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Proteomics Analysis of Nutrient Components in the Foot of the Razor Clam *Solen Grandis*

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Abstract

In this paper, the label-free quantitative proteomics technology was adopted to analyze and identify the proteins contained in the foot of the razor clam *Soles grandis* and explore its nutritional components. Through the proteomics research on *S. grandis*, 1,276 proteins were identified. Among them, the number of annotated proteins in *S. grandis* was 1,110. The results showed that the protein with the highest abundance in *S. grandis* was myosin, and the types of proteins with relatively high abundances were related to movement and participated in the formation of the cytoskeleton and multiple physiological functions of cells. The subcellular localization analysis of the expression products of the corresponding functional genes revealed that the angiotensin-converting enzyme (ACE) was present in the cytoplasm; purine nucleoside phosphorylase 1 (PNP1), purine nucleoside phosphorylase 2 (PNP2) and purine nucleoside phosphorylase 3 (PNP3) were all present in the cytoskeleton; sphingosine-1-phosphate lyase (SGPL) was distributed in the cytoplasm, mitochondria and cytoskeleton of cells; and indoleamine 2,3-dioxygenase 2 (IDO2) was distributed in the cytoskeleton of cell.

Keywords: *Soles Grandis*; Muscle; Nutrient Components; Label-Free Proteomics; ACE Peptide

Introduction

Proteomics captures the most direct functional outputs of genes, revealing a complexity that far exceeds genomic annotations [1]. Integrated analysis of transcriptomic and proteomic data provides critical insights into dynamic gene expression patterns at both RNA and protein levels. Proteomics, characterized by its large-scale data capacity, aims to comprehensively map the entire proteome of cells, tissues, and organisms, thereby providing the most robust dataset for characterizing biological systems [2]. This discipline encompasses nearly the full spectrum of protein expression within biological systems, enabling systematic identification of protein isoforms and functional characterization. Label-free quantitative proteomics, coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS), facilitates high-throughput protein identification by analyzing enzymatically digested peptides. Relative quantification of proteins is achieved through computational analysis of peptide signal intensities, circumventing the need for isotopic labeling. Compared to isotope-labeling techniques, label-free approaches offer distinct advantages, including reduced sample input requirements and enhanced suitability for analyzing complex protein mixtures [3]. Label-free proteomics was chosen over other methods because it determines protein abundance without the use of stable isotope labeling. It exhibits the highest proteome coverage and shows good reproducibility.

Great progress has been made in the proteomics of Marine invertebrates [4-6]. Recent advances in marine bivalve proteomics have predominantly focused on elucidating physiological adaptation mechanisms under various environmental challenges, including heavy metal stress [7], cryo-adaptation [8], thermal responsiveness [9], larval metamorphosis [10], biomineralization processes [11-13], and hypoxia tolerance [14]. In the context of marine animal meat quality assessment, proteomic approaches have been strategically employed to decode the molecular basis of myofibrillar protein formation, optimize textural properties, and establish quality evaluation systems [15].

The razor clam *S. grandis* (Mollusca: Bivalve), a commercially valuable aquaculture species, is widely distributed and favored for its high yield, superior taste, and exceptional meat yield [16-18]. As a premium protein source with high protein and low lipid content [19-22], it also harbors essential small-molecule metabolites. So far, some researches have been carried out mainly in the artificial breeding of the razor clam [23-24], reproductive biology [25], molecular systematics [26] and genetic diversity [27]. Compared with other shellfish, there are relatively few studies on the nutrition and activity of razor clam [28-29]. Therefore, the analysis of the protein composition of the razor clam at the proteomic level will help to better develop and utilize this marine economic animal [30-31]. Here, we employed label-free quantitative proteomics and bioinformatics to analyze the foot muscle proteome of *S. grandis*, identifying key metabolic pathways, functional proteins, and associated genes. This study provides critical insights for exploiting marine bivalve protein resources.

Materials and Methods

Materials

The living samples of *S. grandis* utilized in this article were purchased from the Quanzhou Farmers' Market in Fujian Province, China.

Sample Preparation

Add lysis buffer (7 M urea, 2% SDS, 0.1% PMSF, 65mM DTT) to the foot tissue samples, sonicate to lyse the cells, and centrifuge to collect the supernatant. Protein concentration was measured using the BCA assay. Take 50μg of protein and dilute it to 50μL, then add 1μL of 1 M DTT and incubate at 55 °C for 1 hour. Add 5μL of 1 M iodoacetamide (IAA) and incubate in the dark at room temperature for 1 hour. Precipitate proteins by adding 300μL of pre-chilled acetone for 2 hours. The resulting pel-

let was digested overnight with Trypsin (Promega,USA).

Protein Digestion

The protein concentration of the supernatant was measured using a protein quantification kit. A total of 50μg of extracted protein was suspended in 50μL of solution, followed by the addition of 1μL of 1 M dithiothreitol (DTT) for reduction at 55°C for 1 hour. Subsequently, 5μL of 20mM iodoacetamide (IAA) was added for alkylation in the dark at 37°C for 1 hour. Proteins were precipitated overnight at -20°C using 300μL of pre-chilled acetone. The precipitated proteins were washed twice with cold acetone and then resuspended in 50mM ammonium bicarbonate. Finally, the proteins were digested with sequencing-grade modified trypsin (Promega, Madison, WI) at a substrate-to-enzyme ratio of 50:1 (w/w) for 16 hours at 37°C.

High pH Reverse Phase Separation

All peptide mixtures from the samples were redissolved in buffer. After redissolution, the mixtures were separated using an Ultimate 3000 system (Thermo Fisher Scientific, MA, USA) coupled with a reversed-phase column (XBridge C18 column, 4.6 mm × 250 mm, 5μm; Waters Corporation, MA, USA) under high-pH conditions. A linear gradient elution was applied over 40 minutes, increasing from 5% to 45% acetonitrile (ACN). Ammonium hydroxide was added to adjust the solution to pH 10.0. The column was equilibrated at a flow rate of 1mL/min and a column temperature of 30 °C for 15 minutes. A total of 6 fractions were collected. Each fraction was dried using a vacuum concentrator and stored for subsequent analysis.

DDA: nano-HPLC-MS/MS Analysis

The separated peptides were analyzed using a TripleTOF 5600 tandem mass spectrometer (SCIEX, Framingham, MA, USA). Data acquisition was performed under high-sensitivity mode with an accumulation time of 250 ms per scan and a mass range of 350-1,500 m/z for primary scans. Based on the primary scan data, ions in the first-stage mass spectra were sorted by intensity, and those exceeding 150 counts per second (cps) were selected for fragmentation and secondary mass spectrometry (MS/MS) analysis. The criteria for secondary scan selection were as follows: (1) m/z range: 350-1,250; (2) Charge number: 2-5; (3) Dynamic exclusion: Each precursor ion was excluded from repeated fragmentation within half of its peak duration. After acquiring primary mass spectra, secondary MS scans were performed with an accumulation time of 50 ms per scan.

Database Search, Data Analysis and Protein Function Annotation

The raw mass spectrometry data were merged, analyzed, and searched against a database, utilizing either UniProt or a custom database specific to the research group [30]. During the database search, the results were compared against a contaminant database to assess potential sample contamination. This step is essential in proteomic studies of marine organisms due to the complex marine environment, where samples are highly susceptible to microbial contamination. Database search parameters: Fixed modification: Carbamidomethylation (Cysteine, C), Variable modification: Oxidation (Methionine), False discovery rate (FDR): Set to 1% at both the precursor ion and peptide levels.

Two large databases, GO and KEGG, were searched to annotate and classify the functions of proteins. The SignalP-5.0 web service (available at https://services.healthtech.dtu.dk/service.php?SignalP-5.0) was used to predict signal peptide sequences in a subset of the identified proteins. Additionally, the amino acid sequences of homologous proteins from closely related species of *S. grandis* were retrieved from the NCBI database. These sequences were used to perform a local BLAST analysis to identify proteins in *S. grandis* that share conserved domains. The Conserved Domain Database (CDD) on the NCBI website was then utilized to verify whether the exported protein sequences from *S. grandis* contained the expected structural domains. Proteins lacking the corresponding domains were excluded from further analysis.

Data Analysis

The purpose of database searching is to provide functional annotations for as many proteins as possible in the sample, enabling qualitative analysis of the proteome. To ensure the reliability of the results, a false discovery rate (FDR) threshold of less than 1% was applied as a filtering criterion. Additionally, unique peptides defined as peptides identified exclusively from a single protein sequence or a group of identical sequences were required to meet the condition of having ≥1 unique peptide per protein. Only peptides and proteins that met these stringent criteria were used for subsequent analysis. This approach ensures high confidence in protein identification and functional annotation.

Protein data were blasted to the eukaryotic orthologous groups (KOG), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. KOG is a database of orthologs of eukaryotes. The GO database describes protein function in terms of molecular function, biological pathways and cell components, reflecting relevant biological information. Protein GO enrichment analysis and KEGG metabolic pathway analysis were performed to identify the difference in GO enrichment between giant and constricted Solenoids in the three categories, and the difference in metabolic level was analyzed from the data.

Results

Sample Protein Concentration

The protein concentration and total protein were determined by BCA protein quantitative kit. The results showed that the protein concentration was 16.60 μg/μL and the total protein was 3.32mg.

25μg protein was taken from the sample to observe the SDS-PAGE band pattern, as shown in Figure 1. The concentration and total amount of protein met the requirements of subsequent experiments, and the SDS separation bands were clear and abundant, indicating the existence of high protein abundance.

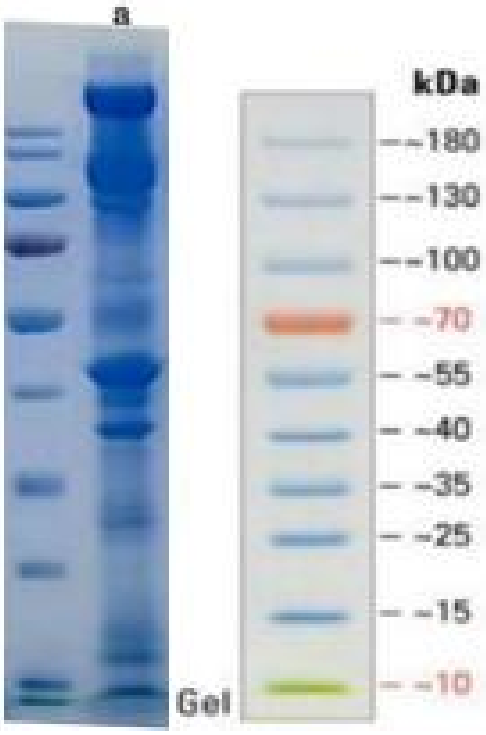

Figure 1: SDS-PAGE gel electrophoretic diagram of proteins of *S. grandis* (a)

Protein Composition and Quantification

The peptides were analyzed by mass spectrometer, the raw data were derived, and the transcriptome sequencing and assembly database established by MASCOT search showed that there were 17,264 peptides, 11,168 unique peptides and 1,276 proteins in the protein secondary spectrum of *S. grandis*. The reliability of the results was guaranteed under the condition of peptide FDR≤1%. In addition, the statistical results of unique peptide segments were shown in Figure 2.

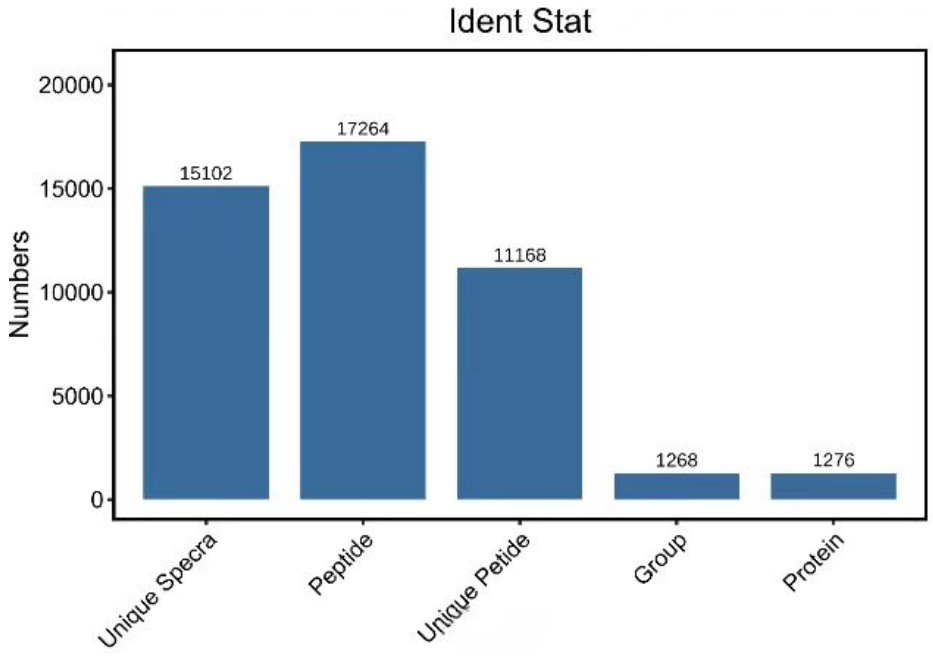

Figure 2: The results of peptide identification of *S. grandis*

The statistics of the number of peptide segments are shown in Figure 3. The horizontal coordinate is the number of peptide segments, and the vertical coordinate is the number of proteins corresponding to the number of peptide segments.

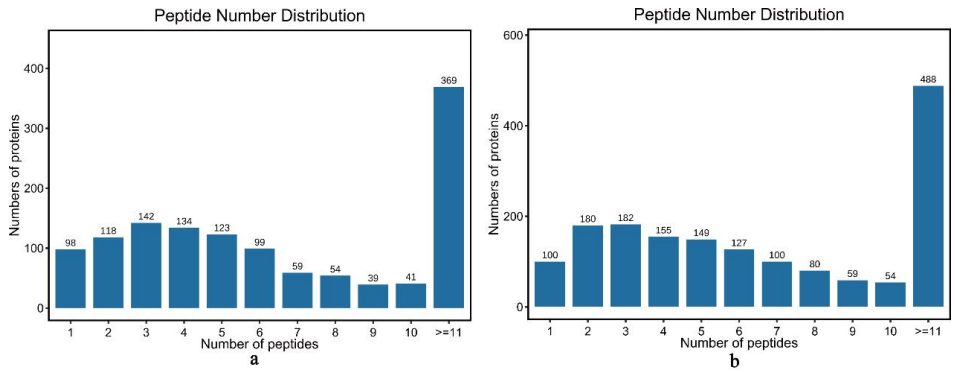

Figure 3: The results of peptide number distribution of *S. grandis*

The statistical results of the length distribution of all peptide segments are shown in Figure 4. The horizontal coordinate in the figure is the length of peptide segment, the number marked on the horizontal coordinate is its amino acid unit, and the vertical coordinate represents the number of peptide segments. According to the statistical results of peptide length distribution, the shortest peptide segment contained 3 amino acid residues, and the longest peptide segment contained 30 amino acid residues.

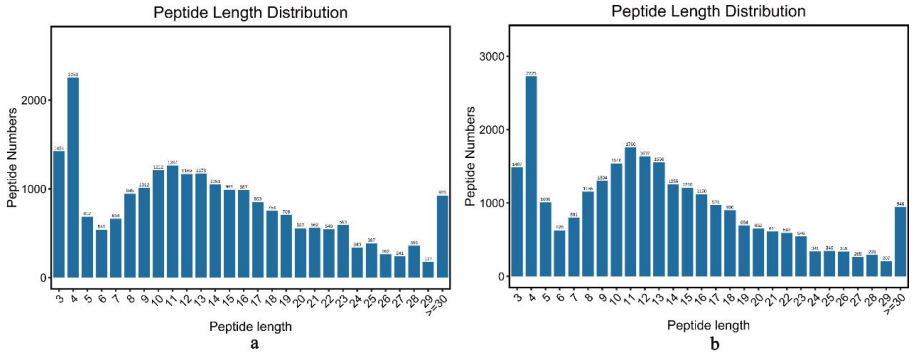

Figure 4: The results of peptide length distribution of *S. grandis*

When the amino acid residues of peptide segment were 3 and 4, the number of peptide segments was the highest, and the proportion of peptide segments was more uniform in other amino acid residues (Figure 4).

The area of the peptide coverage analysis represents the abundance of the corresponding protein, and higher peptide coverage indicates that the abundance of these proteins is likely to be relatively high in the species. According to the results shown in Figure 5, protein proportions with different coverage can be seen, different color blocks represent different sequence coverage ranges, and the numbers and percentages after color blocks represent the number of proteins and their proportion. The results of the razor clam proteome showed that the protein coverage was 30.67% when the peptide coverage was 0-10%. Then, when the peptide coverage was 10-20%, the coverage was 22.82% (Figure 5).

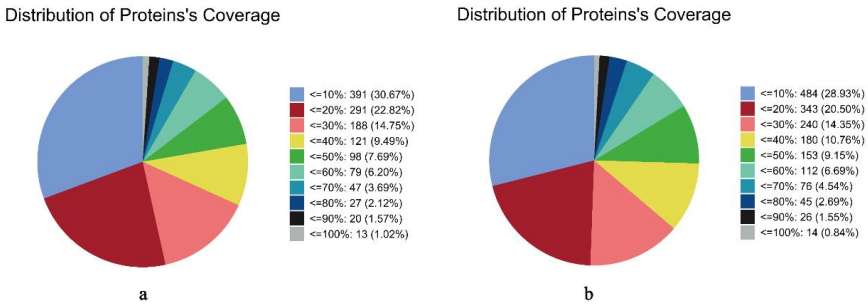

Figure 5: The sequence coverage of *S. grandis* peptide segment

Protein Function Annotation

All identified proteins were annotated by BLASTP against commonly used protein databases (Pfam, GO, KEGG, and KOG) to obtain their functional information. The results showed that 1,110 (86.99%) proteins in S. grandis were functionally annotated, with the highest number of annotations (987 proteins) mapped to the KOG database.

The identified proteins were annotated using KEGG, and gene expression information was integrated as a comprehensive interaction network for in vivo metabolic analysis. The results from the proteomic data of *S. grandis* revealed that 689 proteins were mapped to the KEGG database, participating in 303 metabolic pathways. These annotated pathways were classified into six major categories: Molecular Processes, Environmental Information Processing, Genetic Information Processing, Human Diseases, Metabolism, and Organismal Systems.

Pathways enriched in Organismal Systems were the most abundant, followed by those associated with Human Diseases. In the organic system pathway, the endocrine system, immune system and environmental adaptation were involved in the most proteins, 120,95 and 86, respectively. The number of proteins involved in the nervous system, digestive system and circulatory sys-

tem was also higher (78,62 and 56, respectively).

The number of proteins involved in sensory system, aging system, secretion system, development and regeneration was relatively small, which were 36, 30, 29, 20 and 8, respectively. There were 97, 68 and 58 proteins involved in carbohydrate, amino acid and energy metabolism, respectively.

However, the number of proteins involved in other amino acid metabolism, lipid metabolism, nucleotide metabolism, coenzyme factor and vitamin metabolism, degradation and metabolism of exogenous substances, metabolism of terpenoids and polyketones, polysaccharide biosynthesis and metabolism, and biosynthesis of other secondary metabolites were relatively small, which were 25, 24, 20, 18, 9, 7, 5 and 3, respectively. No protein is enriched into the network of drug development. The statistics of the results are shown in Figure 6.

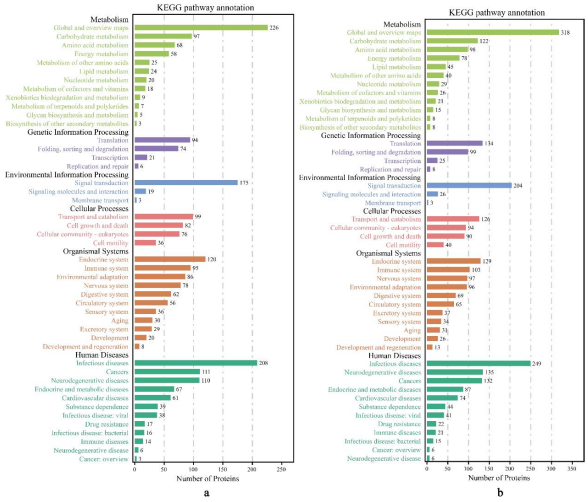

Figure 6: The KEGG enrichment pathway map of *S. grandis*

GO annotation was carried out on 1,276 proteins in *S. grandis*. The results showed that 987 proteins were involved in 57 branches of 3 major categories, including 26 items (terms) in the category of biological processes, 19 items in the category of cell composition, and 12 items in the category of molecular functions. In GO functional annotation, proteins are mainly involved in biological processes such as cellular processes, single tissue processes and metabolic processes. The protein is mainly enriched in the cell. It has the functions of binding, catalytic activity, and molecular structure activity, as shown in Figure 7.

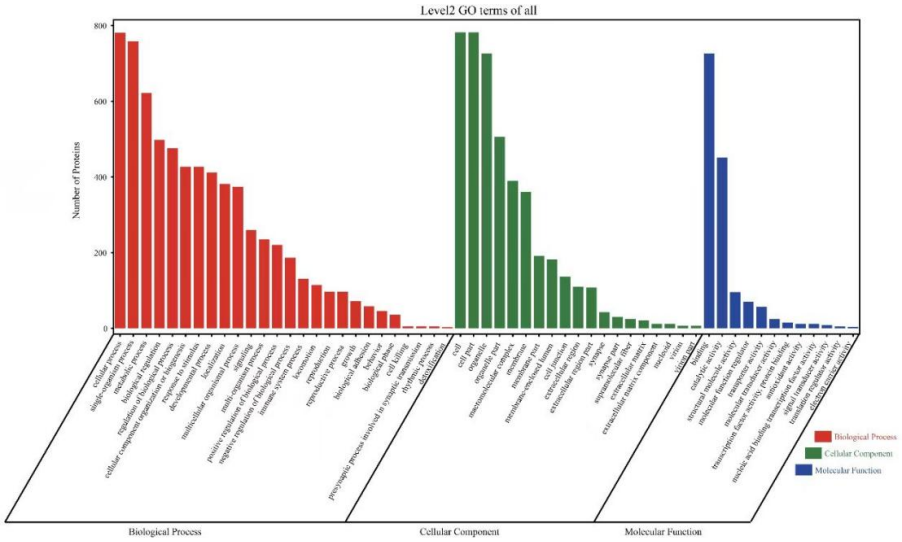

Figure 7: The GO enrichment pathway map of *S. grandis*

The proteins of S. grandis were annotated using the KOG database, classified, and statistically analyzed to identify proteins with conserved functions shared with ancestral orthologs. The results revealed that 1,354 proteins in S. grandis were assigned to 25 KOG functional categories. Among these, the largest category was "Signal transduction mechanisms" with 188 proteins, followed by "Post-translational modification, protein turnover, and chaperones" with 170 proteins, and "General function prediction only" with 154 proteins. Proteins involved in "Energy production and conversion" and "Carbohydrate transport and metabolism" numbered 103 and 56, respectively. Notably, proteins associated with "Defense mechanisms" were relatively scarce, with only 15 identified. Details are shown in Figure 8.

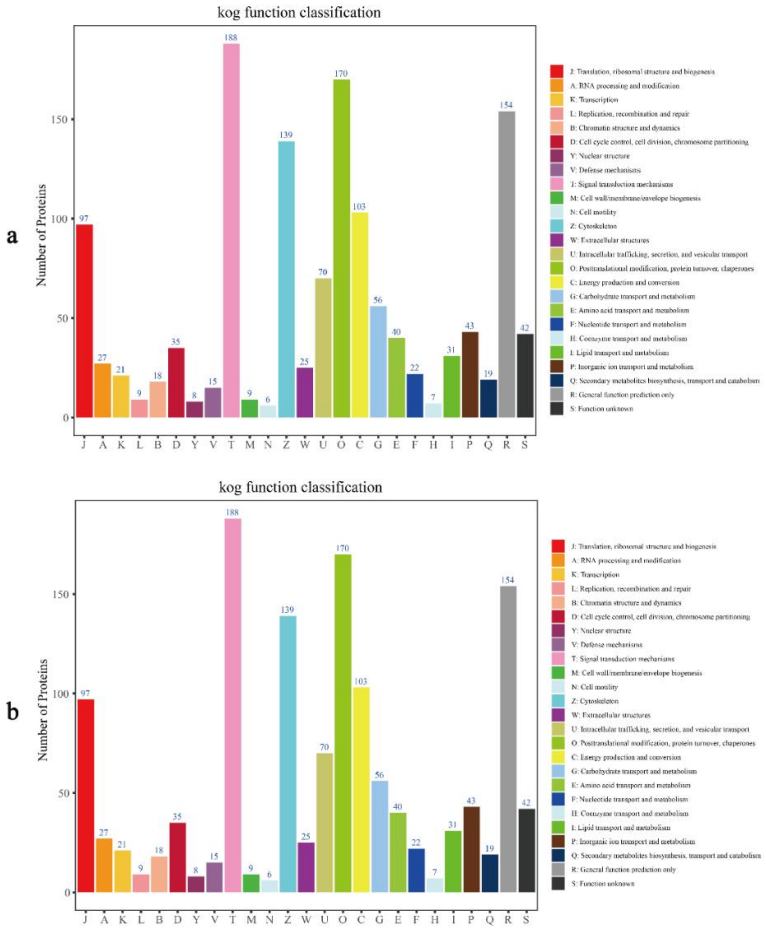

Figure 8: The KOG enrichment pathway map of *S. grandis*

Analyses of different proteins between *S. grandis* and *Sinonovacula constricta*

The peak area in protein mass spectrometry can, to some extent, reflect protein abundance, where a higher protein abundance corresponds to a larger peak area in the mass spectrum. Proteins were ranked based on their peak areas, and the top 10 are summarized in Table 1. Among these, actin and myosin were the most abundant protein types in both *S. grandis* and the Chinese razor clams *Si. constricta* [31]. Specifically, myosin was the most abundant protein in *S. grandis*, whereas actin exhibited the highest abundance in *Si. constricta*. Both species contained arginine kinase in their foot tissues. Additionally, *S.grandis* also expressed fructose-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase.

Table 1: Protein abundance sort of *S. grandis* and *Si. Constricta*

S. grandis			*Si. constricta*		
Protein ID	Symbol	Annotation	Protein ID	Symbol	Annotation
Unigene0000212	MYH16	Myosin	Unigene0001481	act-4	Actin

Unigene0029558	PMY	Myosin	Unigene0008779	MYH16	Myosin
Unigene0070942	cher	Filamin	Unigene0005600	PMY	Myosin
Unigene0028110	act-4	Actin	Unigene0001276	FLNC	Filamin
Unigene0042379	ACTA2	Actin	Unigene0007077	TRO	Tropomyosin
Unigene0066687	Smp_194770	Arginine kinase	Unigene0045759	cher	Filamin
Unigene0015486	Flnc	Filamin	Unigene0024274	gxcB	Transgelin
Unigene0067504	FBPA	Fructose-bisphosphate aldolase	Unigene0028708	unc-22	Twichin
Unigene0067365	GAPDH	Glyceraldehyde phosphate dehydrogenase	Unigene0020903	Smp_194770	Arginine kinase
Unigene0000629	act-2	Actin	Unigene0037878	Rlc-a	Myosin

Subcellular localization of functional gene expression products

Subcellular localization analysis of the corresponding products of nine functional genes was performed using WoLFPSORT to determine the specific intracellular locations of the proteins or their expression products. The results are shown in Table 2. Angiotensin-converting enzyme (ACE) of the razor clam was found in the cytoplasm; purine nucleoside phosphorylase 1 (PNP1), purine nucleoside phosphorylase 2 (PNP2), and purine nucleoside phosphorylase 3 (PNP3) were all localized to the cytoskeleton; sphingosine-1-phosphate lyase (SGPL) was distributed in the cytoplasm, mitochondria, and cytoskeleton; and indoleamine 2,3-dioxygenase 2 (IDO2) was localized to the cytoskeleton.

Table 2: Protein composition, subcellular localization and annotation of *S. Grandis*

Product ID	Subcellular localization	Description	E value
SgACE	cytoplasm	angiotensin-converting enzyme	0
SgPNP1	cytoskeleton	Purine nucleoside phosphorylase	4E-119
SgPNP2	cytoskeleton	Xanthine dehydrogenase	0
SgPNP3	cytoskeleton	Xanthine dehydrogenase	1E-89
SgSGPL1	cytoplasm	gamma glutamyhransferase	1E-156
SgSGPL2	mitochondria	gamma glutamyhransferase	0
SgSGPL3	cytoskeleton	cysteine sulfinic acid decarboxylase	0
SgSGPL4	cytoskeleton	cysteine sulfinic acid decarboxylase	0
SgIDO2	cytoskeleton	L-amino-acid oxidase	1E-112

Discussion

The material used in this study was the foot muscle tissue of *S. grandis*, with actin and myosin identified as the most abundant proteins in the proteomic data. The primary locomotion of *S. grandis* is mediated by its foot muscles, and the high-expression proteins identified through proteomics are closely related to its motor functions. The interaction between actin and myosin is fundamental to muscle contraction [35-37]. In all cells, actin filaments and non-muscle myosin interact in a similar manner, facilitating cellular movement. The myosin heads on myosin filaments contact surrounding actin filaments, and through ATP hydrolysis, the actin filaments in the foot are pulled, generating macroscopic muscle movement [38-39]. Actin proteins derived

from seafood by-products have been found to have beneficial activities in health promotion, disease prevention, and therapeutic interventions [40]. These proteins have a broad spectrum of applications, ranging from health pharmacology to food supplements and pharmaceuticals [41]. The processing and utilization of proteins and protein hydrolysates from fish by-products and underutilized fish species are also being explored for their bioactive properties and peptide sequences [42-43]. Additionally, the development of seafood processing by-products into high-quality products is ongoing [44]. Myosin proteins from seafood processing have health benefits [40,41,45-47]. These proteins can be transformed into more marketable and functional forms through extraction or hydrolysis [40]. They are derived from fish by-products and processing wastes [40,45]. Hydrolysates from fish proteins have been linked to effects against cardiovascular diseases, cancer, diabetes, and aging [47].

The high-abundance proteins in *S. grandis* include fructose-bisphosphate aldolase, a conserved enzyme in the glycolytic pathway that exhibits stress-responsive properties under adverse conditions. In marine organisms, this enzyme can trigger acquired immune responses and has been utilized as a broad-spectrum vaccine to combat pathogens in aquaculture, thereby improving aquatic product quality [48]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), another key enzyme in glycolysis, is ubiquitously expressed in eukaryotes and demonstrates stable expression across tissues. Its gene is frequently employed as a reference gene for fluorescence-based quantitative assays. Both enzymes play crucial roles in the vital biological activities of *S. grandis* [49].

Among the nine functional gene products identified in *S. grandis*, ACE is ubiquitously localized in the cytoplasm and may correlate with the presence of antihypertensive lectin-inhibitory peptides commonly found in marine bivalves [50-52]. Taurine in *S. grandis* is widely distributed in the cytoplasm, mitochondria, and cytoskeleton. Consistent with other aquatic invertebrates, bivalves typically exhibit high taurine content under physiological conditions. Taurine plays a critical role in infant neural development, enhancing neurotransmission and visual function [53-54], while also demonstrating therapeutic effects against iron-deficiency anemia by facilitating intestinal iron absorption and modulating gut microbiota composition [55]. These properties underscore its widespread application as a food additive. Tryptophan is predominantly localized within the cytoskeletal framework. This essential amino acid exerts antioxidant activity and alleviates stress responses in animals. Tryptophan and its metabolites enhance immune function by regulating immunoglobulin production and lymphocyte proliferation. Additionally, it modulates growth performance through appetite regulation and intestinal motility optimization. Tryptophan further influences protein synthesis mechanisms; binding to nuclear tryptophan receptors promotes hepatic nucleoprotein synthesis, thereby accelerating anabolic processes [56-59].

Conclusion

This study employed label-free quantitative proteomics to systematically characterize the proteome of *S. grandis*, integrating qualitative and quantitative analyses to identify high-abundance proteins and annotate their biological functions. Proteomic datasets were cross-referenced with public databases for comprehensive functional prediction. Notably, immune-responsive proteins were underrepresented in the identified proteome, with the majority corresponding to constitutively expressed housekeeping proteins involved in fundamental physiological maintenance. Key differentially expressed proteins, including ACE and their enriched functional pathways not only elucidate the proteomic landscape underlying cellular activities in *S. grandis* but also provide mechanistic insights into its nutritional biochemistry.

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