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Identification of proteins from the accessory sex gland of *Eriocheir sinensis* by two-dimensional electrophoresis and mass spectrometry

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Abstract

Accessory sex glands play an important role in male reproduction; however, the function of their secretions has not been completely elucidated. This study investigated the function of the accessory sex glands of the crustacean, *Eriocheir sinensis*, by identifying proteins secreted during early and peak developmental periods using two-dimensional electrophoresis and mass spectrometry. Most of the proteins had a low molecular weight and were concentrated in the region of pI 4–7 on the gel. After digestion, 13 differentially expressed proteins were analyzed by mass spectrometry. Among these 13, nine were preliminarily identified and could be categorized into four functional groups: spermatozoa capacitation, anti-oxidant, metabolism, and transcription and post-transcriptional regulation. The characteristics of these proteins confirmed that accessory sex gland secretions play an important role in the physiological status of sperm. This proteomics analysis of accessory sex gland can form the foundation for further studies of the function of accessory sex gland secretions.

Key words: Sperm physiology, crustacean, peptide mass fingerprinting, anti-oxidant, reproduction

Introduction

Accessory sex glands such as the seminiferous tubules and epididymus play an important role in male reproduction. Immature spermatozoa, recently produced in the seminiferous tubules, transit though the epididymis where they become motile and undergo a series of transformations. These changes include alterations in the composition of the sperm’s membrane lipids and proteins, ion exchange between the extra- and intra-cellular environments, and cytoskeleton rearrangements (Olsson et al., 2002; Dacheux et al., 2003; Sullivan et al., 2002). The epididymal epithelium secretes proteins that potentially affect the sperm’s maturation (Dacheux et al., 2003) as well as other aspects of its physiology while they are stored in the cauda epididymidis before ejaculation (Henault et al., 1995). These proteins may determine important attributes of fertilizing ability (Riffo & P’Arraga, 1997; Yuan et al., 2003) of sperm [including motility (Curi et al., 2003; Elzanaty et al., 2002), oocyte binding, penetrating ability (Amann & Griel, 1974) as well as capacitation, acrosome reaction (AR) (Einspanier et al., 1993; Yanagimachi, 1994; Aumuller et al., 1997) and DNA integrity (Chen et al., 2002)].

*Eriocheir sinensis* is a traditional savoury food and comprises one of the economically important aquatic...
species of China (Chen et al., 2007). It is a catadromous crustacean with a life-span of about 2 years. It has a single reproductive season and dies shortly after reproduction (Rudnick et al., 2003). With the development of intensive culture, precocious puberty in this crab is very common, and the rate of precocious puberty approaches 20–98%, but never over 10% under natural conditions (Lieshi Zhang, 2001; Chunying Yuan, 2004). Precocious puberty induces early reproductive ec dysis, which results in the miniaturization of mitten crab with consequent economic loss. To date this problem has become the main obstacle affecting the aquaculture of the species. In recent years, many questions involving cultural precocious puberty have been addressed via studies of the reproductive biology of E. sinensis, which is a decapod freshwater crab (Aquacop, 1983). The mitten crab has well-developed accessory sex glands in post-sexual maturity at the junction of the ejaculatory duct and seminal vesicles. The secreted accessory sex glands together with spermatophores enter the spermatheca during mating. The development of accessory sex gland usually starts in August and matures before mating. These organs develop rapidly and can fill the body cavity in only 2–3 months. Why this organ develops so rapidly and whether it has analogous important function in fertilization in mammals is the question upon which we focus. These physiological phenomena must be critical to the spermatozoa modifier in preparation for fertilization. Early studies of E. sinensis in our laboratory have shown that accessory sex glands can break down spermatophores to release free spermatozoa which is accompanied by even higher acrosin activity. To better understand the function of rapidly developed accessory sex glands and the proteins involved in reproduction in mitten crab, this study evaluates the secretions of accessory sex glands using two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization (MALDI) – time of flight (TOF) – mass spectrometry (MS). These techniques were selected because 2-DE is the only tool that can separate tens of thousands of proteins simultaneously (Wasinger, 1995; Rabilloud, 2002), and it has high resolution and reproducibility (Gorg et al., 2000). MS is currently the most accurate and effective means for identifying proteins (Aebersold & Mann, 2003; Griffin et al., 2001; Mann et al., 2001) and can be used to obtain the corresponding amino acid sequences.

Materials and Methods

Chemicals and materials

Im mobilized pH gradient (IPG) strips (pH 3–10, linear, 17 cm), IPG buffer (pH 3–10), urea, thiourea, CHAPS, DTT, IAA, bromophenol blue, Tris, glycerol, low melting point agarose, acrylamide, bis-acrylamide, AP, Temed, 10 × electrophoresis buffer, mineral oil, Bio-safe Coomassie G-250 stain, Protein IEF cell, electrophoresis apparatus, GS710 scanner, PDQuest analysis software, and quantity one software were purchased from Bio-Rad Laboratories. Trifluoroacetic acid (TFA), trypsin, α-cyano-4-hydroxycinnamic were bought from Sigma Chemical Company. Sequazyme peptide mass standard kit, Voyager-De Maldi-TOF-MS and steel target with 96 wells were the products of Applied Biosystems (USA). All buffers were made using high-purity MiliQ water.

Experimental materials and sample preparation

Sexually mature E. sinensis with accessory sex glands in early and peak stages were purchased from the Changfeng market in Shanghai, China. Ten crabs in the early stage of reproductive development were dissected to collect the accessory sex glands as early stage samples, and three crabs in peak stage as the peak stage samples. The collected samples were washed twice in ice-cold Ca²⁺-FASW (0.4 mol/L NaCl, 15 mmol/L KCl, 8.6 mmol/L boric acid, 4.8 mmol/L NaOH, 41 mmol/L MgSO₄, pH7.4) and homogenized; the accessory sex glands from E. sinensis were centrifuged at 15,000 g for 30 min at 4°C. The protein of the supernatant was dissolved in 1 mL Ca²⁺-FASW and then extracted with 1 mL of lysate buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% pharmalyte 3–10, 40 mM Tris, 40 mM DTT) for 1 h at 4°C. After centrifugation at 12,000 g 4°C for 1 h, protein concentrations were determined by a modified Bradford assay (Marra et al., 2006; Pitarch et al., 2002; Pitarch et al., 2003). The supernatant was aliquoted and stored at −80°C.

2-DE: Isoelectric focusing (IEF)

IEF was performed using IPG dry strips (17 cm, pH 3–10). About 200 µL of protein samples (2.4 mg) and 100 µL of loading sample buffer (8 M urea, 4% CHAPS, 65 mM DTT, 0.5% IPG buffer, 0.001% bromophenol blue) were combined for a total volume of 300 µL, and dropped into a hydration groove. The IPG dry strip was left at room temperature for 1 h and then covered with 2–3 mL of mineral oil. The setting procedure for IEF was 50 V for 12 h (sample loading), 250 V for 0.5 h, 1000 V for 1 h, 9000 V for 5 h, and 9000 V for 50,000 V. Hours (20°C; for specific procedures see Table 1).

SDS-PAGE

After IEF, the strip was equilibrated in 6 mL...
Table 1. Procedure for isoelectric focusing

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Voltage</th>
<th>Mode</th>
<th>Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hydration</td>
<td>50</td>
<td>Active hydration</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>Desalination</td>
<td>250</td>
<td>Line</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>Desalination</td>
<td>1000</td>
<td>Rapid</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>High voltage</td>
<td>9000</td>
<td>Line</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Focus</td>
<td>9000</td>
<td>Rapid</td>
<td>50,000 V. h</td>
</tr>
<tr>
<td>6</td>
<td>Maintain</td>
<td>500</td>
<td>Line</td>
<td>Random time</td>
</tr>
</tbody>
</table>

Equilibration buffer I [0.375M Tris-HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS, 0.12 g DTT (added before use)] for 15 min, and in equilibration buffer II (same as buffer I but 0.15 g iodoacetamide was added before use instead of DTT) for 15 min. The strip was removed and then immersed in 1× electrophoresis buffer. Next, the IPG strip was placed above the surface of a 10% SDS-gel and covered with low melting point agarose for 15 min. Finally, vertical SDS-PAGE was performed at 15°C, with constant power at 50 V per gel for the first 30 min and 200 V per gel until the end of electrophoresis.

**Staining and image analysis**

Separated proteins were stained with Bio-safe Coomassie blue G-250 stain and three replications were performed for each sample. Destained gels were scanned and digitized by a Bio-Rad GS710 scanner, and then analyzed by PDQuest 8.0 analysis software. Combined with artificial correction, the significant differences in protein expression were detected using the criteria of three times the dot-staining intensity and volume changes in area.

**MS identification of differentially expressed proteins**

Protein spots of interest were excised from the gel with a scalpel and placed in a 1.5 mL siliconized Eppendorf centrifuge tube. To wash away the soluble Coomassie blue, a 200 μL destain solution (200 mM NH4HCO3 with 40% acetonitrile) was added to the tube and incubated for 30 min at 37°C. Then the gels were dried in a Speed Vac (40°C) for 30 min. The dried gels were rehydrated with 20 μL cold working trypsin solution (20 μg/mL) and 50 μL trypsin diluents (40 mM NH4HCO3 in 9% acetonitrile). The rehydrated gels were incubated at 37°C for 16 – 24 h. Gel slices were soaked in 50 μL of 50% acetonitrile and 0.1% trifluoroacetic acid for 30–60 min with gentle agitation. The supernatant was transferred to a second clean 500 μL microcentrifuge tube. This procedure was repeated 2–3 times, followed by drying and condensing remaining extracted peptides in an N2 flow. The dried sample was reconstituted by adding 500 μL acetonitrile, 100 μL 3% trifluoroacetic acid containing 10 mg/mL α-Cyano-4-hydroxycinnamic acid. The solution was spotted on a stainless steel target with 96 wells and dried at room temperature. The resulting crystallized protein was used for the MALDI-TOF-MS analysis according to the methods of Abbas et al. (2005).

The differentially expressed protein spots were selected on 2-DE maps of accessory sex gland of *E. sinensis* at early and peak stages. The selected spots were excised and identified with MALDI-TOF-MS using a MS sequazyme peptide mass standard kit for external calibration and a matrix trypsin cut from the peak as an internal correction. Parameters were set as follows: reflection mode, nitrogen laser (337 nm, 0.5 ns pulse width, 20 Hz repetition rate), delayed ion extraction 100 ns, 70% grid voltage, vacuum degree 4e-008, single scan of MS signals accumulating 200 times, and positive spectra determination.

**Database searching**

Sequence similarity searches based on MALDI-TOF-MS/peptide mass fingerprinting (PMF) were performed with Mascot software (Matrixscience Company; http://www.matrixscience.com) using default parameters for the NCBInr, SWISS-PRO and MSDB databases. Parameters were set as follows: all entries; allow error, 150 ppm, fixed modification, carbamidomethylation; variable modification, methionine oxidation; mass values, MH+; and maximum allowed missed cleavage, 1.

**Results**

Figs. 1 and 2 show maps of the proteins of the accessory sex glands of *E. sinensis* during the early and peak developmental periods after separation with 2-DE. About 50 spots can be detected on each map using the same parameters. The apparent molecular weight and pI of these proteins have a wide distribution, but most of them are focused in the region of pI 4–7. After correction with PDQuest 8.0 software and manual analysis, 13 spots from Figs. 1 and 2 that were distinct, reproducible, and had high-resolution were selected as having significant differences between early and peak periods (Fig. 3). Protein spots 1 to 5 were present only during peak development; spots 6 to 13 were seen during both periods but were markedly more dense in the peak compared to early development (Table 2).

The 13 distinct protein spots were excised from gels for MALDI-TOF-MS analysis. The majority of PMFs
obtained had low background, high peak signals were between 800–3000 m/z. Fig. 4 shows the PMF of protein spot 12.

Once the PMFs were obtained, they were used to search the NCBInr, SWISS-PROT and MSDB databases. Using the molecular weight and pI, 9 proteins were preliminarily identified based on peptide mass matching according to the amino acid sequences and match coverage (Table 3). These proteins were then divided into four categories according to function: (1) spermatozoa capacitation-related proteins, such as spots 2 and 7; (2) anti-oxidant proteins (e.g. spot 12); (3) metabolism-related proteins, including spots 1 and 13; (4) transcription and post-transcriptional regulation proteins, such as spots 4, 5, 9, and 10. Spot 11 is a hypothetical protein and its function is unknown, and matching proteins for spots 3, 6, and 8 were not found in the database.

**Discussion**

In this study, we used 2-DE to separate proteins of the accessory sex glands of *Eriocheir sinensis* at early and peak stages and identified 13 highly expressed...
Table 3. Database search results for 13 differentially expressed proteins

<table>
<thead>
<tr>
<th>Spot ID</th>
<th>NCBI ID</th>
<th>Peptides matched</th>
<th>Sequence covered (%)</th>
<th>Theoretic Mr/pl</th>
<th>Protein name</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gi</td>
<td>126652121</td>
<td>7/8</td>
<td>15</td>
<td>60141/4.95</td>
<td>Dipeptide ABC transporter</td>
</tr>
<tr>
<td>2</td>
<td>ARGB_PSYWF</td>
<td>6/8</td>
<td>17</td>
<td>31993/5.51</td>
<td>N-acetylglutamate kinase</td>
<td>Spermatozoa capacitation</td>
</tr>
<tr>
<td>3</td>
<td>Unidentified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>gi</td>
<td>119936850</td>
<td>15/39</td>
<td>32</td>
<td>53384/8.17</td>
<td>Similar to helicase-like protein 2</td>
</tr>
<tr>
<td>5</td>
<td>RRP3_NEUCR</td>
<td>8/11</td>
<td>14</td>
<td>56975/8.97</td>
<td>ATP-dependent rRNA helicase rrp-3</td>
<td>Transcription</td>
</tr>
<tr>
<td>6</td>
<td>Unidentified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>gi</td>
<td>183220276</td>
<td>14/56</td>
<td>43</td>
<td>26094/6.55</td>
<td>Putative transcriptional regulator, Crp family</td>
</tr>
<tr>
<td>8</td>
<td>Unidentified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>gi</td>
<td>57094590</td>
<td>21/70</td>
<td>51</td>
<td>32198/5.10</td>
<td>Similar to heterogeneous nuclear ribonucleoprotein C</td>
</tr>
<tr>
<td>10</td>
<td>gi</td>
<td>119571321</td>
<td>18/32</td>
<td>17</td>
<td>100950/6.45</td>
<td>Chromodomain helicase protein 1-like</td>
</tr>
<tr>
<td>11</td>
<td>gi</td>
<td>153813998</td>
<td>28/114</td>
<td>62</td>
<td>31762/8.86</td>
<td>Hypothetical protein RUMTOR_00205</td>
</tr>
<tr>
<td>12</td>
<td>gi</td>
<td>158148206</td>
<td>9/29</td>
<td>34</td>
<td>21691/4.84</td>
<td>Thio-specific antioxidant peroxidase</td>
</tr>
<tr>
<td>13</td>
<td>gi</td>
<td>167215166</td>
<td>13/27</td>
<td>22</td>
<td>59034/5.09</td>
<td>Phosphoglucomutase/ phosphomannomutase</td>
</tr>
</tbody>
</table>
proteins in the peak period. Because all the accessory sex gland organs of mitten crabs were used for samples preparation, some of these proteins are probably from the epithelial of accessory sex glands. According to their functions, the 13 differentially expressed proteins identified by MS could be grouped into following categories, which will be discussed below: spermatozoa capacitation-related proteins (spot 2 and 7), anti-oxidant proteins (spot 12), metabolism-related proteins (spot 1 and 13) and transcription and post-transcriptional regulation proteins (spot 4, 5, 9 and 10).

Spot 2 was identified as N-acetylglutamate kinase (NAGK), in which N-Acetylglutamate synthase (AGS) and glutamate N-acetyltransferase (GAT) are the key enzymes in the synthesis of arginine that serves as an important precursor for the synthesis of protein, polyamines, urea, and nitric oxide. The NAGK is the key second enzymes in the biosynthesis of L-arginine (Sakanyan et al., 1996), which plays an important physiological role in goat spermatozoa and has been shown to enhance cell metabolism (Patel et al., 1998, 1999) and protect against lipid peroxidation (Srivastava et al., 2000). Nitric oxide (NO) is derived from L-arginine by a reaction catalyzed by the enzyme NO synthase (NOS; Palmer et al., 1988) and plays a decisive role in regulating multiple functions within the male and female reproductive systems. The enzyme, NOS, is mainly present in the sperm acrosome and tail region suggesting that NO may be related to sperm motility, as well as the AR and capacitation (Herrero et al., 2001; Revelli et al., 2001). A similar role has been demonstrated for NO in human spermatozoa where this reactive oxygen species (ROS) has been shown to trigger events closely related to capacitation such as phosphorylation of tyrosine (Herrero et al., 2000) and serine/threonine (Thundathil et al., 2003). Therefore, NAGK can be indirectly but closely related to spermatozoa capacitation. Another spermatozoa capacitation-related protein (spot 7) was identified as a putative transcriptional regulator, belonging to the Crp (cAMP receptor protein) family, which can be activated and converted to cAMP dependent protein kinases (PKA). Materials that can promote the synthesis of cAMP, also known as the ring of adeno-nucleoside phosphorylase, can also elevate spermatozoa capacitation and reaction (Cornett, 1978). In support of this, one study using bacteria-cation Nigeria and the carrier A23187 to stimulate AR in sea urchin sperm showed that cAMP increased before the AR (Garbers, 1981). Furthermore, cAMP was found to play a major role in the maturation process of sperm in the epididymis; cAMP gradually increased as sperm mature in the epididymis (Hoskins, 1974) and exogenous cAMP promoted sperm movement (Morton, 1974). Sperm metabolism has also been shown to be stimulated by cAMP by increasing sperm sugar consumption and respiratory rate (Hoskins, 1973). These physiological effects of cAMP depend on cAMP receptor protein (Crp), from which it may be inferred that they are the most important spermatozoa capacitation-related proteins. In the sperm of mice, humans, cows and pigs, PKA/cAMP signaling pathways can regulate protein tyrosine phosphorylation, which is the major biochemical reaction that occurs during spermatozoa capacitation (Urner & Sakkas, 2003). In E. sinensis, the spermatophores of the seminal vesicles, together with accessory sex gland proteins, come into the spermatheca during mating. Sperm gradually dissociate from the spermatophores and undergo capacitation and AR throughout fertilization. N-acetylglutamate kinase and cAMP receptor protein may play a key role in the regulating this process, but the details of this procedure, especially the signal pathway regulating this procedure, require more experimental investigation.

The microenvironment in the male reproductive tract has to provide enzymatic and non-enzymatic strategies to protect the spermatozoa from excessive oxidative stress, thereby preserving sperm function and DNA integrity (Vernet et al., 2004). In the post-ejaculatory phase and in the female genital tract, spermatozoa have to depend on antioxidants, metal ions and proteins provisions in male accessory sex gland secretions for protection (Wai-sum O et al., 2006). Our study found a protein (Spot 12) belonging to a family of peroxidase proteins, namely thio-specific antioxidant peroxidase (TSA/AhpC). This new type of peroxidase with cysteine as the primary site of catalysis is also referred to as “thiol peroxidase” and was discovered in prokaryotes and eukaryotes (Lim et al., 1994; Cha et al., 1995; Kang et al., 1998; Jeong et al., 2000; Park et al., 2000). The member of the TSA/AhpC family eliminates H₂O₂ and alkyl hydroperoxides using a thiol-reducing equivalent (Kim et al., 1988; Chae et al., 1993; Lim et al., 1993; Lim et al., 1994). Sperm in mitten crab are enveloped by a spermatophore wall which will dissolves after sperm enter the spermatheca. Sperm are then in direct contact with semen, accessory sex glands secretions and spermatheca secretions. Without the protection of the spermatophore wall, sperm are vulnerable to oxygen free radicals in the semen and, therefore, the role of anti-oxidants are particularly important to sperm at this time. Such anti-oxidant proteins secreted by the accessory sex glands may protect sperm membranes from oxidative damage, as well as to protect the integrity of the DNA.

We identified other metabolic regulation, transcription regulation and post-transcriptional regulation proteins with higher expression during the peak than...
that in early developmental period. Spot 1 was identified as dipeptide ABC transporter, which uses the hydrolysis of ATP to energise diverse biological systems which are involved in the export or import of a wide variety of substrates ranging from small ions to macromolecules. The major function of ABC import systems is to provide essential nutrients. Spot 13 was identified as phosphoglucomutase/phosphomannomutase (PGM/PMM), which induces the generation of glucose-6-phosphate and mannose-6-phosphate (Joshi et al., 1964). Glucose-6-phosphate is an important intermediate product of metabolism and connects many glucose metabolism processes (anaerobic glycolysis, aerobic oxidation, pentose phosphate way, glycogen synthase, glycogenolysis). Adenosine triphosphate (ATP) generated through anaerobic glycolysis and aerobic oxidation of glucose is necessary to maintain sperm motility and hyperactivated motility (William & Ford, 2001), and for phosphorylation and cAMP synthesis which though PKA/cAMP signaling pathways regulate protein tyrosine phosphorylation (Urner & Sakkas, 2003). Therefore, we infer that this enzyme is most likely to be involved in the metabolism of sperm and capacitation, and provides energy for normal metabolism and fertilization. Our study also found that a large number of proteins relating to cell proliferation and protein synthesis were abundantly expressed during peak development, which may be from the epithelia of accessory sex glands. Transcription and post-transcriptional regulation proteins include chromodomain helicase protein 1-like (CHD1L) (spot 10), ATP-dependent rRNA helicase (spot 5), helicase-like protein 2 (spot 4) and heterogeneous nuclear ribonucleoprotein C (hnRNP)(spot 9). The first three are helicases, which are enzymes that can follow two directions, the nucleic acid phosphate ester skeleton and specifically direct the movement of motor proteins. Heterogeneous nuclear ribonucleoprotein C is usually active in a variety of RNP complexes and plays an important role in regulating transcription, precursor mRNA splicing, mRNA export, mRNA degradation and other biological processes (Moore et al., 1995).

In conclusion, this study investigated the protein products of genes that were differentially expressed in accessory sex glands of *E. sinensis* during early and peak stage. The identified 13 differently expressed proteins may not be the all proteins in the two samples due to the limitation of 2D analysis. The spermatozoa of *E. sinensis* is an aflagellate sperm, so there must be a distinctive modulation mechanism and functional method to complete fertilization. The traits of these proteins strongly suggest that accessory sex gland secretions play an important role in regulating the physiological status of sperm. Thus, these results can be the basis for further studies of the function of accessory sex gland secretions. Such a study in the different kind of spermatozoa found in *E. sinensis* will supplement our understanding the mechanism of animal fertilization biology.

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