M. galloprovincialis</i> <i>Pinctada fucata</i> | [47,48] | 44% (5.0E-30)27% (1.3E-6) | Additional file 16 |
| Lotgi1 173138 | BMSP (fragment)Pif (fragment) | <i>M. galloprovincialis</i> <i>Pinctada fucata</i> | [47,48] | 37% (1.6E-33)27% (3.2E-13) | Additional file 16 |
| Lotgi1 238526 | BMSP 100 | <i>Mytilus galloprovincialis</i> | [48] | 21% (4.0E-7) | Additional file 16 |
| Lotgi1 133595 | dermatopontin | <i>Biomphalaria glabrata</i> | [49] | 31% (6.6E-17) | Figure 7 |
| Lotgi1 233583 | ependymin-related protein | <i>Haliotis asinina</i> | [6] | 27% (6.5E-9) | Additional file 15: Figure SA |
| Lotgi1 235548aa170-540 | gigas-in-2 | <i>Crassostrea gigas</i> | [9] | 26% (8.6E-4) | Additional file 15: Figure SB |
| Lotgi1 132911 | Kunitz-type protease inhibitor KCP_HALAI | <i>Haliotis asinina</i> | [6] | 56% (3.6E-18) | |
| Lotgi1 233461 | nacrein B4/B3/A1/B2 | <i>Pinctada margaritifera</i> | [7] | 36-38% (1.6E-9 - 5.2E-6) | |
| Lotgi1 238082 | nacrein-like protein | <i>Mytilus californianus</i> | [10] | 25% (4.1E-13) | Additional file 15: Figure SC |
| Lotgi1 239188(aa1-420) | nacrein B2/B3/A1/B4; aa421-633 very acidic, with similarity to such proteins as aspein | <i>Mytilus californianus</i> | [10] | 27-33%(4.1E-6 - 3.3E-5) | |
| Lotgi1 229175(aa1-156) | perlucin_like | <i>Mytilus galloprovincialis</i> | [10] | 26% (1.3E-4) | Figure 8 |
| Lotgi1 235529(aa1-165) | perlucin_like | <i>Mytilus galloprovincialis</i> | [10] | 31% (1.0E-4) | Figure 8 |
| Lotgi1 174065 | perlustrin | <i>Haliotis laevigata</i> | [50] | 33% (0.076) | Figure 9 |
| Lotgi1 238970 | perlustrin | <i>Haliotis laevigata</i> | [50] | 39% (1.1E-7) | Figure 9 |
| Lotgi1 143247 | perlwapin | <i>Haliotis laevigata</i> | [51] | 31% (0.003) | |
| Lotgi1 201804 | perlwapin | <i>Haliotis asinina</i> | [6] | 35% (1.2E-5) | |
| Lotgi1 239125 | perlwapin | <i>Haliotis laevigata</i> | [51] | 40% (4.3E-9) | |
| Lotgi1 228264 | Pif (fragment) BMSP (fragment) | <i>Pinctada fucata</i> <i>M. galloprovincialis</i> | [47,48] | 28% (5.8E-5)29% (1.1E-11) | Additional file 17 |
| Lotgi1 232022 | PifBMSP | <i>Pinctada fucata</i> / <i>Mytilus galloprovincialis</i> | [47,48] | 24% (3.3E-15)32% (5.0E-12) | Additional file 17 |
| Lotgi1 239574(~aa300-650) | BMSP Pif | <i>Mytilus galloprovincialis</i> / <i>Pinctada fucata</i> | [47,48] | 22% (5.9E-9)28% (4.6E-4) | Additional file 17 |
| Lotgi1 237510 | P86860Pif | <i>Mytilus californianus</i> <i>Pinctada fucata</i> | [10,48] | 28% (2.0E-9)44% (1.0E-11) | |
| Lotgi1 166196(aa1-400) | tyrosinase | <i>Pinctada fucata</i> | [52,53] | 35% (5.7E-5) | Additional file 15: Figure SD |
| Lotgi1 231009 | UP2 | <i>Haliotis asinina</i> | [6] | 28% (2.9) | Additional file 15: Figure SE |

For complete lists of matrix proteins see Additional file 1 and Additional file 2. ¹, identified in database searches against complete databases (UniProt Knowledgebase, NCBI non-redundant protein sequences) the suggested homolog was usually not the best match, but the best mollusc shell match. ², sequence identity in regions of sequence similarity identified by database searches; E values for the FASTA results are shown in brackets. Accessions in bold belong to the 26 most abundant proteins with average empAI > 1000 (Additional file 1 and Additional file 2).

homolog BMSP [48] (Table 2; Additional file 16 and Additional file 17). Pif is synthesized as a large precursor cleaved into two products, Pif97 and Pif80. Pif97 contains a von Willebrand type A (VWA) domain and a chitin-binding peritrophin A domain. Pif80, which does not contain any known domain, induces the formation of aragonite. Similarly, BMSP is cleaved into BMSP120,

which contains four VWA domains and a chitin-binding domain, and BMSP100, the calcium carbonate-binding protein. The sequence of Pif80 and BMSP100 were described as completely different [48]. A Pif-related protein was also identified in *P. margaritifera* [7].

Lotgi1|140660 and Lotgi1|173138 were highly abundant in the acid-insoluble matrix and moderately abundant in

| | | |
|---------------|-----|---|
| Lotg11 133595 | 1 | MLKLFVCLLVVLPATVA--WMTEYDKPFLKECPKQSVYVIKSOHSNSRE |
| B6RB40_HALDI | 1 | MMFPTAFLLLSLGAAVTSSYINKFDKPFPLFCPLGESIASWESYHSNYYE |
| DERM_BIOGL | 1 | AYINLPGQPFDFQCPAGQVIRSVSVYDVLLE |
| Lotg11 133595 | 49 | DRVWDYRCRHSSKISTTSEI--NPYDGTILFQCPRK GALVGVQSYHSNH |
| B6RB40_HALDI | 51 | DRRYRFTCQQTPOLEFNCQWHNKVNNFKAPISFOCQHDVAITGVSSYHRNR |
| DERM_BIOGL | 33 | DRQWEPGCRTEENVVOTCSTSGYANDFGLPLSYSCPGKIVLTGIRSYHDNQ |
| Lotg11_133595 | 96 | HEDRIWSFKCCDVHLTRGAN-CLFTKSFVNQYDAVMDYKITTANNYITGV |
| B6RB40_HALDI | 101 | PEDREFKFLCCSLQGSPLYLAHCVKTD-FVNDWDQRLTFRV-PIRHFLKGV |
| DERM_BIOGL | 83 | IEDRRFTFRCCDVMSKATTG-CHVSEQ-VNQFNGPMLLEVSAGQA-IKGA |
| Lotg11_133595 | 145 | YSEHNNHREDRVVRFETCSIIVH |
| B6RB40_HALDI | 149 | YSTYSRYKDRRWQFETCSIQ- |
| DERM_BIOGL | 130 | ISQHDVAFEDRVVRFKLCCK |

Figure 7 Comparison of Lotg11|133595 to dermatopontin. The sequence of Lotg11|133595 is compared to the sequence of *Biomphalaria glabrata* dermatopontin [49] and to the unpublished sequence of *Haliotis discus* dermatopontin submitted to EMBL by H.-S. Kang, M. De Zoysa and J. Lee. Peptides sequenced by MS/MS are shown in red. The N-glycosylation site of *B. glabrata* dermatopontin is shaded green. The *Biomphalaria* sequence is the sequence of the mature protein determined by Edman degradation and therefore lacks a secretion signal peptide.

the acid-soluble matrix (Additional file 1 and Additional file 2). The sequence of Lotg11|140660 contained two predicted VWA domains, but no signal peptide. Lotg11|173138 contained no VWA domain, no signal sequence, but a chitin-binding domain. As often observed with major proteins, the peptides were detected in all slices of the gel. However, there was an unequivocal tendency towards slices from the high molecular weight region (see, for instance, Additional file 13) indicating that both entries possibly represented cleavage products of a larger protein. Lotg11|238526 was one of the most abundant proteins in the acid-insoluble *Lottia* shell proteome and a much less abundant, but still major, protein of the acid-soluble matrix (Additional file 1 and Additional file 2). The sequence showed a low similarity to the aragonite-binding part of BMSP. The overall sequence identity was 21%, but in the C-terminal ~100 amino acid-long sequence it rose to 40% (Additional file 16). Because these three entries occurred at the same abundance level and were more similar to BMSP than to Pif (Table 2), we believe that they belong together and may represent fragments of a possible *Lottia* BMSP homolog.

Lotg11|228264 was part of both consensus proteomes but was much less abundant than the presumed BMSP fragments described before (Additional file 1 and Additional file 2). This protein contained a signal sequence, a VWA domain, and a chitin-binding domain. The difference in abundance to the previously described fragments indicated that this protein was a possible Pif homolog rather than a possible BMSP homolog, although it was as similar to BMSP as to Pif in database searches. Lotg11|232022 was a minor protein of the acid-insoluble consensus proteome and also occurred in fractions A and C of the acid-soluble matrix. It contained a predicted VWA domain and a chitin-binding domain, but no signal sequence (Additional file 1 and Additional file 2). The sequence aligned to Pif in the same region as Lotg11|228264 and

may be a minor Pif-related protein of the shell matrix (Additional file 17). Lotg11|239574 was a major protein of both consensus proteomes. The sequence contained a secretion signal and a predicted chitin-binding domain. The chitin-binding domain was preceded by a Thr-rich motif (aa300–370; 59% Thr). This arrangement of chitin-binding domain and Thr-rich motif was very similar to Lotg11|228264 and Lotg11|232022 (Additional file 17). Our results indicate that the *Lottia* shell matrix may contain at least three Pif-related proteins occurring at different abundances. We did not identify the aragonite-binding part of any of these possible Pif homologs. However, the sequence of this part of Pif does not contain a known domain structure and may be poorly conserved between species [Suzuki et al., 2009; 2011], probably rendering identification by database searches difficult.

Both Prosite and InterProScan predict a second chitin-binding domain immediately after the published chitin-binding domain of *Mytilus galloprovincialis* BMSP and *Pinctada fucata* Pif. This domain was also predicted in all of the *Lottia* BMSP- and Pif-related proteins described above. In contrast to the regular invertebrate chitin-binding domain with six cysteines there was a cysteine doublet intercalated between regular Cys3 and Cys4 of the normal pattern (Additional file 16 and Additional file 17). This was reminiscent of cysteine patterns in plant chitin-binding domains, where a cysteine doublet is inserted between Cys2 and Cys3 [64,65]. Therefore it is not clear whether these sequence motifs are really chitin-binding domains and consequently they were not considered in the respective figures (Additional file 16 and Additional file 17).

Lotg11|237510 was a major protein in the acid-soluble and a less abundant protein in the acid-insoluble consensus proteome (Additional file 1 and Additional file 2). This protein showed similarity to the recently described chitin-

Table 3 Other proteins with a possible or established link to biomineralization

| Accession | Protein | Comment |
|----------------------------|--------------------------|---|
| Lotgi1 230492 | Similar to calcineurin | 30% identity in a 120aa overlap (Fasta E value: 0.37) with <i>Pinctada fucata</i> calcineurin (C1ITK0_PINFU) [59,60]; EFh; |
| Lotgi1 205401 | Carbonic anhydrase | Minor protein; possibly intracellular |
| Lotgi1 66515 | Carbonic anhydrase | Major protein in acid-soluble shell proteome; possibly intracellular |
| Lotgi1 159694 | Chitin-binding | Minor protein, 4 chitin-binding peritrophin A domains and 4–6 SRCR (scavenger receptor-related) domains |
| Lotgi1 160173 | Chitin-binding | Major protein, secreted; 2–3 chitin-binding peritrophin A domains |
| Lotgi1 231395 | Chitin-binding | Sequence contains predicted secretion signal sequence followed by two chitin-binding peritrophin A domains |
| Lotgi1 226726 | Chitin-binding | Major protein in acid-soluble, minor in acid-insoluble consensus proteome; chitin-binding_3 domain |
| Lotgi1 231869 | Chitin-binding | Major protein in acid soluble proteome; 10 chitin-binding peritrophin A domains organized in two blocks separated by four Pro-rich extensin-like motifs (aa470–600; 29% Pro, 16% Thr, 12% Gln, 12% Asn) |
| Lotgi1 232880 | Chitin-binding/chitinase | Major protein in acid-insoluble proteome; several SEA domains; chitin-binding peritrophin domain (aa2140–2200) with some similarity to chitinases |
| Lotgi1 234405 | Chitin-binding | Major protein in acid soluble proteome; four chitin-binding peritrophin A domains preceded by a predicted secretion signal sequence |
| Lotgi1 238400 | Chitin-binding | Major protein in acid-insoluble proteome; predicted secretion signal sequence, VWA domain and Chitin-binding peritrophin A domain |
| Lotgi1 209107 | Chitinase | Lysosomal; chitin degradation; major protein |
| Lotgi1 181237 | Chitin deacetylase | Minor secreted protein |
| Lotgi1 156599 | FAM20C/DMP4 | Extracellular matrix protein; minor |
| Lotgi1 109908Lotgi1 176394 | Osteonectin/SPARC/BM-40 | Overlapping fragments; extracellular matrix protein; major in acid-soluble matrix, minor in acid-insoluble matrix; Additional file 15: Figure SF |

Accessions in bold belong to the 26 most abundant proteins with average emPAI > 1000 (Additional file 1 and Additional file 2). For complete lists of matrix proteins see Additional file 1 and Additional file 2.

binding protein P86860 of different *Mytilus* species [10] (Table 2) but part of it (aa1–100) was also predicted to be similar to Pif in database searches.

Tyrosinase-like proteins

Lotgi1|166196 encoded a minor protein of the acid-insoluble consensus proteome that was predicted to contain a secretion signal sequence and a tyrosinase domain. Database searches indicated similarity of ~ aa1–400 of this protein to several molluscan tyrosinases previously shown to occur in shells [7,52], or to be synthesized by mantle cells [17,53] indicating the shell as destination (Additional file 15: Figure SD). In addition the sequence was very similar to other molluscan tyrosinase database entries, the known localization of which are either not in shells or was not reported. The C-terminal half of Lotgi1|166196 contained nine repeats of the type GPPVNP (aa393–462). Tyrosinase was suggested to function in periostracum formation of *Pinctada fucata* [53]. A second, unrelated, putative tyrosinase was found in Lotgi1|234481, but this protein was of low abundance, did not contain a secretion signal sequence, and was only identified in acid-insoluble fractions A and C.

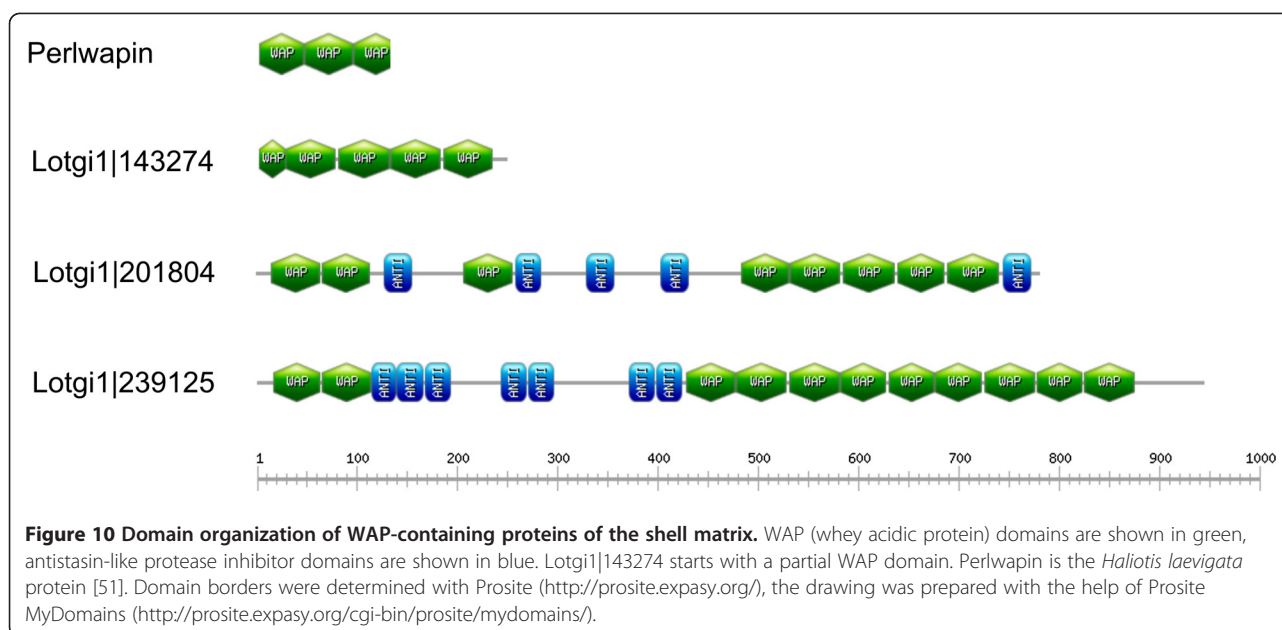
Miscellaneous proteins

Lotgi1|171918 contained a sequence with high similarity to the protease inhibitor antistasin. However, the sequence was also similar to aa660–950 of the *Haliotis rufescens* shell protein lustrin A [66]. Two other entries, Lotgi1|231010 and Lotgi1|237013 matched to aa980–1420 of lustrin A in database searches. However, these matches were not convincing and were probably due to similarities in amino acid composition. Most importantly, the typical cysteine pattern of the lustrin A cysteine-rich repeats was not conserved in all of these *Lottia* sequences.

Lotgi1|132911 contained a fragment of a Kunitz-type protease inhibitor sequence similar to a recently published *Haliotis asinina* shell protein (Table 2) [6]. Lotgi1|231009, one of the most abundant proteins in the acid-soluble shell matrix, showed some similarity to the *Haliotis asinina* protein UP2 (Uncharacterized Protein 2; Table 2; Additional file 15: Figure SE) [6].

Other proteins of possible interest in biomineralization

Lotgi1|230492 contained a sequence with 30% identity in a ~120aa overlap with *Pinctada fucata* calcineurin B [59]



[76]. *Lottia* osteonectin was a major protein in the acid-soluble shell matrix proteome and a minor one in the acid-insoluble fraction (Additional file 1 and Additional file 2). Lotgi1|109908 contained the C-terminus of the protein, the N-terminus was identified in the first 135 amino acids of Lotgi1|176394 (Additional file 15: Figure SF). Related proteins were reported from *Haliothis discus* and *Pinctada fucata* (unpublished, UniprotKB/TrEMBL accessions F2Z9K1_PINFU and F2Z9K2_HALDI, submitted by H. Miyamoto and F. Asada) and the sequences were included in the sequence alignment (Additional file 15: Figure SF) together with the human sequence [77]. A possible role in molluscan biomineralization is unknown at present.

Conclusions

The *Lottia gigantea* shell matrix turned out to contain a rather diverse set of proteins, comparable in complexity to the few other invertebrate shell matrix proteomes analyzed in-depth at present [21-23]. Among the 569 proteins identified by high-resolution mass spectrometry-based proteomics were at least 23 with a clear similarity to previously identified bivalve or gastropod shell matrix proteins. Others showed characteristics shared with previously known shell proteins, such as long stretches of acidic amino acids, of glycine, proline, or other amino acids. This made unequivocal recognition of homology difficult, if not impossible. However, such features as similar amino acid composition or preservation of domain structures may at least suggest functional equivalence. In addition we have identified many previously unknown proteins that may eventually turn out to play an important role as framework components or in regulation of matrix assembly and crystallization

of the mineral. Despite the long list of identified proteins we do not expect to have identified all *Lottia* shell matrix proteins. Some may have been missed because of a lack of specific cleavage sites while others may not be represented adequately in the present draft of the database. Other known proteins may have been identified but were not recognized because of a low preservation of amino acid sequence. Nevertheless, we hope that this set of data, the most comprehensive list of mollusc shell matrix proteins available at present, may provide a starting point for the functional characterization of these proteins by researchers interested in biomineralization processes.

Additional files

Additional file 1: *Lottia gigantea* acid-soluble matrix proteins.

Doc-file containing a list of all accepted protein identifications, their distribution in matrices obtained after different sodium hypochlorite treatments, the number of unique peptides, emPAI values and previously known or predicted subcellular occurrence.

Additional file 2: *Lottia gigantea* acid-insoluble matrix proteins.

Doc-file containing a list of all accepted protein identifications, their distribution in matrices obtained after different sodium hypochlorite treatments, the number of unique peptides, emPAI values and previously known or predicted subcellular occurrence.

Additional file 3: Proteins identified in acid-soluble matrix A.

Xls-file containing MaxQuant output data such as Lotgi1 entries grouped together because of sequence identity, number of sequence-unique and non-unique peptides, sequence coverage, protein length and molecular weight, PEP values and distribution among gel slices.

Additional file 4: Peptides identified in acid-soluble matrix A.

Xls-file containing MaxQuant output data concerning peptides, such as peptide sequence, mass, score, PEP and distribution among gel slices.

Additional file 5: Proteins identified in acid-soluble matrix B. Xls-file containing MaxQuant output data such as Lotgi1 entries grouped

together because of sequence identity, number of sequence-unique and non-unique peptides, sequence coverage, protein length and molecular weight, PEP values and distribution among gel slices.

Additional file 6: Peptides identified in acid-soluble matrix B. Xls-file containing MaxQuant output data concerning peptides, such as peptide sequence, mass, score, PEP and distribution among gel slices.

Additional file 7: Proteins identified in acid-soluble matrix C. Xls-file containing MaxQuant output data such as Lotgi1 entries grouped together because of sequence identity, number of sequence-unique and non-unique peptides, sequence coverage, protein length and molecular weight, PEP values and gel slice origin of proteins.

Additional file 8: Peptides identified in acid-soluble matrix C. Xls-file containing MaxQuant output data concerning peptides, such as peptide sequence, mass, score, PEP and distribution in gel slices.

Additional file 9: Proteins identified in acid-insoluble matrix A. Xls-file containing MaxQuant output data such as Lotgi1 entries grouped together because of sequence identity, number of sequence-unique and non-unique peptides, sequence coverage, protein length and molecular weight, PEP values and gel slice origin of proteins.

Additional file 10: Peptides identified in acid-insoluble matrix A. Xls-file containing MaxQuant output data concerning peptides, such as peptide sequence, mass, score, PEP and distribution of peptides among gel slices.

Additional file 11: Proteins identified in acid-insoluble matrix B. Xls-file containing MaxQuant output data such as Lotgi1 entries grouped together because of sequence identity, number of sequence-unique and non-unique peptides, sequence coverage, protein length and molecular weight, PEP values and gel slices yielding peptides of the respective proteins.

Additional file 12: Peptides identified in acid-insoluble matrix B. Xls-file containing MaxQuant output data concerning peptides, such as peptide sequence, mass, score, PEP and distribution of peptides among gel slices.

Additional file 13: Proteins identified in acid-insoluble matrix C. Xls-file containing MaxQuant output data such as Lotgi1 entries grouped together because of sequence identity, number of sequence-unique and non-unique peptides, sequence coverage, protein length and molecular weight, PEP values and gel slice origin of peptides for protein identification.

Additional file 14: Peptides identified in acid-insoluble matrix C. Xls-file containing MaxQuant output data concerning peptides, such as peptide sequence, mass, score, PEP and distribution among gel slices.

Additional file 15: Selected sequence alignments. Doc-file showing sequence alignments of ependymin-related protein, gigasin-2, nacrein-like protein, tyrosinase, UP2, and osteonectin to similar proteins identified in this study.

Additional file 16: Sequence analysis of BMSP-related *Lottia* proteins. Doc-file showing the alignment of BMSP-related protein sequences to *Mytilus galloprovincialis* BMSP (A) and the domain distribution in these sequences (B).

Additional file 17: Sequence analysis of Pif-related *Lottia* proteins. Doc-file showing the alignment of Pif-related protein sequences to *Pinctada fucata* Pif (A) and the domain distribution in these sequences (B).

Abbreviations

Aa: Amino acid; BMSP: Blue Mussel Shell Protein; CA: Carbonic anhydrase; CLECT: C-type lectin; IGFBP: Insulin-like growth factor-binding protein; emPAI: Exponentially modified protein abundance index; FDR: False discovery rate; HCD: Higher-energy collision-induced decomposition; PAGE: Polyacrylamide gel electrophoresis; PEP: Posterior error probability; WVA: Von Willebrand type A; WAP: Whey acidic protein.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KM conceived the study, performed sample preparation and data acquisition. EEG collected and mechanically cleaned *Lottia* shells and helped with database search and annotation. MM supplied methodological expertise. All authors took part in the design of the study and were critically involved in manuscript drafting. All authors read and approved the final manuscript.

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