江苏省吕四港潮间带贝类的蛋白质组分析

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摘要：潮间带滩涂是江苏省海岸线的重要特征，腹足类、双壳贝类海洋生物资源丰富。该文基于 shotgun 蛋白质鉴定技术研究江苏省吕四港地区主要贝类的蛋白质组成。采用 nano-LC MS/MS 技术从 9 种贝类生物中共鉴定了 265 个蛋白质，其中角螺中鉴定的蛋白质最多(105 个)，从毛蚶中鉴定的蛋白质最少(39 个)。这些蛋白质以细胞骨架蛋白为主，如微管蛋白、肌动蛋白、原肌球蛋白、肌球蛋白重链及组蛋白；另外还鉴定出核糖体蛋白、精氨酸激酶、ATP 合成酶、延伸因子、热休克蛋白等。采用 PCA 分析法将蛋白质按照细胞成分与分子功能进行统计分析，结果表明缢蛏、泥螺与角螺的蛋白质组成相似，而竹蛏、文蛤、杂色蛤和四角蛤蜊的蛋白质组成相似。Heatmap 和聚类分析结果表明，杂色蛤和西施舌被聚为一类，而缢蛏、泥螺、角螺、文蛤、毛蚶和四角蛤蜊被聚为另一类，竹蛏与这两类距离较远。该文研究建立了江苏沿海潮间带主要贝类中蛋白质组成特点比较分析的方法，有利于江苏沿海贝类资源开发利用。

关键词：蛋白质组；贝类；江苏；主成分分析；潮间带
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Proteomic Profiling in Shellfish From Lüsi Aquaculture in the Intertidal Zone Area of the Jiangsu Province

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ABSTRACT: Jiangsu province is rich in intertidal marine resources, especially gastropod and bivalve shellfish. In this study, we performed shotgun proteomic analysis on shellfish from Lüsi, Jiangsu to identify protein content. In total, 265 proteins were identified in nine species of shellfish using nano LC-MS/MS. The highest number of proteins were identified in the gastropod *Hemifusus tuba* (HF) (105 proteins), whereas the bivalve *Scapharca subcrenata* (SSC) produced the fewest (39 proteins). The most abundant proteins are constituents of cytoskeleton, including actin, tubulin, tropomyosin, myosin heavy chain, as well as histone proteins. Additional proteins were also identified, including ribosomal proteins, enzymes such as arginine kinase and ATP synthase, and housekeeping proteins such as elongation factor and heat shock protein. Proteins were classified based on cellular components and molecular function using the principal component analysis (PCA). Results of PCA analysis revealed that *Sinonovacula constrzcta* (SC), *Bullacta exarata* (BE) and *Hemifusus tuba* (HF) showed similar protein profiles, while*Solen strictus* (SS), *Meretrix meretrix* (MM), *Ruditapes philippinarum* (RP), and *Mactra veneriformis* (MV) also showed similar protein profiles. Hierarchical clustering results of heat map studies showed that RP and *Mactra antiquate* (MA) were classified into one group. SC, BE, HF, MM, SSC, and MV were classified into another group. SS showed the furthest distance from this group. We established a method for analyzing and characterizing the protein composition characteristics of predominant shellfish species in Jiangsu providence's intertidal zone area, which provides basic data for the development of shellfish resources.

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Jiangsu is a coastal province, located in the northern latitude (30°45′ to 35°20′) of the east coast of China. The total area of intertidal zone habitat in the Jiangsu province is approximately 6 580 km^2. This intertidal zone occupies roughly a quarter of the entire intertidal zone area of China. It is a suitable marine environment for shellfish to grow and has been widely used in aquaculture production. According to the *China Fishery Statistics Year Book* of 2015, the total yield of seawater aquaculture products in Jiangsu was 18.13 million tons in 2014. Of that total yield, shellfish represent the main aquaculture product in Jiangsu. This accounts for approximately 68.3% of the total commercial seawater aquaculture industry.

Used as both food and medicine, shellfish have been included in the records of Chinese *materia medica* for thousands of years. For example, *Meretrix meretrix* Linnaeus (MM), the edible clam, was first documented in *Shennong Bencao Jing* (about 200 B.C.—200 A.D.), and the bivalve *Mactra veneriformis* (MV) was first documented to have medicinal use in *Bencao jingjizhu* (about 480—498 A.D.). Recently, studies have shown that consuming shellfish promotes immune regulation, produces anticoagulant properties, and protects the vascular system [1-2]. Chemical analysis has shown that shellfish are rich in nutrients such as proteins, polysaccharides, and nucleosides [3-5]. Investigations also showed that purified polysaccharides of MV were polymerized by D-glucose [5]. The content of nucleosides and nucleotide bases in Jiangsu shellfish changed during different harvest times[3, 6-7]. Enzymatic peptides with antioxidant activity and ACE inhibitory activity have been identified [4,8]. However, proteomic profiling of shellfish is lacking in the literature, but altogether necessary for a comprehensive analysis of proteins in shellfish. This information would be essential regarding the quality and quantity of nutrients in these commercially important marine species.

In the present study, the proteomic profiles of shellfish from the Jiangsu Lüsi intertidal zone area were investigated by nano-LC MS/MS. The proteomic results revealed the composition characteristics of prominent shellfish species grown in Jiangsu intertidal zone aquaculture.

1 Materials and methods

1.1 Samples and protein extraction

Shellfish samples were collected from Lüsi's aquaculture region. Lüsi is the best shellfish-producing area in the Jiangsu province. Specimens of shellfish were identified for species identification by Professor Xihe Wan (Institute of Oceanology and Marine Fisheries, Jiangsu). After collection, samples were starved in an aquarium for 24 h to evacuate their gut contents. Subsequently, internal tissue was excavated from the shell and stored at −20 ℃. The tissue was dried at 50 ℃ for 3 days, and then powdered. Detailed information of shellfish used in this study is shown in Figure 1 and Table 1. Collection regions are shown in Figure 2.

Figure 1　Pictures of shellfish samples

Figure 2　Location information of shellfish samples collection

Table 1　Summary of the tested samples of shellfishes

No.	Species	Collection region
1	*Solen strictus* (SS)	
2	*Sinonovacula constrzcta* (SC)	
3	*Bullacta exarata* (BE)	
4	*Hemifusus tuba* (HF)	Lüsi, Nantong, Jiangsu
5	*Meretrix meretrix* (MM)	N 31°59′12″, E 121°40′58″
6	*Ruditapes philippinarum* (RP)	
7	*Scapharca subcrenata* (SSC)	
8	*Mactra veneriformis* (MV)	
9	*Mactra antiquate* (MA)	

20 mg of shellfish powder was immersed in 4 mL of 2% sodium dodecyl sulfate, 50 mmol/L sodium phosphate (pH 7.8), 20 mmol/L dithiothreitol (DTT) and incubated overnight at 65 ℃. Then, aliquots of the soluble extract were incubated at room temperature for an additional 0.5 h following the addition of 40 mmol/L iodoacetamide. Proteins were precipitated from the soluble extract by addition of three vol-

umes of ethanol. Protein samples were rinsed with 70% ethanol, then with freshly prepared 0.1 mol/L ammonium bicarbonate, and were finally resuspended in fresh 0.1 mol/L ammonium bicarbonate adjusted to 2 mol/L urea. To each suspension, bovine L-1-tosylamido-2-phenylethyl chloromethylketone-treated trypsin (Worthington, Lakewood, CO, USA) was added to 1% (w/v). After 6−8 h at 37 ℃, samples were stirred overnight at room temperature.

1.2 Protein identification using nano LC-MS/MS

A splitless Ultra1D Plus (Eksigent, Dublin, CA, USA) system was coupled with the Triple TOF 5600 using a Nanospray III source (AB SCIEX, Concord, ON, CA). Nano-LC gradients were delivered at 300 nL/min. Buffer A was 2% acetonitrile and 0.1% formic acid; buffer B was 98% acetonitrile and 0.1% formic acid. The nano-LC gradient program delivered an acetonitrile gradient over 60 min (5%−30% buffer B over 40 min, 30%−60% buffer B over 6 min, 60%−90% buffer B over 3 min, hold buffer B at 90% for 5 min, and 90%−5% B in 6 min). Peptides were separated on a fused silica capillary emitter (inner diameter, 75 μm, New Objective, Woburn, MA, USA) packed in-house with 5 μm C_{18} resin (New Objective). Positive ion MS and sequential precursor ion fragmentation acquisitions were carried out on a TripleTOF 5600 System (AB SCIEX, Concord, ON, USA) controlled with Analyst TF 1.6 software with MS/MS^{ALL} mode activated to carry out the series of product ion scans defined by the mass range from m/z 200−2 000 at an accumulation time of 300 ms. Helium was used as the collision gas for collision-induced dissociation (CID), and collision energy for each MS/MS step was (50±30) eV.

All raw data files (*.wiff) were collectively searched using ProteinPilot Software v4.1 (AB SCIEX, Foster City, CA, USA) against the Mollusca_uniprot-taxonomy_6447 database (downloads on March 15th, 2013). Spectra were also searched against an equal number of decoy sequences to estimate the false discovery rate (FDR) of the integrated tools in ProteinPilot. The specified enzyme was trypsin. Up to two missed cleavages were permitted in this study. Oxidation of methionine (+15.994 9) and acetylation of the protein N-terminus (+42.010 6) were specified as variable modifications and carbamidomethylation of cysteine (+57.021 5) was specified as a fixed modification. All other parameters were default settings, including a fragment ion tolerance of 0.5 Da and a maximum precursor ion tolerance of 6 ppm after recalibration. Identified peptides were filtered to curate a dataset with an FDR of less than 1% at both the peptide and protein levels. Protein identifications were accepted if the probability was over 90% (as assigned by the Protein Prophet algorithm) and included at least two identified peptides.

1.3 Ontology analysis of identified proteins and statistical analysis

GI numbers of identified proteins were matched to the UniProtKB database (www.uniprot.org) to obtain Gene Ontology (GO) annotation using the molecular functions and cellular component categories. Data from each sample were analyzed separately. Hierarchical clustering and principal component analysis (PCA) were performed using SPSS 16.0 and Simca-P.

2 Results and discussion

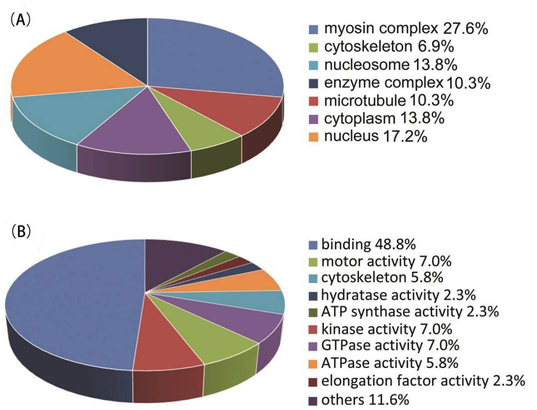
2.1 Protein identification

In this study, 265 proteins were identified in *Solen strictus* (SS), *Sinonovacula constrzcta* (SC), *Bullacta exarata* (BE), *Hemifusus tuba* (HF), MM, *Ruditapes philippinarum* (RP), *Scapharca subcrenata* (SSC), MV, and *Mactra antiquate* (MA) according to their MS/MS spectrum using the nano-LC MS/MS approach. The highest number of proteins was identified in HF (105 proteins), while SSC produced the fewest (39 proteins). Most identified proteins, including actin, tubulin, tropomyosin, myosin heavy chain, were structural constituents of cytoskeleton or involved in motility. Myosin heavy chain is required for the function of myosin, and tropomyosin binds to actin to regulate the interaction between myosin and actin filaments [9]. Some other proteins, such as arginine kinase, ATP synthase, elongation factor, heat shock protein (HSP), fructose-bisphosphate aldolase, and ribosomal protein were also identified. These proteins are involved in binding, cellular metabolism and maintenance, or included in structural components of the ribosome. Arginine kinase and ATP synthase are enzymes that phosphorylate ADP, restoring cellular ATP levels for energy production and storage [10]. HSP is involved in cellular stability, triggered by various environmental stress conditions [11]. Elevated cellular levels of HSP can prevent the aggregation of stress-damage proteins to strengthen stress tolerance in organisms [12]. Ribosomal proteins are the components of the ribosomal subunits that involved in the cellular process of translation in conjunction with rRNA.

GO analysis indicated that many of the identified proteins were related to the cytoskeletal protein myosin (9.8%−13.3%) or other enzymes in the cytoplasm (5.3%−11.5%). Many of the identified proteins were related to binding activity (46.9%−56.7%) in molecular function analysis. These results suggest that most proteins in shellfish function in cytoskeleton, cell motility, metabolic stability, energy storage, and proteins synthesis.

2.2　Functional classification of proteome profiles

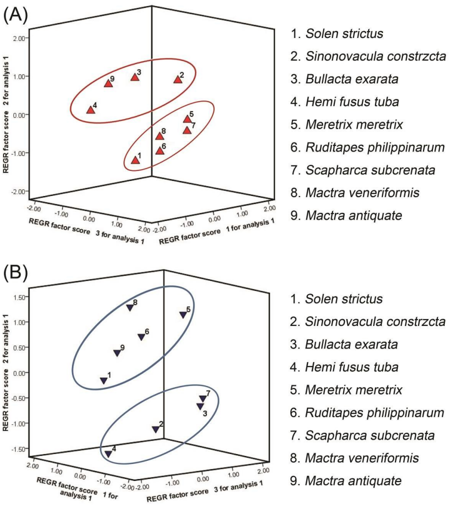
In the case of SS, 53 proteins were identified, among which approximately 40 % were analyzed as known cellular components. As shown in Figure 3, the majority of these proteins were located as components of the myosin complex (eight proteins), nucleus (five proteins), cytoplasm (four proteins), nucleosome (four proteins), enzyme complex (three proteins), and cytoskeleton (two proteins). Among the identified proteins in molecular function analysis, over 48% were assigned to binding activity, and other groups included those with kinase activity (six proteins), motor activity (six proteins), GTPase activity (six proteins), cytoskeletal activity (five proteins), ATPase activity (five proteins), hydratase activity (two proteins), elongation factor activity (two proteins), and ATP synthase activity (two proteins). The cellular components and molecular functions of proteins identified in the other eight shellfishes were similarly assigned.



Note: Protein set (A) was classified according to cellular components, while (B) was classified according to molecular function

Figure 3　Gene Ontology analysis of identified proteins of *Solen strictus*

PCA of cellular components reduced nine categories into three principal components (PC1, PC2 and PC3) accounting for more than 80% of the entire variance (Figure 4A). Based on cellular components, SS, MM, RP, SSC, and MV were classified into one group, while SC, BE, HF, and MA were classified into another group. This suggests that the bivalve shellfish species, SS, MM, RP, SSC, and MV showed similar protein profiles at the level of protein cellular components. Furthermore, PCA of the molecular function reduced ten categories into three principal components (PC 1, PC 2 and PC 3) accounting for > 76% of the variance (Figure 4B). SC, BE, HF, and SSC were classified into one group, while SS, MM, RP, MV and MA were classified into another group. According to PCA, in both levels of protein cellular components and molecular functions, BE and HF showed similar protein profiles, and SS, MM, RP, and MV also showed similar protein profiles.



Note: Samples in (A) were classified according to cellular components, (B) were classified according to molecular function

Figure 4　Principal component analysis of shellfish based on the GO analysis

Furthermore, as shown in Figure 5, actin, myosin, tubulin, histone, and filamin were identified in each shellfish sample. According to the Unused ProtScore of identified proteins, a heat map of protein compositions in each shellfish sample was established. Unused ProtScore reflects the amount of unique peptides related to the identified proteins. Hierarchical clustering of differentially proteins was based on Unused ProtScore of each protein. Hierarchical clustering results of nine shellfish samples showed that RP and MA could be classified into one group, while SC, BE, HF, MM SSC and MV could be classified into a different group. SS showed the furthest distance from this group. As shown in the heat map, actin, myosin, histone, and filamin were common proteins in the nine shellfish with different Unused ProtScore.

3　Conclusion

In this study, proteins from nine shellfish were identified using the nano-LC MS/MS technique. Protein composition of the predominant shellfish species of the Jiangsu intertidal zone area was elucidated. SC, BE, and HF showed similar protein profiles, while SS, MM, RP, and MV also showed a similar protein profile. Our study shows that the major proteins in shellfish are involved in motility, glycol-metabolism, and amino acids synthesis, indicating the bioactive and nutritional value in shellfish from Jiangsu, a commercially important region.

Figure 5　Heat maps according to the Unused ProtScore of identified proteins

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