

Extraction and identification of membrane proteins from black widow spider eggs

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ABSTRACT

The eggs of oviparous animals are storehouses of maternal proteins required for embryonic development. Identification and molecular characterization of such proteins will provide much insight into the regulation of embryonic development. We previously analyzed soluble proteins in the eggs of the black widow spider (*Latrodectus tredecimguttatus*), and report here on the extraction and mass spectrometric identification of the egg membrane proteins. Comparison of different lysis solutions indicated that the highest extraction of the membrane proteins was achieved with 3%-4% sodium laurate in 40 mmol/L Tris-HCl buffer containing 4% CHAPS and 2% DTT (pH 7.4). SDS-PAGE combined with nLC-MS/MS identified 39 proteins with membrane-localization annotation, including those with structural, catalytic, and regulatory activities. Nearly half of the identified membrane proteins were metabolic enzymes involved in various cellular processes, particularly energy metabolism and biosynthesis, suggesting that relevant metabolic processes were active during the embryonic development of the eggs. Several identified cell membrane proteins were involved in the special structure formation and function of the egg cell membranes. The present proteomic analysis of the egg membrane proteins provides new insight into the molecular mechanisms of spider embryonic development.

Keywords: *Latrodectus tredecimguttatus*; Egg; Membrane protein; Extraction; Identification

INTRODUCTION

In oviparous species, the egg is a storehouse of maternal proteins required for fertilization and the initiation of zygotic development (Calvert et al, 2003; Yue et al, 2011). During

hatching, nutrients are transported from the egg components to the developing embryo. The proteins in the egg, which usually form lipoproteins with lipids, are crucial for the development, growth, and survival of the embryo (Laino et al, 2013; Wu et al, 2009; Zhao et al, 1994). Identification and molecular characterization of such proteins can provide insight into the regulation of embryonic development. Therefore, egg proteome analysis has become a hotspot in the field of proteomics. Up to now, many proteomic studies have been performed on eggs of the domestic chicken (Mann 2007; Rose & Hincke, 2009). Mann et al (2008) identified 119 proteins in egg yolk, 78 proteins in egg white, and 528 proteins in the decalcified eggshell organic matrix, whereas Farinazzo et al (2009) identified 255 yolk proteins in their study.

Cell membranes and cellular inner membranes are critical components of egg structure and function, and are involved in the partitioning of organelles, protecting the integrity of the genome and proteome, and providing defense against foreign molecules and external conditions that may damage or destroy the eggs. Membrane proteins are not only the main components of biological membranes but also the main executors of membrane functions. It has been estimated that membrane proteins account for about 30% of cellular proteins (Wu et al, 2003). Some proteomic analyses have focused on the membrane proteins of eggs. Due to the fact that most membrane proteins are hydrophobic and difficult to dissolve in aqueous buffer, the extraction of egg membrane proteins is often performed with the help of detergents and/or other

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additives (Mann et al, 2006; Rose & Hincke, 2009). For example, SDS-containing lysis solution was used to solubilize proteins in the hen eggshell membrane (ESM), followed by SDS-PAGE and LC-MS/MS, revealing 62 proteins, including 53 not previously reported (Kaweewong et al, 2013). Egg proteomes of other species, such as the silkworm (Fan et al, 2013) and snail (Sun et al, 2012), have also been analyzed. However, reports on spider egg proteomes are limited.

The black widow spider is an oviparous animal. The adult female constructs 7-8 egg sacs containing about 450 eggs each from June to October, with 1-3 weeks in between (Bonnet 2004). Compared with the eggs of other species, the eggs of the black widow spider received particularly early attention due to their inherent toxicity (Kobert 1889; Buffkin et al, 1971; Russell & Maretć, 1979). In our previous work, we conducted a proteomic analysis of the water-soluble proteins in the eggs of the black widow spider (*Latrodectus tredecimguttatus*) and identified 157 proteins, concluding that the molecular basis and mechanism for egg toxicity were different from those of spider venom (Li et al, 2012). Our present work analyzed the membrane proteins in the spider eggs based on detergent-assisted extraction and mass spectrometric identification to more comprehensively understand the egg proteome of the spider *L. tredecimguttatus*.

MATERIALS AND METHODS

Reagents

Dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, SDS, 3-[(3-cholamidopropyl) dimethylamino] propanesulfonate (CHAPS), acrylamide, bisacrylamide, glycine and Tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium laurate (SL) was from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Trypsin was from Promega (Madison, WI, USA). Protein assay kit was from Solarbio (Beijing, China). All other reagents were products of the highest grade available.

Extraction of egg membrane proteins

The eggs were homogenized in ddH₂O with a mortar and pestle and the resulting homogenate was centrifuged at 15 400 g for 15 min at 4 °C. After the supernatant was removed, the pellet (membrane debris) was repeatedly homogenized and extensively washed to eliminate the soluble components, and was then lyophilized. For extracting proteins from the egg membrane debris, different lysis solutions (40 mmol/L Tris-HCl/4% CHAPS/2% DTT, containing 1%-4% of SDS or SL, pH 7.4) were used and compared. Briefly, 50 mg aliquots of the lyophilized membrane debris were separately placed in eight Eppendorf tubes, with the eight different lysis solutions added, respectively. After intermittent oscillation with a vortex oscillator for 30 min, the mixtures were centrifuged at 15 400 g for 15 min. The supernatants were separately collected and the pellets were extracted twice. The obtained supernatants were separately pooled and the total volume of the extraction for each tube was adjusted to 2.5 mL. Protein content was determined with the protein assay kit using BSA as the standard according to the manufacturer's instructions.

SDS-PAGE of egg membrane proteins

SDS-PAGE of the egg membrane proteins was performed according to Laemmli (1970) under denaturing conditions on a 10% polyacrylamide separating gel overlaid with a 5% stacking gel. The sample solution (6 µL) was mixed with 3 µL of loading sample buffer (500 mmol/L Tris-HCl, 4% SDS, 100 mmol/L DTT, 20% glycerol, a trace of bromophenol blue, pH 6.8,) and then centrifuged at 10 000 g for 10 min. The proteins in the supernatants were loaded and separated through gel electrophoresis, which was run at 20 mA on the stacking gel and at 40 mA on the separating gel. After completion of electrophoresis, the resolved proteins in the gel were fixed with 10% acetic acid/40% methanol and visualized by staining with Coomassie brilliant blue G-250.

In-gel digestion

Digestion of the separated proteins in the gel was performed according to Chen et al (2006). Briefly, the lane gel was cut into slices about 2-3 mm wide and then broken into small pieces, followed by washing with 25 mmol/L NH₄HCO₃ and 50% ACN/25 mmol/L NH₄HCO₃ sequentially. The gel pieces were dehydrated in 100% ACN and then dried in a Speed Vac. In-gel digestion was performed with trypsin (1 µg enzyme/slice) in 25 mmol/L NH₄HCO₃ containing 10% ACN with incubation overnight at 37 °C. The released peptides were extracted twice by adding 100 µL of 67% ACN containing 5% formic acid with ultrasonication. The supernatants were pooled and then concentrated in a Speed Vac.

nLC-MS/MS analysis

Tryptic digests prepared by in-gel digestion were analyzed by an Easy-nLC system (Proxeon Biosystems, Odense, Denmark) coupled with a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA) and HCTultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with an autosampler and a C18 reverse phase column (PepMap, 75 µm i.d., 15 cm long, Sunnyvale, CA, USA). For nLC separation, solvent A (0.1% formic acid) and solvent B (0.1% formic acid in ACN) were used. The peptides were eluted using a gradient from 0 to 35% B in 38 min, 35% to 90% B in 15 min, and 90% to 100% B in 5 min. The flow rate was 200 nL/min. The peptides eluted from the column were online directed into the mass spectrometer. The LC-MS system was controlled by Data-Dependent Automatic Acquisition (DDA), using positive ion detection mode. Peptide ions were detected in the MS scan, and the seven most abundant ions in each MS scan were selected for collision-induced dissociation (CID) using data-dependent MS/MS mode over the m/z range of 350-1800.

Data processing and protein identification

Raw mass spectrometry data were processed with Xcalibur v.2.1 (Thermo Scientific) and Proteome Discoverer v.1.3 beta (Thermo Scientific). For protein identification, database searches were performed using the in-house sequence algorithm in the Proteome Discoverer software against UniProt/Swiss-Prot and UniProt/TrEMBL, with the parameters set as follows: enzyme, trypsin; allowance of up to two missed

cleavages; MS accuracy, 0.015; MS/MS accuracy, 0.05; fixed modification, carbamidomethylation (C); variable modification, oxidation (Met). Proteins were identified at the 95% confidence level ($P < 0.05$). The theoretical molecular weight (MW) and isoelectric point (pI) of the identified proteins were retrieved from the output files of Proteome Discoverer. Further information on the subcellular location and function of identified proteins were retrieved from <http://www.uniprot.org/uniprot>.

RESULTS

Extraction and separation of egg membrane proteins

Due to the water-insolubility of the membrane proteins in black widow spider eggs, we used eight mixed lysis solutions containing different concentrations (1%-4%) of SL or SDS to extract this set of proteins. The protein extraction efficiencies were

compared and demonstrated different extraction abilities, as shown in Table 1. As the concentration of the detergent increased, the protein extraction rate gradually increased. SL-containing lysis solutions achieved extraction rates varying from 1.28% to 2.33%, whereas the protein extraction rate of SDS-containing lysis solutions was 1.33% to 1.97%. Figure 1 shows the dynamic changes in extraction efficiency based on the protein concentration of the extract. Comparatively, when the concentrations of SDS and SL were 1% and 2%, the protein extraction efficiencies of the two lysis solutions were not significantly different ($P > 0.05$). However, when the concentrations of the detergents increased to 3% and 4%, the extraction efficiency of the SL-containing mixed lysis solutions was higher than that of the SD-containing mixed lysis solutions ($P < 0.05$). The highest extraction of egg membrane proteins was obtained with the mixed lysis solution containing 3%-4% SL.

Table 1 Comparison of the extraction efficiencies of different mixed lysis solutions

Item	SL concentration (%)				SDS concentration (%)			
	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0
Membrane sample (mg)	50	50	50	50	50	50	50	50
Extract volume (mL)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Proteins extracted (mg)	0.638	0.803	1.105	1.165	0.663	0.673	0.853	0.985
Extraction rate (%)	1.28	1.61	2.21	2.33	1.33	1.35	1.71	1.97

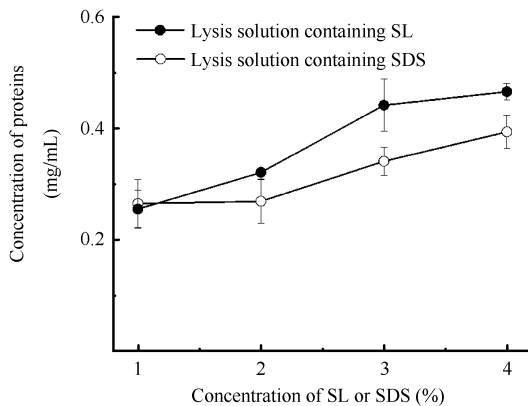


Figure 1 Effect of different mixed lysis solutions on the extraction of egg membrane proteins

The experiments were performed in triplicate.

Prior to nLC-MS/MS analysis, the extracted membrane protein mixtures were separated in parallel lanes with SDS-PAGE (Figure 2), which showed that the protein distribution profiles were similar between the two extraction conditions. As the concentration of the detergents increased, the protein content of the extract exhibited an increasing trend. However, when the concentrations of the detergents were greater than 2%, the extraction of proteins greater than about 60×10^3 was obviously enhanced while that of the proteins below 60×10^3 remained relatively stable, indicating that the efficient extraction of higher molecular weight proteins, which are generally more hydrophobic, needed detergents at higher concentrations.

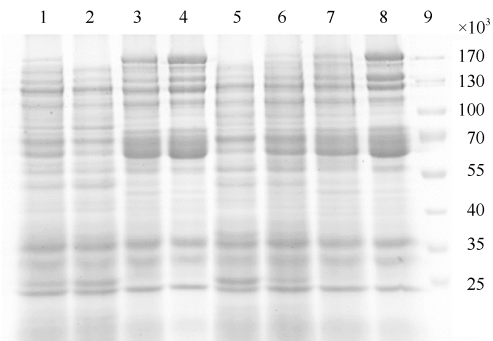


Figure 2 SDS-PAGE image of egg membrane proteins extracted by different mixed lysis solutions

Lanes 1-4: proteins extracted by lysis solutions containing 1%, 2%, 3%, and 4% SDS, respectively; Lanes 5-8: proteins extracted by lysis solutions containing 1%, 2%, 3%, and 4% SL, respectively; Lane 9: protein molecular weight marker.

Identification the membrane proteins

After the proteins in the gel slices were in-gel digested and the recovered tryptic peptides were analyzed with nLC-MS/MS, the acquired data were used to search against protein databases. As a result, a total of 39 proteins with membrane-localization information were identified after de-redundancy and removal of the proteins with no membrane localization information (Table 2). The data in Table 2 and Figure 3 show that the identified membrane proteins were distributed in the MW range of 14.6×10^3 - 352.9×10^3 , with more than 60% of the identified membrane proteins in the MW range of 20×10^3 - 80×10^3 . The

39 membrane proteins were distributed in the pI range of 4.74-10.04. About 77% of the proteins were acidic (pI<7), with proteins with a pI value between 5 and 6 accounting for 53.85% (Figure 4). Table 2 also lists the number of unique peptides identified for each protein. The proteins keratin, hornerin, and ATP synthase had relatively higher numbers of unique peptides.

According to their biological functions, the 39 identified

membrane proteins could be divided into four groups, though this functional classification was not strict as proteins usually have multiple functions: (i) Metabolic enzyme; (ii) Structure; (iii) Regulation; and (iv) Transport (Figure 5; Table 2). Nineteen membrane proteins (accounting for 48.72%) were metabolic enzymes, involved in substance and energy metabolism, including acetyl-coenzyme A carboxylase carboxyl transferase,

Table 2 Information on the membrane proteins identified from the spider eggs

Protein name	MW ($\times 10^3$)	pI	No. of pep
Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	35	6.34	1
ATP synthase subunit alpha	55.2	5.62	7
ATP-dependent Clp protease ATP-binding subunit ClpX	46.8	5.22	1
ATP synthase subunit beta	50.4	5.1	1
ATP synthase gamma chain	31.4	8.34	2
Carbamoyl-phosphate synthase large chain	117.5	5.26	1
ATP synthase epsilon chain	14.6	5.11	1
Succinate dehydrogenase flavoprotein subunit	64.4	6.27	1
Glycerol kinase	55.5	5.49	2
Serine hydroxymethyltransferase 1	44.9	6.11	1
Coproporphyrinogen-III oxidase, aerobic	35.3	5.8	1
Adenylate kinase	23.3	6.42	1
Aspartate carbamoyltransferase.	36.3	7.27	1
Nucleoside diphosphate kinase	15	5.57	1
Adenylosuccinate synthetase	46.9	5.21	1
Probable polyketide synthase 18	339.3	6.77	1
Aspartate-tRNA ligase	66.2	5.54	1
Dual oxidase	174.4	6.99	2
Polyribonucleotide nucleotidyltransferase	74.9	5.4	7
Keratin, type I cytoskeletal 10	58.8	5.21	11
Keratin, type I cytoskeletal 9	62	5.24	27
Keratin, type II cytoskeletal 6A	60	8	4
Keratin, type II cytoskeletal 2 epidermal	65.4	8	32
PREDICTED: microtubule-actin cross-linking factor 1, isoforms 1/2/3/5 isoform 1	352.9	5.68	3
PREDICTED: microtubule-actin cross-linking factor 1, isoforms 1/2/3/5 isoform 2	332.6	5.89	3
Hornerin	282.2	10.04	10
Plakophilin-1	80.1	8.95	1
Desmoglein-1	113.7	5.03	1
Junction plakoglobin	81.7	6.14	2
Desmocollin-1	99.9	5.43	1
Dynamin-like 120 kDa protein, mitochondrial	111.6	7.87	1
Keratin, type II cytoskeletal 1	66	8.12	17
14-3-3 protein epsilon	29.2	4.74	1
Ras-related protein Rab-3A	24.9	5.03	1
Neuronal growth regulator 1	37.9	6.52	1
60 kDa chaperonin	56.8	5.08	7
Protein translocase subunit SecY	47.8	9.82	1
Protein translocase subunit SecA	102.8	5.74	1
AP-4 complex subunit beta-1	82.3	5.44	1

No. of pep: Number of unique peptide for an identified protein.

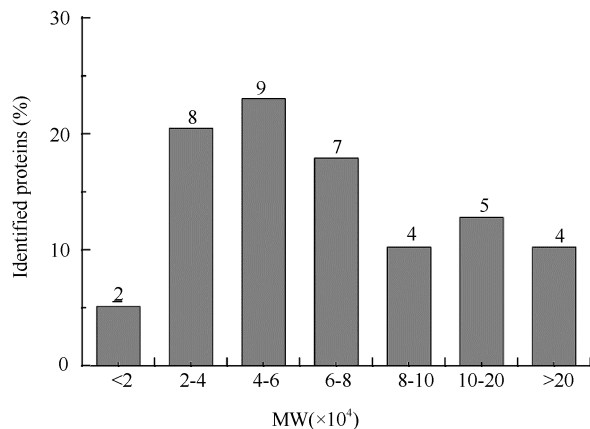


Figure 3 Distribution of the identified membrane proteins as a function of molecular weight (MW)

Values on the top of the column are the number of identified proteins.

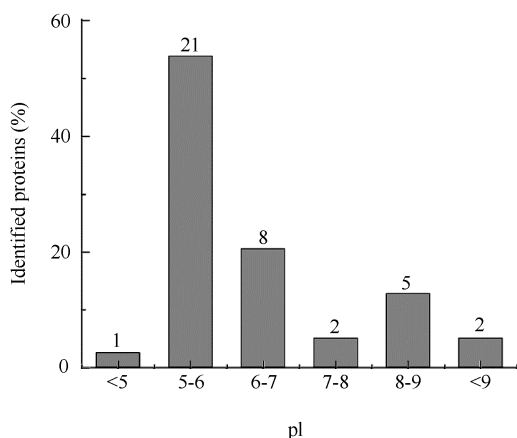


Figure 4 Distribution of identified membrane proteins as a function of isoelectric point (pI)

Values on the top of the column are the number of identified proteins.

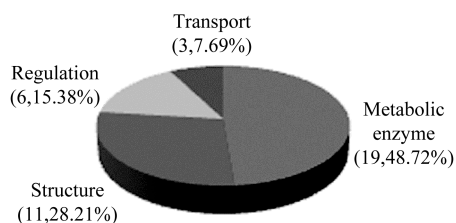


Figure 5 Functional classification of the identified membrane proteins

ATP synthase and carbamoyl-phosphate synthase. Eleven proteins (28.21%) were classified into the “Structure” group, and included membrane-localized cytoskeleton components and cell membrane proteins related to cell adhesion and cell junction, such as hornerin, plakophilin-1, junction plakoglobin, and some special kinds of keratins. Most proteins in this group not only acted as a structural component but also exerted other biological functions (see Discussion). Six regulatory proteins and three transport proteins were unambiguously identified, and

were primarily involved in the regulation of enzyme activity and signal pathways as well as transmembrane transport of proteins.

Of the 39 identified membrane proteins, 14 had unambiguous cell membrane-localized annotation in the protein databases. These cell membrane proteins were involved in catalytic, structural and regulatory functions. At least eight identified cell membrane proteins, including hornerin, plakophilin-1, junction plakoglobin and neuronal growth regulator 1, were involved in membrane cornification and recognition and communication between eggs, suggesting that the cell membrane of these spider eggs had specificities.

DISCUSSION

Contemporary proteomic techniques provide an effective means to comprehensively analyze all proteins in a cell, tissue or organism. However, membrane protein analysis has comparatively lagged behind that of soluble proteins due to their poor solubility in aqueous buffers, which limits their solubilization, extraction and enzymolysis (Rabilloud 2003; Santoni et al, 2000; Wu & Yates, 2003). To improve the extraction of membrane proteins, particularly high-hydrophobicity integral membrane proteins, a series of additives can be supplemented to the buffer alone or in combination, including detergents, chaotropes, aqueous-organic solvents, and organic acids (Masuda et al, 2008; Speers & Wu, 2007). SDS is the most often used detergent because it has a stronger ability than other commonly used detergents to disrupt biological membranes and extract membrane proteins (Masuda et al, 2008; Botelho et al, 2010). However, SDS is difficult to remove from the samples and therefore interferes with the following proteolysis and mass spectrometric analysis, thus limiting its application to membrane proteomics (Yu et al, 2003; Botelho et al, 2010). Recently, another detergent, sodium laurate (SL), was found not only to lyse membranes and extract membrane proteins as efficiently as SDS, but was also compatible with proteases and mass spectrometry as it can be conveniently removed from samples by phase transfer after acidification (Lin et al, 2013). In the present study, we used SDS- and SL-containing lysis solutions to extract the membrane proteins from black widow spider eggs. Because the eggs used were near hatching, their cell membranes were cornified to a certain extent, which further hampered the lysis of membranes and the extraction of membrane proteins. Therefore, to enhance extraction efficiency, other additives (CHAPS and DTT) were added to the Tris-HCl buffer to constitute a basic lysis solution. The results demonstrated that the mixed lysis solution containing higher concentrations of SL showed a certain advantage over the SDS-containing mixed lysis solution in the extraction of egg membrane proteins.

Membrane proteins perform a variety of cellular functions, including catalyzing metabolic reactions, transporting molecules and ions across the membrane, relaying signals in metabolic regulation and allowing cells to identify and interact with each other (Almén et al, 2009). In the present study, we identified 39 membrane proteins with corresponding functions. These proteins were distributed in wide MW (14.6×10^3 - 352.9×10^3) and

pl (4.74-10.04) ranges, suggesting diversity in both structure and function. Nearly half of the identified membrane proteins were metabolic enzymes, the majority of which were involved in energy metabolism and synthetic metabolism and included ATP synthase, succinate dehydrogenase, adenylate kinase, nucleoside diphosphate kinase, acetyl-coenzyme A carboxylase carboxyl transferase, aspartate carbamoyltransferase, and adenylosuccinate synthetase. The identification of these metabolic enzymes indicated that the relevant metabolic processes were active during the embryonic development of the spider egg. All metabolic processes in a cell are elaborately regulated and many membrane proteins participate in these regulation processes.

Six identified proteins had regulatory activities and were classified into the "Regulation" group, although they also had other biological functions. For example, 14-3-3 protein epsilon in this group is a member of the 14-3-3 protein family and is involved in the regulation of a large spectrum of cellular processes, including metabolism, signal transduction, and cell development (Dunaway et al, 2005; Paul & van Heusden, 2005). Many experiments have demonstrated the importance of 14-3-3 proteins in embryonic development. Wu & Muslin (2002) used an unphosphorylated peptide inhibitor of 14-3-3, R18, to determine the role of 14-3-3 proteins in *Xenopus* embryonic development, and demonstrated the requirement for 14-3-3 in mesodermal specification. Inhibition of 14-3-3 resulted in embryos with axial patterning defects and reduced expression of mesodermal marker genes. These phenotypic defects were caused by impaired fibroblast growth factor signaling in R18-injected embryos. To evaluate the role of individual 14-3-3 proteins in vertebrate embryonic development, Lau & Muslin (2009) utilized an antisense morpholino oligo microinjection technique in *Xenopus laevis* embryos, and showed that embryos lacking specific 14-3-3 proteins displayed unique phenotypic abnormalities. Keratin, type II cytoskeletal 1 was another identified membrane protein classified into this group, which not only acts as a structural constituent of the cell membrane but also as a high affinity receptor (Pixley et al, 2011).

Eleven membrane proteins were categorized into the "Structure" group, including several keratins closely related to embryo development. Lu et al (2005) demonstrated that two type I and two type II keratin genes were already transcribed in the 2-cell stage embryo, and type II keratins preceded type I keratins during early embryonic development. Hornerin was a constituent of the cell membrane and was identified based on 10 unique peptides, only lower than that of several keratins, suggesting that the concentration of hornerin in the sample was fairly high. The relative concentration of a protein identified by mass spectrometry is directly related to the number of identified peptides, neglecting the possible effects of other factors such as enzymatic digestion constraint, detection mass range of the mass spectrometer and differential post-translational modification. Therefore, the number of identified unique peptides assembled into a protein may reflect the protein's relative abundance (Fan et al, 2013; Jin et al, 2008; Li et al, 2010). Hornerin can bind Ca^{2+} and was rich in the cell membrane in our present study, suggesting that it may play an

important role in the cornification of cell membrane because Ca^{2+} is known to trigger the process of cornification (Hennings et al, 1980). Another identified cell membrane protein, desmocollin-1, can also bind Ca^{2+} and is involved in cornification (Ishida-Yamamoto et al, 2011). During embryo development, calcium is a major nutritional requirement (Johnston & Comar, 1955). It is speculated that the existence of such Ca^{2+} -binding proteins in the cell membrane not only enrolls Ca^{2+} to strengthen the cell membrane, but also stores Ca^{2+} for later stages of embryo development. It is worth mentioning that we identified several cell membrane proteins with cell adhesion and cell junction functions, including plakophilin-1, desmoglein-1 and junction plakoglobin, suggesting recognition and communication between eggs in the same egg sac, although the eggs existed dispersedly. In addition, the identification of polyribonucleotide nucleotidyltransferase and several proteins with protein transport activity, as well as chaperonin, suggested that processing and translation of mRNA was active during the embryonic development of the eggs. A batch of ribosomal proteins and regulatory factors for protein synthesis were identified, together with membrane proteins from the eggs, supporting this speculation (data not shown).

In summary, to efficiently extract and identify the membrane proteins of black widow spider eggs, we comparatively employed different lysis solutions to lyse the biological membranes and extract membrane proteins, followed by SDS-PAGE and nLC-MS/MS analysis. The mixed lysis solution containing SL showed a certain advantage over that containing SDS when the concentrations of the detergents were higher (3%-4%). A total of 39 membrane proteins involved in structure, catalysis, metabolism regulation, signal transduction or cell communication were identified, which is consistent with the functions of biological membranes. Nearly half of the identified membrane proteins were metabolic enzymes involved in various cellular processes, particularly energy metabolism and biosynthesis, suggesting that relevant metabolic processes were active during the embryonic development of the eggs. The identification of cell membrane proteins is helpful for revealing the special structure and functions of egg cell membranes.

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