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Full Length Research Paper

Detection and partial identification of proteins in pearls formed in *Hyriopsis cumingii* (Lea)

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Pearl and nacre (mother of pearl) have similar chemical compositions. However, more than 20 proteins have been identified in nacre, yet none have been detected in pearl thus far. This study aimed to detect and identify protein in pearl. Two batches of pearls formed in *Hyriopsis cumingii* (Lea) were purchased from two pearl farms. They were ground into a powder of >10,000 mesh followed by ultra-sonication and extraction in water for 4 h at room temperature. The solution was centrifuged and the supernatant was saved as pearl powder water extract. A portion of the extract was heated at 121°C for 20 min. TCA precipitation and tricine–SDS-PAGE were conducted on both the heated and non-heated extracts. After silver nitrate staining, the heated extract demonstrated a distinct protein signal, but the non-heated extract did not. The protein band from each of the two heated extracts was excised from the gel and subjected to tryptic digestion and RP-nano-HPLC-ESI-MS/MS analysis. A MASCOT search of the results indicated that one protein had significant sequence homology to a putative vitelline envelop receptor for lysine in the common marine mussel *Mytilus edulis*, and the other to the putative imaginal disc growth factor (IDGF) of *Diaprepes abbreviatus*.

Key words: Pearl, nacre, *Hyriopsis cumingii* (Lea), RP-nano-HPLC-ESI-MS/MS analysis, MASCOT search, vitelline envelop receptor for lysine, imaginal disc growth factor.

INTRODUCTION

Pearl powder, a traditional Chinese mineral medicine, has been used to treat palpitations, convulsions, insomnia, epilepsy, ulcers, and eye diseases in China for thousands of years. It has also been used for the prevention of myopia, prevention of aging, promotion of wound healing, and as a beauty treatment (Xu et al., 2001; Dai et al., 2010; Shao et al., 2010). Pearl powder is manufactured from the pearls formed in *Pteria martensii* (Dunker), *Hyriopsis cumingii* (Lea), *Cristaria plicata* (Leach), etc. It is rich in amino acids and has high calcium bioavailability (Chen et al., 2008). In Taiwan, some people use pearl powder as a regular health food supplement, or during pregnancy and the postpartum period (Chen et al., 2008; Chung et al., 2009). Studies have demonstrated that pearl powder products have antioxidant and anti-radiation properties (Shao et al., 2010). The water and acetic acid extracts of pearl powder from *H. cumingii* (Lea) have been found to stimulate fibroblast mitosis, collagen deposition and tissue inhibitor of metalloproteinase-1

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(TIMP-1) production *in vitro*, all of which are important processes of wound healing (Dai et al., 2010).

Traditionally, pearl powder is processed by wet-milling. Briefly, pearls are boiled in water for 2 h. After cooling to room temperature, the pearls are milled in pure water, followed by buoyancy sedimentation. The supernatant in the buoyancy separation is collected and dried in hot air to become powder. The sediment is then re-milled in water and the supernatant in the buoyancy separation is collected and dried. This cycle is repeated several times to collect more powder and the entire process take about a week. It is suspected that organic molecules such as proteins would be degraded or lost in the process (Chen et al., 2008). Up till now, no protein has been reported in pearl or pearl powder.

Nacre (mother of pearl) is the inside lustrous layer of many molluscan shells. It has a similar chemical composition to pearl. The predominant chemical component of pearl and nacre is calcium carbonate ($CaCO_3$) in the form of aragonite; however, pearl contains more organic substances and trace elements than nacre (Shen et al., 2006).

Due to its unique physical properties, nacre is a very important biogenic composite material. It has high mechanical strength and plasticity similar to many ceramics (Evans, 2008). Studies of nacre have been focused on its biocompatibility, osteogenetic activity, and bone-repairing activity (Evans, 2008). For instance, nacre powder or chips of *Pinctada maxima* have been shown to stimulate the proliferation of bone forming cells in sheep and humans (Westbroek and Marin, 1998; Lamghari et al., 2001; Berland et al., 2005) and the proliferation of cutaneous fibroblasts in rats (Lopez et al., 2000; Liao et al., 2002). The nacre water extract of *P. maxima* has been shown to stimulate the differentiation of fibroblasts into osteoblast-like cells and the proliferation of bone marrow stromal cells and osteoblasts *in vitro* (Almeida et al., 2000; Mouriès et al., 2002). Water-soluble proteins constitute only about 0.03% (w/w) of nacre powder (Be´douet et al., 2007). Despite this, low-molecular weight peptides and more than 20 distinct proteins have been detected in the nacre extracts of *P. maxima*, *Pinctada fucata*, *Pinctada margaritifera, Haliotis rufescens*, *Haliotis laevigata, Pinna nobilis* and *Unio pictorum* (Kono et al., 2000; Marin et al., 2000; Yan et al., 2007; Yano et al., 2007; Bédouet et al., 2006; Bédouet et al., 2007; Marie et al., 2008; Evans, 2008). Some of these proteins have been shown to possess *in vitro* biochemical activity, including perlustrin with growth factor binding activity (Weiss et al., 2000), P95 with calcium binding activity (Marie et al., 2008), and nacrein with carbonic anhydrase activity, which converts carbon dioxide into bicarbonate ion (Kono et al., 2000).

In addition, more than 10 proteins have been shown to carry activities that either promote or block calcium carbonate crystallisation *in vitro* (Evans, 2008). In these studies, the nacreous powder was prepared by grinding the dried inner nacreous layer of shells, which was obtained by either immersing the shells in dilute NaOH for 24 h and then mechanically removing the outer layer of the dried shells or, oppositely, mechanically separating the outer and inner layers by abrasion followed by dissociating the outer layer in dilute sodium hypochlorite solution (Marin et al., 2000; Yan et al., 2007). The nacre powder was extracted by water, acetic acid, or other extraction buffers and proteins could be detected in the extracts (Kono et. al., 2000; Weiss et al., 2000; Be´douet et al., 2007; Marie et al., 2008; Dai et al., 2010). It has been reported that both nacre and pearl of *H. cumingii* (Lea) could stimulate osteoblast proliferation *in vitro*, but the proliferation proceeded more quickly and smoothly on pearl pieces than on nacre chips (Shen et al., 2006). It is thus hypothesized that pearl of *H. cumingii* (Lea) may also contain proteins pertinent to osteogenesis, biomineralization, and/or other biochemical functions. To test this hypothesis, a suitable preparation method for pearl powder (and the extract) is required for protein detection.

A room-temperature super extraction system (RTSES) for ultrasonic extraction of molecules from solid particles has been developed by MesoPhase Technologies (Tainan, Taiwan; Figure 1). The system contains an ultrasound-producing element with a free end and a fluid circulation line. Between the free end of the ultrasound-producing element and the fluid circulation line, there is an internal section where, with the passage of fluid in the fluid circulation line, the intensity of the ultrasonic waves is concentrated and acoustic cavities in the fluid are created, causing the crushing of the particles in the fluid. As the fluid circulates, particles in the fluid are repeatedly crushed in this internal section and their size is reduced, leading to continuous extraction in the fluid. Effective extraction and retention of the components in the particles can be expected (Kao, 2006). The system operates at room temperature and does not require any organic solvents. As a result, large molecules in the extract would retain their activities and no organic wastes or contaminants are present in the extract or during the extraction course.

In this study, pearl powder water extracts were prepared by this ultrasonic extraction system using water as both the ultrasound transmitting medium and the extraction fluid. Two proteins were detected from the extracts. The proteins were subjected to mass spectrometry analysis, and homologies to proteins in the database were found.

MATERIALS AND METHODS

Pearls and pre-treatment

Two batches of pearls formed in freshwater pearl mussel [*H. cumingii* (Lea)] were obtained from two separate pearl farms in the Zhejiang province of China. The pearls were cleaned in deionised water, dried at 40°C and ground into powder of >10,000 mesh (less than 1.5 micron) by an air impacting grinder. The powder was used

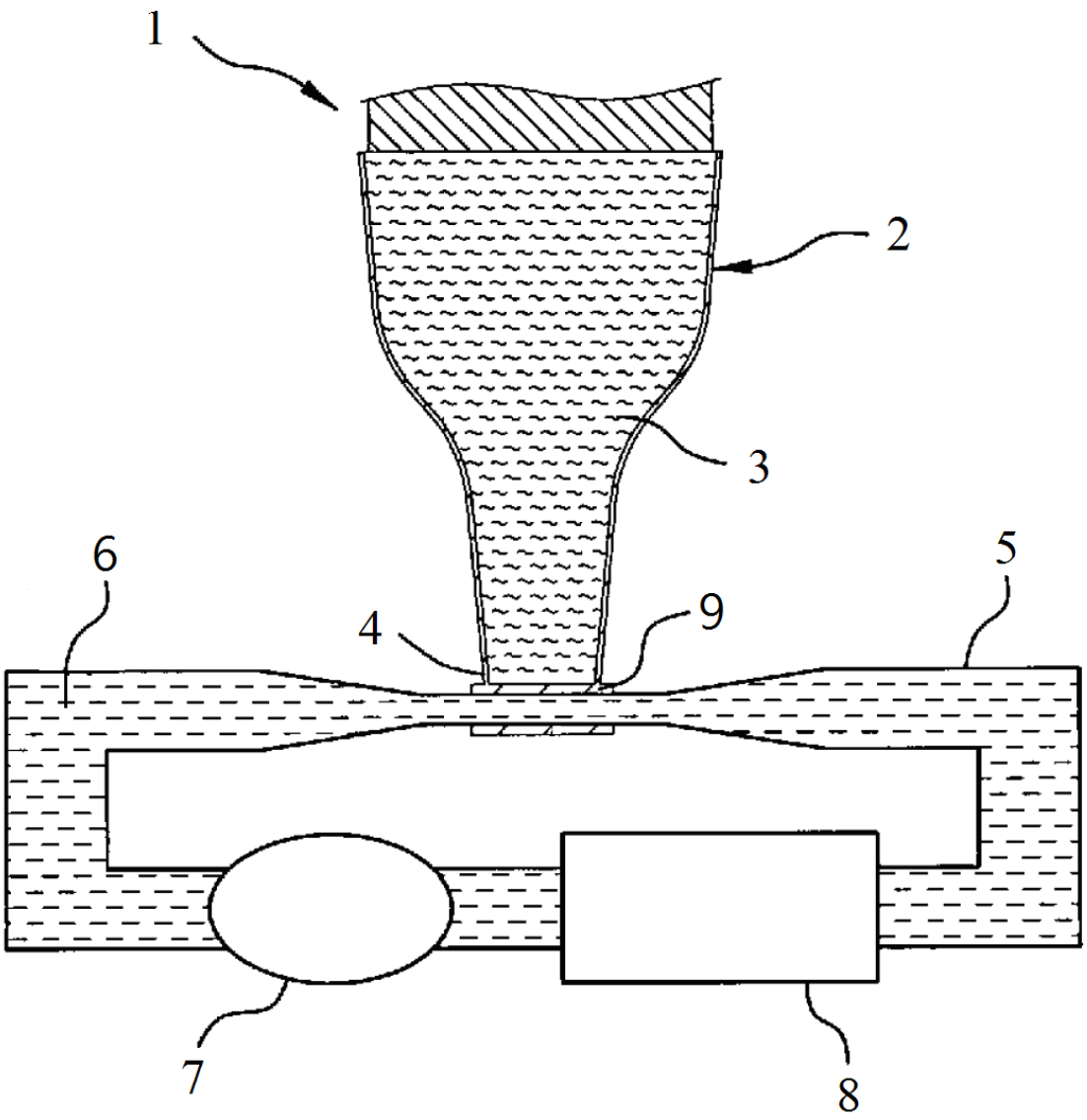


Figure 1. The room-temperature super extraction system (modified from Kao, 2006). The system contains two connected parts. The top part is an ultrasound generator (1) linked to a bell (2) that contains water (3) as a sound-transmitting medium and has a free end (4). The bottom part is a fluid circulation line (5) that contains particles to be extracted in extraction fluid (6), a drive mechanism (7), and a cooling mechanism (8). Between the free end (4) of the bell (2) and the fluid circulation line (5), there is an internal section (9) where, with the passage of extraction fluid (6) in the fluid circulation line (5), the intensity of the ultrasonic waves is concentrated and acoustic cavities in the fluid are created, leading to crushing of the particles in the extraction fluid (6).

for preparation of pearl powder water extract and acetic acid extract.

Pearl powder water extract

One kilogram of the powder was re-suspended in 10 L of pure water and extracted by RTSES (Kao, 2006; Figure 1) for 4 h. After the extraction, the size of the pearl powder was determined to be 70 to 300 nm by a laser particle size analyser (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). The solution was centrifuged at 3,000 × g for 10 min, and the supernatant was collected as pearl powder water extract. One litre of extract was further heated at 121°C for 20 min, whereas the remainder was not. The extracts were stored at -80°C before analysis.

Pearl powder acetic acid extract

Fifty grams of the powder were re-suspended in one litre of 10% acetic acid and stirred at 4°C for 20 h. The solution was then centrifuged at 3,000 × g for 10 min and the supernatant was collected as pearl powder acetic acid extract. The extract was stored at -80°C before analysis.

TCA precipitation

Ice cold 100% trichloric acid (TCA) was added to 200 ml of pearl powder extracts (water extract or acetic acid extract) to 10% final concentration and incubated at 4°C for 1.5 h. The solution was centrifuged at 14,000 × g for 20 min at 4°C and the supernatant was discarded. The pellet was washed with 95% ethanol and air-dried.

Electrophoresis

The dried pellets from TCA precipitation of the pearl powder extracts (water extract or acetic acid extract) were dissolved in 60 µL protein sample dye [75 mM Tris (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol] and boiled for 10 min. The solution was centrifuged at 15,000 × g for 10 min, and 20 µL of the supernatant was analysed on tricine-SDS-PAGE (Schagger, 2006). After electrophoresis, gels were stained for protein visualisation according to the method of Giulian et al. (1983). Briefly, gels were soaked in fixing solution [40% (v/v) ethanol, 10% (v/v) acetic acid] for 30 min, followed by soaking in sensitising solution [30% (v/v) ethanol, 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulfate, 0.125% (v/v) glutaraldehyde] for 30 min, three times in ddH_2O for 5 min, in silver solution [0.25% (w/v) silver nitrate, 0.015% (v/v) formaldehyde] for 20 min, two times in ddH_2O for 1 min, and in developing solution [2.5% (w/v) sodium carbonate, 0.0074% (v/v) formaldehyde) for 1 to 5 min to display the signals. To stop the staining reaction, the gels were soaked in stop solution [1.5% (w/v) Na_2EDTA] for 10 min. All of the staining reactions were carried out in the dark at room temperature on an orbital shaker with slow shaking (50 rpm).

Proteomics analysis

Protein bands appearing on the stained gels were excised, digested with trypsin, and subjected to RP-nano-HPLC-ESI-MS/MS (reverse phase-nano-high performance liquid chromatography-electrospray ionisation-tandem mass spectrometry) by the Proteomics Research Core Laboratory of National Cheng Kung University according to the method described by Wang et al. (2010). The tryptic peptides was fractionated on a nano-HPLC system (LC Packings, Amsterdam, Netherlands) coupled with an ion trap mass spectrometer (LCQ DECA XP Plus, ThermoFinnigan, San Jose, CA) equipped with an electrospray source. RP-HPLC was done on a C18 reverse phase column (ZORBAX 300SB-C18, 3.5 µM (bead size) × 75 µM (i.d) × 15 cm, Agilent, Wilmington, DE) at a flow rate of 0.2 µL/min with 5% acetonitrile / 0.1% formic acid (v/v, eluent A) and 80% acetonitrile / 0.1% formic acid (v/v, eluent B).

Database search and protein identification

The mass spectra data of the fragmented peptides were searched against a National Center for Biotechnology Information (NCBI) non-redundant database, using the MASCOT search engine (http://www.matrixscience.com) of the Expasy server. The number of maximal missed cleavage sites for tryptic cleavages was set to 2. The mass tolerance of the precursor peptide ion was set to 1 Da and the fragment ion tolerance was set to 1 Da. Search parameters included: Database, NCBI; taxonomy, other metazoa; enzyme, trypsin; modification, carboxymethyl (C, K), deamidation (N, Q), oxidation (M), pyro-Glu (D, E). Proteins identified were validated based on the MASCOT/ MOWSE score, quality of the MS/MS spectra, gas phase fragmentation chemistry, and on the number of fragment ions. Identification of a protein was considered to be significant according to the MOWSE score ($P<0.05$). The proteins identified had to meet one of the following criteria for validation:

(1) Have significant hits to 2 rank-one peptides that were either in different sequences or in the same sequence, but with different modifications.

(2) Have significant hits to 3 peptides that were either in different sequences or in the same sequence, but with different modifications or a combination.

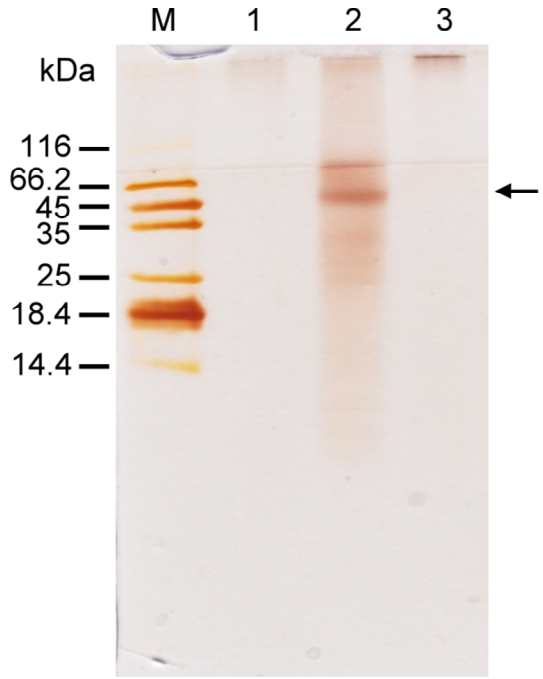
RESULTS AND DISCUSSION

Initially, pearls formed in *H. cumingii* (Lea) were purchased from one pearl farm in the Zhejiang province of China, washed, dried, and ground into powders of >10,000 mesh (less than 1.5 micron). Because several studies used acetic acid solution for making nacre powder extracts and successfully detected the presence of proteins in the extracts (Weiss et al., 2000; Be´douet et al., 2007; Marie et al., 2008; Dai et al., 2010), pearl powder acetic acid extract was prepared and subjected to TCA precipitation and tricine SDS-PAGE. However, silver nitrate staining did not reveal any polypeptide signals (Figure 2A).

Next, 1 kg of the powder of >10,000 mesh was extracted in 10 L water for 4 h by ultrasound using RTSES. After the extraction, the solution was centrifuged and the supernatant was collected as pearl powder water extract. Two hundred millilitres of the pearl powder water extract was TCA-precipitated and analysed via tricine-SDS-PAGE. Still, no protein signal was detected after silver nitrate staining (Figure 2A). A portion of the extract was used for sterilisation at 121°C for 20 min. The solution was centrifuged and 200 ml of the supernatant was used for TCA precipitation and tricine-SDS-PAGE. A distinct band with apparent protein size between 45 and 66.2 kDa was clearly observed. A band at the boundary between the spacer gel and the separation gel was also observed (Figure 2A). This is the first time that protein has been detected in pearl powder extract, even though the protein content of pearl powder water extract was estimated to be 12% using a colometric coomassie brilliant protein determination kit (Dai et al., 2010). It seems that the amount of protein in the pearl powder extract was so little that the protein could not be precipitated by TCA. Denaturation of the proteins by heating the extract at 121°C for 20 min somehow helped the proteins to be aggregated and precipitated by TCA. Figure 3 shows the flow chart for the detection of proteins in pearl formed in *H. cumingii* (Lea).

The band of proteins with sizes between 45 and 66.2 kDa was excised from the gel, digested with trypsin, and analysed by mass spectrometry. The peptide mass fingerprint (PMF) data was searched with the MASCOT tool. Only one protein in the NCBI database was identified and validated (Figure 2B). One peptide representing a sequence of 15 amino acids from residue 144 to 158 of the putative vitelline envelop receptor for lysine in common marine mussel *Mytilus edulis* (accession no:

A.



B.

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  1 FNPHSTNSNF SSAEFKVFWG FTTAHLMNEY EPSQISCIAE GNGTENVAAK
 51 DIDEDYLPVN HELVKSLGGD YAGSATLKLI DILGEEIDTR SVPMSKKVEL
101 LLTVDTADYQ GVIPYDCRAV SKDLGTSYRF LLAGCGDGTI IPKNKGFTTK
151 TIAGGNEKTA TSPFFKIFKL LETTKQGGIS YECSFTVCND TCDGSSCAMR
201 NKRSVDSLAE IEEQEKVKTP L
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Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Score	Rank	Sequence
144 - 158	783.8486	1565.6826	1564.8209	0.8617	57	1	K.NKGFTTKTIAGGNEK.T
144 - 158	783.9248	1565.8351	1565.8049	0.0302	42	1	K.NKGFTTKTIAGGNEK.T Deaminated (NQ)
144 - 158	783.9844	1565.9542	1565.8049	0.1493	46	1	K.NKGFTTKTIAGGNEK.T Deaminated (NQ)

Figure 2. (A) Tricine-SDS-PAGE of the unheated pearl powder water extract (lane 1), heated pearl powder water extract (lane 2), and pearl powder acetic acid extract (lane 3) prepared from pearls of one pearl farm. The molecular mass of size markers (lane M) is shown on the left of the gel. (B). Proteomics analysis of the protein in the tricine-SDS-PAGE gel in A (indicated by an arrow) digested with trypsin. The MS/MS spectra were used for searching the NCBI database with MASCOT. Matches to the putative vitelline envelop receptor for lysine in common marine mussel *Mytilus edulis* (accession no: gi 222531723) were observed.

gi 222531723; molecular weight: 24,071 Da) (residues 144 to 158) was identified. Vitelline envelop receptor for lysine is a protein in the egg envelope of mussels where spermatozoa bind to and release lysine to create a hole in the envelope through which the spermatozoan passes to reach the egg for fertilization (Swanson and Vacquier, 1997). Transcripts of the gene have been detected in the mantle of *M. edulis* and the cDNA was cloned and sequenced (GenBank accession no. FM995161.1; Sedik et al., 2010).

Furthermore, pearls of *H. cumingii* (Lea) from another pearl farm in Zhejiang province of China were again purchased. Preparation of the pearl powder water extract and detection of proteins in the extract were performed by the same method (Figure 3). A band of protein with sizes between 25 and 35 kDa was detected after silver nitrate staining of the gel (Figure 4A). This protein band was excised, trypsin-digested, and analysed by mass spectrometry. Only one protein in the database was identified and validated by MASCOT search with the PMF data (Figure 4B). Two peptides of the putative imaginal disc growth factor (IDGF) of *Diaprepes abbreviatus* (accession no: gi 55978158; molecular weight: 49,436 Da) (residue 70 to 90; residue 127 to135) were identified.

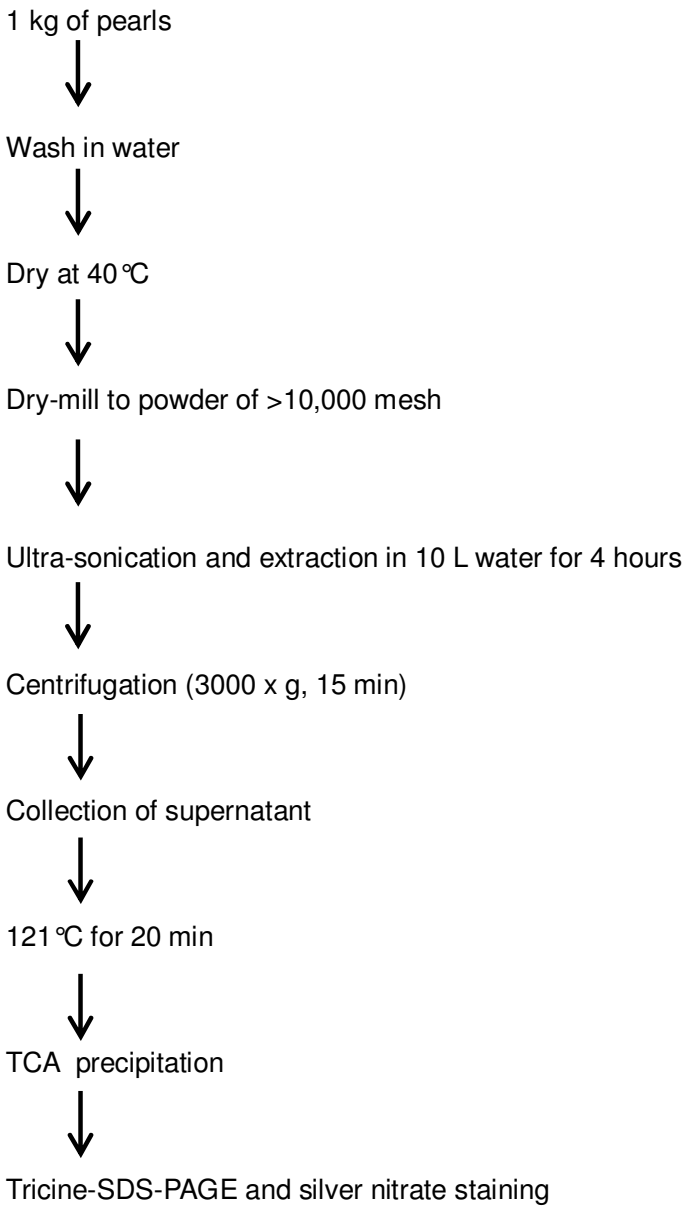
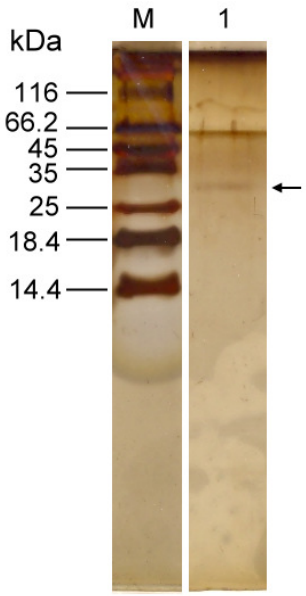


Figure 3. Flowchart for the detection of proteins in pearls formed in *Hyriopsis cumingii* (Lea).

IDGF, the first reported polypeptide growth factor from invertebrates, was originally isolated from the culture medium of *Drosophila* wing-disc C1.8+ cells with the ability to stimulate the proliferation, polarisation, and motility of insect imaginal disc cells in the presence of insulin (Kawamura et al., 1999). The imaginal disc cells are progenitor cells in larva that would undergo differentiation and proliferation in larva and pupa, finally developing into different organs in the adult. The IDGF from *Mamestra brassicae* (MbIDGF) was later found to be capable of stimulating proliferation of two cell lines of the insect in the absence of insulin (Zhang et al., 2006). It is suspected that the pearl protein we identified with sequence homology to IDGF of *D. abbreviatus* may be involved in the development of *H. cumingii* (Lea). It may also be involved in the activity of pearl of *H. cumingii* (Lea) to stimulate osteoblast proliferation described by Shen et al. (2006) and/or fibroblast mitosis described by Dai et al. (2010).

Only 6 and 5% sequences of the proteins identified by the MASCOT search showed homology with the sequences of proteins detected in the first and the second pearl powder water extracts, respectively (Figures 2 and 4). In an attempt to increase the sequence coverage, another mass spectrometry analysis was performed with the two protein bands from the two pearl powder water

A.



B.

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  1 MECVKIVLLA IFALASFTGK TESATDSKLV CYYDSRAYNR PGNGKFDIPF
 51 LEPALQFCTH LIYGYAGIRE DNFKISPLNE PLDINKQNYR HITDLKRKYP
101 GLRVLLSVGG NNDVTGEGSE KNLKYRTLLE SVESRLAFVN SAHDLVKNYG
151 FDGLDLSWEF PENKPKKIRN AVSSWFSKIK HKIVGESVVD EKAEEHKEQF
201 TALVRELKNV FRHDGLLLTV SVLPNVNSSV YFDPRQLAPN IDFATLEAFD
251 YRTPQRNPKE LDYVAPLYEL LDRKVDENAD YQVRYWLGGG LPANKLILGI
301 PTYGRAWKLN DDSGLTGVPP LLTDGAADPG PYSNEAGLLS YPEICSKIAT
351 PKEIQAGYLG KLRKTNDPTK RYGSYAYRLP DSNGENGIWV GFEDPDTVGN
401 KAAYSKAKGL GGIAIVDLTL DDFRGTCSQD HFPLLRAAKF RL
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Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Score	Rank	Sequence
75 - 90	639.6091	1915.8056	1914.9686	0.8370	32	1	K.ISPLNEPLDINKQNYR.H
127 - 135	517.1663	1032.3180	1032.5451	-0.2271	30	1	R.TLLESVESR.L

Figure 4. (A) Tricine-SDS-PAGE of the heated pearl powder water extract prepared from pearls of the other pearl farm. The molecular mass of size marker (lane M) is shown on the left of the gel. (B) Proteomics analysis of the protein in the tricine-SDS-PAGE gel in A (indicated by an arrow) digested with trypsin. The MS/MS spectra were used for searching the NCBI database with MASCOT. Matches to the putative imaginal disc growth factor (IDGF) of *Diaprepes abbreviatus* (accession no: gi 55978158).

extracts (Figures 2 and 4), but the same result was obtained. This is not surprising as no genomic data on freshwater mussels are available nor are any pearl protein sequences. In fact, this is the first report to demonstrate the presence of a distinct protein in pearls from any mollusc species. Although cDNA sequences of three genes from *H. cumingii* (Lea), theromacin and cytochrome oxidase subunits I and II genes, have been reported (Campbell et al., 2005; Shirai et al., 2010; Xu et al., 2010), this is also the first report for the successful detection of distinct proteins produced by *H. cumingii* (Lea).

In conclusion, the extraction and detection of pearl proteins is challenging. This is the first report that distinct proteins were successfully detected in pearl and partially identified. We used a combination of air-type dry-milling and ultrasound extraction to produce our pearl powder extract, and a combination of heating, TCA precipitation, tricine-SDS-PAGE, and silver nitrate staining to detect protein in the extract. No other solvent except pure water was used for the extraction. The heating step greatly improved the recovery of detectable proteins. Proteomics analysis of the two proteins bands detected on tricine-SDS-PAGE gels from two independent pearl samples

revealed homologies to a putative vitelline envelop receptor for lysine in the common marine mussel *M. edulis* and the putative imaginal disc growth factor (IDGF) in *D. abbreviates.* The difference of proteins detected in the two pearl samples can be explained by differences in the cultivation environments of the two pearl farms, as well as differences in the seasons and the ages of *H. cumingii* (Lea) while harvesting the pearls. The randomness of the experiment could also contribute to the difference.

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