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The seminal plasma proteome of the giant panda

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Abstract

For the ex-situ conservation of giant pandas, both collecting and preserving semen are important methods. The seminal plasma is rich in nutrients and bioactive substances, such as proteins, carbohydrates, lipids, amino acids, and hormones, which play an important role in the reproduction and reproductive health of the species. This is the first study to analyze the seminal plasma proteins of giant pandas through proteomics and identified 1125 proteins. These proteins are related to protein turnover, translation, and metabolism. The seminal plasma proteins of giant pandas were then compared to those of humans, pigs and sheep, with many unique proteins found in giant panda samples. Among these proteins, the WD40 repeat-containing proteins have been identified and implicated in sperm function and fertility. Understanding the composition and function of proteins in the giant panda seminal plasma proteome can provide valuable insights into their reproductive biology and help develop strategies to improve their reproductive success in captivity, which is essential for giant panda conservation.

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Abstract

For the *ex-situ*conservation of giant pandas, both collecting and preserving semen are important methods. The seminal plasma is rich in nutrients and bioactive substances, such as proteins, carbohydrates, lipids, amino acids, and hormones, which play an important role in the reproduction and reproductive health of the species. This is the first study to analyze the seminal plasma proteins of giant pandas through proteomics and identified 1125 proteins. These proteins are related to protein turnover, translation, and metabolism. The seminal plasma proteins of giant pandas were then compared to those of humans, pigs and sheep, with many unique proteins found in giant panda samples. Among these proteins, the WD40 repeat-containing proteins have been identified and implicated in sperm function and fertility. Understanding the composition and function of proteins in the giant panda seminal plasma proteome can provide valuable insights into their reproductive biology and help develop strategies to improve their reproductive success in captivity, which is essential for giant panda conservation.

KEYWORDS

Giant panda, seminal plasma, proteome, WD40 repeats proteins

The giant panda (*Ailuropoda melanoleuca*) is a unique and vulnerable species endemic to China. It is not only a flagship species for global biodiversity conservation, but also serves as a political and diplomatic ambassador for China, and a cultural icon. Conserving the giant panda is of particular ecological, social, political and economic significance.

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In recent years, both *in-situ* and *ex-situ* conservation measures have been implemented to different degrees of success, however, the species is still vulnerable to extinction. For the captive population, the low proportion of naturally mating male giant pandas is a major limiting factor for their reproductive efficiency, utilization, and genetic diversity. With artificial insemination techniques, this problem can be overcome and genetic management can also be facilitated, making the preservation and efficient use of individual giant panda semen significant for maintaining the entire captive population.

Seminal plasma is a fluid that is produced by the male reproductive system and is a mixture of secretions from the testis, epididymis and male accessory sex glands. Sperm motility is an index tightly associated with male fertility(Jia et al., 2021). Seminal plasma proteins have important roles in sperm functionality, and different mechanisms including micro-vesicle transport of proteins are involved in the regulation of sperm biology. Due to the role of seminal plasma, specific proteins present in seminal plasma may be used as discriminant variables with the potential to predict sperm motility and fertility(Gaitskell-Phillips et al., 2022). The seminal plasma proteome refers to the complete set of proteins in the fluid that makes up semen, including enzymes, transport proteins, and immune system components. The seminal plasma proteome contains thousands of proteins and includes many tissue-specific proteins that might accurately indicate a pathological process in the tissue of origin(Drabovich et al., 2014). Several seminal plasma proteins are associated with male fertility but most of these have not been studied in detail until now(Candenas & Chianese, 2020; Cannarella et al., 2020).

Research on the seminal plasma proteome has been ongoing for many years and has yielded important insights into male reproductive biology and the mechanisms underlying male infertility. Camargo, M. et al used proteomic techniques to analyze the seminal plasma proteome of men with and without a varicocele, a common cause of male infertility (Camargo et al., 2013). The researchers identified several differentially expressed proteins in the two groups, suggesting that these proteins may be involved in the pathogenesis of varicocele-associated male infertility. Another study used mass spectrometry to analyze the seminal plasma proteome of fertile and infertile men(da Silva et al., 2016). The researchers identified several proteins that were present at significantly different levels in the two groups, suggesting that these proteins may be useful biomarkers for male infertility. Recently, Martins, A.D. et al elucidated the potential role of differentially expressed proteins in the seminal plasma as a diagnostic biomarker for primary and secondary infertility, their results showed overexpression of ANXA2, CDC42 and under-expression of SEMG2 proteins in primary infertility; and overexpression of ANXA2 and APP proteins in secondary infertility(Martins et al., 2020). For instance, the correlation between semen protein composition, sperm activity and fertility in animals such as cattle(Westfalewicz et al., 2017), goats(Jia et al., 2021), pigs(Mills et al., 2020) and chicken(Li et al., 2020) has been explored.

Zhu et al quantified 35 metabolome molecules with distinct age-related trends in the giant panda seminal plasma(Zhu et al., 2022). However, the metabolic profile of giant panda seminal plasma has not yet been reported. The aim of the present study was to identify the whole proteome profiles of giant panda seminal plasma using a gel-free, label-free shotgun proteomics approach. Semen from four sexually mature giant pandas (aged between 9 and 16 years, the average was 11.5±3.32) was collected by electroejaculation during the breeding season according to the previous methodology(Cai et al., 2018). Semen was collected into a plastic container and immediately placed in a centrifuge. An aliquot of 0.5 mL of fresh semen was centrifuged at 900 × *g* for 30 min at 4 to separate seminal plasma from spermatozoa. Seminal plasma was then transported at 4 and frozen at -80 until further use. All samples were collected during artificial insemination and cryogenically stored following a standard, routine procedure at the Sichuan Key Laboratory of Conservation Biology for Endangered Wildlife, Chengdu Research Base of Giant Panda Breeding.

All samples were initially sonicated three times in ice and lysed in lysis buffer containing 100 mM NH4HCO3(pH 8), 6 M Urea and 0.2% SDS, followed by 5 min of ultrasonication on ice. The lysate was centrifuged at 12000 *g* for 15 min at 4degC and the supernatant was transferred to a clean tube. Extracts from each sample were reduced with 2mM DTT for 1 h at 56 and subsequently alkylated with sufficient Iodoacetamide for 1 h at room temperature in the dark. Then 4 times the volume of precooled acetone

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was mixed with samples by well vortexing and incubated at -20degC for at least 2h. Samples were then centrifuged, and the precipitation was collected. After washing twice with cold acetone, the pellet was dissolved by a dissolution buffer containing 0.1 M triethylammonium bicarbonate (TEAB, pH 8.5) and 6 M urea. Protein concentration was determined again by Bradford protein assay.

The supernatant from each sample, containing precisely 0.12 mg of protein was digested with Trypsin Gold (Promega) at 1:50 enzyme-to-substrate ratio. After 16 h of digestion at 37degC, peptides were desalted with a C18 cartridge to remove the high urea, and desalted peptides were dried by vacuum centrifugation.

Shotgun proteomics analyses were performed using an EASY-nLCTM 1200 UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher) operating in the data-dependent acquisition (DDA) mode. A sample volume containing 2 μg of total peptides was injected onto a home-made C18 Nano-Trap column (2 cm×100 μm, 3 μm). Peptides were separated on a home-made analytical column (15 cm×150 μm, 1.9 μm), using a 60 min linear gradient from 5 to 100% eluent B (0.1% FA in 80% ACN) in eluent A (0.1% FA in H2O) at a flow rate of 600 nL/min. The detailed solvent gradient is listed as follows: 5-10% B, 2 min; 10-30% B, 49 min; 30-50% B, 2 min; 50-90% B, 2 min; 90-100% B, 5 min. Q-Exactive HF-X mass spectrometer was operated in positive polarity mode with a spray voltage of 2.3 kV and capillary temperature of 320°C. Full MS scans ranging from 350 to 1500 m/z were acquired at a resolution of 60000 (at 200 m/z) with an automatic gain control (AGC) target value of 3×10^6 and a maximum ion injection time of 20 ms. The 40 most abundant precursor ions from a full MS scan were selected for fragmentation using higher energy collisional dissociation (HCD) fragment analysis at a resolution of 15000 (at 200 m/z) with an AGC target value of 1×10^5, a maximum ion injection time of 45 ms, a normalized collision energy of 28%, an intensity threshold of 2.2e4, and the dynamic exclusion parameter of 20 s.

The resulting spectra from each fraction were searched separately against 'P101SC18111984-01-ailuropoda_melanoleuca.fasta' by the search engines: Proteome Discoverer 2.2 (PD 2.2, thermo). The searched parameters were as follows, a mass tolerance of 10 ppm for precursor ion scans and a mass tolerance of 0.02 Da for the product ion scans were used, carbamidomethyl was specified in PD 2.2 as fixed modifications, oxidation of methionine (M) and acetylation of the N-terminus were specified in PD 2.2 as variable modifications and a maximum of 2 miscleavage sites were allowed.

For protein identification, a protein with at least one unique peptide was identified at FDR less than 1.0% on peptide and protein levels, respectively. Proteins containing similar peptides that could not be distinguished based on MS/MS analysis were grouped separately as protein groups. Precursor quantification based on intensity was used for label-free quantification. Gene Ontology (GO) and InterPro (IPR) analysis were conducted using the InterProScan-5 program against the non-redundant protein database (including Pfam, PRINTS, ProDom, SMART, ProSiteProfiles, PANTHER)(Jones et al., 2014), and the databases COG (Clusters of Orthologous Groups) and KEGG (Kyoto Encyclopedia of Genes and Genomes) were used to analyze the protein family and pathway. Based on the related species, the probable interacting partners were predicted using the STRING-db server (http://string.embl.de/). STRING is a database of both known and predicted protein-protein interactions(Franceschini et al., 2013). The enrichment pipeline (Huang da et al., 2009) was used to perform GO, IPR and KEGG enrichment analysis, respectively.

Result

A total of 1125 proteins in the sperm plasma (Table S1, Supporting Information) were identified using the label-free shotgun proteomic approach. Researchers can understand the functional characteristics of different proteins by utilizing these databases to perform functional annotations on identified proteins. In this study, we used four databases (GO, IPR, KEGG and COG) for annotation, most proteins could be annotated (Figure 1A). , Based on the results of the GO annotation analysis of the cellular compartment, there is a broad range of sources for seminal plasma proteins, and the primary sources are intracellular, ribosome and membrane (Figure 1B). The molecular function of these proteins is associated with protein binding, ATP binding, calcium ion binding and so on, they are mainly involved in the oxidation-reduction process,

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proteolysis, translation and carbohydrate metabolic process. We found that these proteins are rich in EF-hand domain, intermediate filament and immunoglobulin-like domain (Figure 1C), which should match their functions. Based on COG and KEGG annotation (Figure 2), it also highlights that these proteins are involved in protein turnover, translation, and metabolism, which may play a major role in the immune and endocrine systems.

As different species may use different versions of proteins, gene names corresponding to proteins were used uniformly for comparative analysis. A comparison of the seminal plasma proteins identified in giant pandas and three other species (human(Saraswat et al., 2017), pig(Perez-Patino et al., 2016), sheep(Soleilhavoup et al., 2014)) revealed that pandas share 25 genes (corresponding to 25 proteins) with the other species, and have 598 unique genes (corresponding to 598 proteins). Subsequent functional enrichment analysis shows that these shared proteins mainly play a role in the ion binding process (Figure 3A), seminal plasma contains a variety of ions including sodium, potassium, calcium, magnesium, and zinc(Sorensen et al., 1999), and the ion binding process is important for the regulation of the pH and osmotic pressure of the semen, as well as for the function and viability of sperm. The COG annotated these proteins in serum albumin and fibronectin, human serum albumin (HSA) is the most abundant seminal plasma protein and an important constituent of seminal plasma (Figure 3B), it has an established role as a sink for cholesterol and is removed from the sperm membrane during capacitation(Kumar et al., 2012), albumin is believed to play a role in the maintenance of sperm motility(Mogielnicka-Brzozowska et al., 2019), and may also have antioxidant properties that protect the sperm from oxidative stress. Fibronectin is a ubiquitous multifunctional glycoprotein and a component of the seminal fluid. It plays a key role in the formation of seminal gel following ejaculation and can bind to cellular components that are exposed when a spermatozoon is damaged and thus helps select abnormal spermatozoa. Overall, both albumin and fibronectin are important components of seminal plasma and may play important roles in the function and viability of sperm. Additionally, researchers are investigating these proteins' potential diagnostic and therapeutic uses in the context of male infertility and other reproductive disorders.

In the comparative analysis, most proteins were unique to giant pandas, which may be related to the unclear annotation of proteins in different species. Functional analysis was also conducted on panda unique proteins, these proteins were enriched in the ribosome pathway (Figure 3C), and were also related to binding function, including heterocyclic compound binding and nucleic acid binding (Figure 3D). As we know, sperm do not have ribosomes and hence do not synthesize proteins, during sperm development, ribosomes are present in the early stages, but they are degraded and eliminated as the sperm matures. Therefore, instead of synthesizing proteins, sperm rely on the proteins that are produced by the cells of the male reproductive system and are present in the seminal fluid. Additionally, these proteins were associated with WD40 repeat and WD40 repeat-containing domains (Figure 3E). The WD40 repeat is a short motif structure consisting of approximately 40 amino acids that are often in a tryptophan-aspartic acid (W-D) dipeptide(Neer et al., 1994). The WD40 domain often comprises several of these repeats and is found in many proteins that are involved in a variety of cellular processes(Li & Roberts, 2001). Previous research has shown that the WD40 repeat containing proteins, including DDB1-CUL4-associated factors (DCAFs), are abundant and conserved proteins that play important roles in different cellular processes, including spermatogenesis (Mistry et al., 2020). Several proteins containing WD40 repeats have been identified in seminal plasma, including beta-catenin, which is involved in cell adhesion and signaling(Takezawa et al., 2011), and ring finger protein 17 (RNF17), which is a ubiquitin ligase that regulates protein degradation(Liu et al., 2011). These proteins are thought to play important roles in sperm function and fertility (Pan et al., 2005; Rivas et al., 2014), although the exact mechanisms are not yet fully understood. In humans, WDR62(Qin et al., 2019), WDR63(Lu et al., 2021), WDR66(Kherraf et al., 2018) have been identified and implicated in abnormalities of the sperm flagellum and male infertility. A recent study found a non-synonymous point mutation in a WD-40 domain repeat of EML5 leads to decreased bovine sperm quality and fertility(Nogueira et al., 2022).

In this study, twelve WD40 repeat-containing proteins were identified, we presented fundamental information about these repeats, and their molecular functions were not directly associated with sperm. Through a literature review, we discovered *PAFAH1B1* plays important roles in spermatogenesis, fertilization and subsequent

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embryonic development in mice (Yao et al., 2015). We believe that these proteins in the seminal plasma are related to the formation and functional performance of sperm, but further research is still needed, especially for giant pandas.

Table 1 Summary of WD40 repeat-containing proteins in seminal plasma.

Uniprot ID	Protein name	Gene symbol	Molecular function
G1LGH1	WD repeat domain 1	*WDR1*	regulation of actin cytoskeleton
G1KZF6	Receptor for activated C kinase 1	*RACK1*	regulation of protein kinase C ac
G1LLT4	Platelet-activating factor acetylhydrolase IB subunit alpha	*PAFAH1B1*	regulation of microtubule cytoske
G1LGH6	Coatomer subunit beta	*COPB2*	a subunit of the Golgi coatomer
D2HI58	Uncharacterized protein	unkown	unkown
G1LI97	Actin-related protein 2/3 complex subunit	*ARPC1A*	regulation of actin filament polyr
G1LI94	unkown	unkown	unkown
D2HX06	Uncharacterized protein	unkown	unkown
G1LEQ8	SEC31 homolog A, COPII coat complex component	*SEC31A*	anterograde transport from the e
D2HX51	Coronin	unkown	unkown
G1LEX2	EMAP like 4	*EML4*	microtubule stabilization, intrace
G1M6T5	Coatomer subunit alpha	*COPA*	retrograde transport from the Go

Overall, research on the seminal plasma proteome has the potential to yield important insights into male reproductive biology and the mechanisms underlying male infertility and other reproductive disorders. As a vulnerable species, the reproduction of giant pandas has always been a matter of great concern. Scientists have been studying ways to increase the breeding rate of captive giant pandas to ensure their population's health and sustainability. By establishing a dataset of the giant panda seminal plasma proteome, and identifying specific proteins and biomarkers associated with reproduction, researchers may be able to develop new diagnostic and therapeutic approaches to improve male panda reproductive health.

Author Contributions:

Conceived and designed the experiments: Kailai Cai, Yuliang Liu and Rou Hou. Performed the experiments: Kailai Cai, Jiasong Chen, Feiping Li, Shenfei Wang, Mengshi Zhang, Xianbiao Hu, He Huang, Junhui An, Donghui Wang, Hairui Wang. Analyzed the data: Tao Wang. Contributed to writing the manuscript: Tao Wang, Kailai Cai and Ayala James.

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Data Availability Statement: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository (Chen et al., 2022; Ma et al., 2019) with the dataset identifier PXD044374.

Conflicts of Interest: The authors declare no conflict of interest.

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Figure 1. The Venn diagram displayed the annotation results of the identified proteins in four databases (A), B and C showing the GO and IPR annotations, respectively. The bar represents the number of proteins.

Figure 2. The COG (A)and KEGG (B) annotation of detected seminal plasma proteins. The bar represents the number of proteins.

Figure 3. The GO (A) and KEGG (B) annotation of panda seminal plasma proteins and three other species (human, pig, sheep). (C) and (D) represent the GO and KEGG annotation of panda-specific seminal plasma proteins compared to three other species, The results of IPR analysis have also been presented simultaneously (E).

Figure 1

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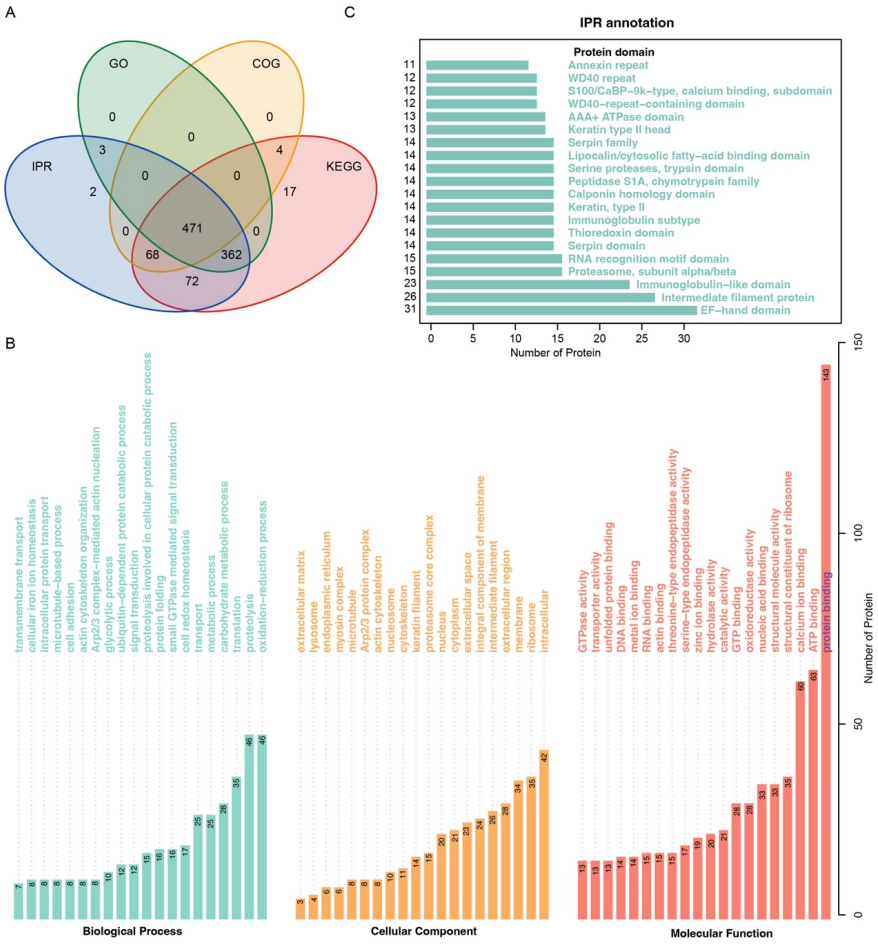

Figure 2

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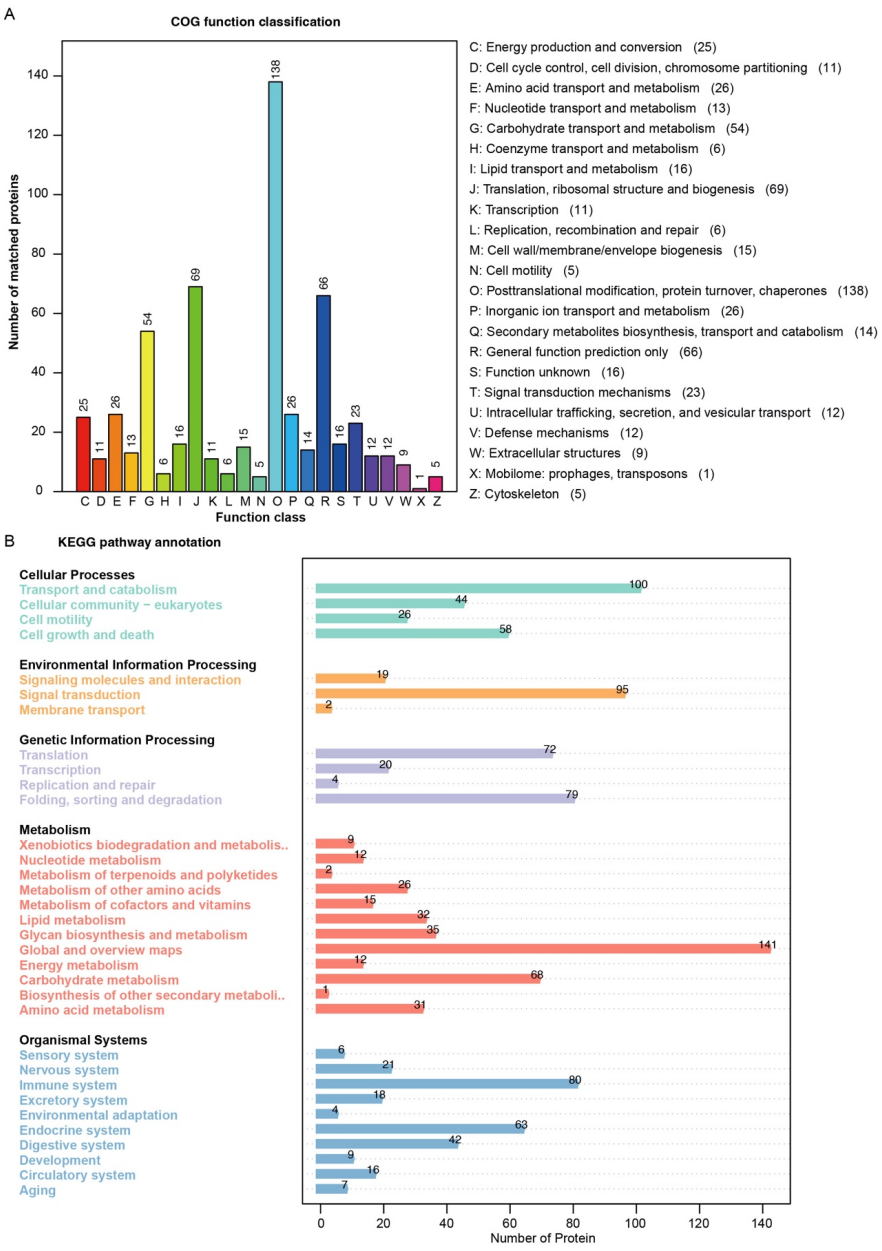

Figure 3

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