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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

A global phosphoproteomics analysis of adult *Fasciola gigantica* by LC–MS/MS

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Abstract

Protein phosphorylation plays key roles in a variety of essential cellular processes. *Fasciola gigantica* is a tropical liver fluke causing hepatobiliary disease fascioliasis, leading to human health threats and heavy economic losses. Although the genome and protein kinases of *F. gigantica* provided new insights to understand the molecular biology and etiology of this parasite, there is scant knowledge of protein phosphorylation events in *F. gigantica*. In this study, we characterized the global phosphoproteomics of adult *F. gigantica* by phosphopeptide enrichment-based LC–MS/MS, a high-throughput analysis to maximize the detection of a large repertoire of phosphoproteins and phosphosites. A total of 1030 phosphopeptides with 1244 phosphosites representing 635 *F. gigantica* phosphoproteins were identified. The phosphoproteins were involved in a wide variety of biological processes including cellular, metabolic, and single-organism processes. Meanwhile, these proteins were found predominantly in cellular components like membranes and organelles with molecular functions of binding (51.3%) and catalytic activity (40.6%). The KEGG annotation inferred that the most enriched pathways of the phosphoproteins included tight junction, spliceosome, and RNA transport (each one contains 15 identified proteins). Combining the reports in other protozoa and helminths, the phosphoproteins identified in this work play roles in metabolic regulation and signal transduction. To our knowledge, this work performed the first global phosphoproteomics analysis of adult *F. gigantica*, which provides valuable information for development of intervention strategies for fascioliasis.

Keywords *Fasciola gigantica* · Mass spectrometry · Phosphoproteomics · Phosphoprotein · Annotation

Background

Fasciola spp. are parasitic food-borne trematodes causing hepatobiliary disease-fascioliasis, leading to extensive liver tissue damage and cirrhosis (Good and Scherbak 2021). Fascioliasis is endemic worldwide infecting 17 million people from 61 countries, and 180 million people are at risk of infection (Mas-Coma et al. 2019; McManus 2020). Meanwhile, these trematodes infect approximately 600 million domestic ruminants with a significantly negative effect on their live weight gains, causing profound damage to animal productivity and economic losses in excess of US$3 billion per year (Cwiklinski et al. 2016; Hayward et al. 2021). As the only drug effective against the infection, triclabendazole (TCBZ) was recently approved to be used by the FDA, and there is currently no therapeutic alternative to treat human fascioliasis. However, someone treated with TCBZ still continue excreting eggs through feces despite a standard care regimen (Marcos et al. 2021). Additionally, emerging hybridization and introgression between *Fasciola gigantica* and *Fasciola hepatica* make the therapy and control of fascioliasis more difficult (Calvani and Slapeta 2021).

Post-translational modifications (PTMs) are biochemical changes to regulate the modified proteins, which are

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indispensable for their functional diversities (Mann and Jensen 2003). Therein, as one of the most important PTMs, reversible phosphorylation at serine, threonine, and tyrosine residues plays key roles in a variety of cellular processes including signal transduction, membrane transport, and metabolic regulation (Humphrey et al. 2015; Day et al. 2016). Protein kinases and protein phosphatases are involved to catalyze the protein phosphorylation/dephosphorylation processes via addition and removal of phosphoryl groups to substrates (Wang et al. 2020; Hirst et al. 2020). Particularly, protein kinases from *Schistosoma* and *Leishmania* as well as *Toxoplasma* had been identified as potential drug targets against these zoonotic parasites (Gaji et al. 2021; Wu et al. 2021; Efstathiou and Smirlis 2021). For *F. gigantica*, the draft genome (size: 1.04–1.13 Gb) was assembled with a high degree of genomic polymorphism. Using DNA library construction and sequencing as well as bioinformatics analysis, a total of 20,858 genes were predicted, and 455 protein kinases were identified in 2020 (Pandey et al. 2020; Das et al. 2020). However, due to the limitations in genome annotation and functional study of these kinases, there is scant knowledge of protein phosphorylation events in *F. gigantica*. Recent progress has been made in the global phosphoproteomics analyses of various parasites (Hirst et al. 2020; Wang et al. 2020; Liu et al. 2020b), which made contributions to understanding the complex biology of these organisms and developing new anti-parasitic candidates.

To identify a large repertoire of phosphoproteins or establish a phosphoproteomics database, the high-throughput analysis of liquid chromatography-tandem mass spectrometry (LC–MS/MS) is currently the most common and powerful analytical platform for analyzing the phosphorylation state of parasites (Wang et al. 2019a; Broncel and Treeck 2020). In the present study, the phosphopeptide enrichment-based LC–MS/MS was used to define the first global phosphoproteomics analysis of adult *F. gigantica.* Combining with functional annotations and biological connections, this work provides an overview and discusses current knowledge on phosphorylation of *F. gigantica* and enables us to develop new intervention strategies to control fascioliasis.

Methods

Parasite preparation

Adult *Fasciola gigantica* were isolated from the bile ducts of naturally infected buffaloes, washed thoroughly with phosphate-buffered saline (PBS, pH 7.4), and then incubated in RPMI 1640 medium at 37 ℃ for 1 h. The flukes were frozen in liquid nitrogen and stored at – 80 ℃ until use.

Protein extraction and digestion

Protein extraction was conducted on three biological repeats from the frozen samples. Each sample was ground into powder and suspended in a lysis buffer (8 M urea in 100 mM triethyl ammonium bicarbonate, pH 8.5) with 1% protease inhibitor cocktail (Merck, Denmark). Subsequently, the sample was ultrasonicated on ice at 30 s bursts for 10 min. The lysate debris were eliminated by centrifugation at 12,000 g for 30 min at 4 ℃, and individual supernatants were collected and conducted to determine the protein concentration by a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, USA). Protein digestion was carried out as previously described (Liu et al. 2020b; Ren et al. 2018), with some modifications. Briefly, 300 µg protein sample was reduced with 20 mM dithiothreitol at 56 ℃ for 30 min, followed by alkylation with 100 mM iodoacetamide at room temperature for 30 min, then dissolved into urea solution with the addition of 25 mM NH_4HCO_3. Finally, the digestion process was conducted on a trypsin (Promega, USA) solution (6 µg trypsin in 40 µL 100 mM NH_4HCO_3) with a 1:100 ratio of trypsin-to-protein mass overnight at 37 ℃.

Phosphopeptide enrichment

After enzymatic hydrolysis, the peptide solution was lyophilized in vacuum and dissolved in a 2,5-dihydroxybenzoic acid (DHB) buffer (Thingholm et al. 2006). Phosphopeptides were enriched using a titanium dioxide (TiO_2) enrichment method (Wang et al. 2020; Larsen et al. 2007), with some modifications. Briefly, TiO_2 beads (Thermo Fisher Scientific, USA) were mixed with peptides and incubated for 40 min at room temperature and then eluted with 1% ammonia solution and 30% acetonitrile. Subsequently, phosphopeptides were freeze-dried and then dissolved with 20 µL 0.1% formic acid (FA). Therein, 6 µL sample was analyzed for LC–MS/MS analysis.

LC–MS/MS

LC–MS/MS analysis was performed using the Q Exactive Mass spectrometer (Thermo Fisher Scientific, USA) with a nanoliter flow rate HPLC liquid system-EASY-nLC1000 (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

The tryptic peptides were injected into the enrichment column EASY column SC001 traps 150 µm*20 mm RP-C18 (Thermo Fisher Scientific, USA) at an isocratic flow of 5 µL/min of 2% CH_3CN containing 0.1% FA (solvent A) for 6 min and then separated by the analytical column EASY column SC200 150 µm*100 mm RP-C18 (Thermo Fisher Scientific,

USA) at an isocratic flow of 400 nL/min. The solvent A and 84% CH_3CN in 0.1% FA (solvent B) were as the eluents. The gradient of solvent B was as follows: (i) 0–110 min, 0–55% solvent B; (ii) 110–118 min, 55–100% B; and (iii) 118–120 min, maintaining at 100% B. Subsequently, the elution products were analyzed by the Q Exactive Mass spectrometer. The spectra of first-grade MS (MS1) were acquired during the scan scope of 300–1800 m/z with a 2.0 kV electrospray voltage, a resolution of 70,000, an automatic gain control (AGC) target of $3e^6$, and a maximum IT time of 50 ms. The spectra of second-grade MS (MS2) were obtained using the following parameter: a resolution of 17,500, a maximum injection time of 40 ms, and an AGC target of $1e^5$. In addition, the mode of MS2 spectra was high-energy collisional dissociation (HCD), and the normalized collision energy of which was set as 30 eV.

Data analysis

All MS/MS data were analyzed using Maxquant1.3.0.5 implementing a Mascot search engine (Matrix Science, London, UK version 2.6.1). The mascot was set up to search the database P17429_fasciola_hepatica_33454_20170815.FASTA (total number of sequences: 33,454; download link: http://www.uniprot.org). The search parameters were set as follows: (i) enzyme: trypsin; (ii) missed cleavage sites: set to 2; (iii) a fixed modification: carbamidomethyl cysteine; and (iv) variable modifications: an oxidation of methionine (M), acetylation of protein N-term, phosphorylation of serine (S), threonine (T), and tyrosine (Y). For proteins and peptides, the filter parameter false discovery rate (FDR) was set as ≤ 0.01.

Bioinformatic analysis

BLAST analysis was performed using Blast2GO (http://www.blast2go.com), and the Gene Ontology (GO) functional categories were performed using the program InterProScan v5.15 (Conesa et al. 2005; Wang et al. 2019b). The InterProScan was utilized for identifying annotated genes features, including protein families, domains, functional sites, and GO terms from the InterPro database (Quevillon et al. 2005; Luo et al. 2021).

In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (http://www.genome.jp/kegg/) were carried out to identify the enriched pathways. The classified pathways were shown in the hierarchical categories according to the data from the KEGG website.

Results

Identification of phosphoproteins and phosphopeptides

In this study, TiO_2-dependent phosphopeptide enrichment method, LC–MS/MS analysis, and Mascot search were used, and a total of 1,030 phosphopeptides were identified, of which 1244 phosphosites represented 635 phosphoproteins of adult *F. gigantica* (Fig. 1A). The identified 1244 phosphosites consisted of 1086 serine phosphorylation (87.3%), 145 threonine phosphorylation (11.7%), and 13 tyrosine phosphorylation (1.0%) (Fig. 1B, Supplementary Table 1). In particular, both hypothetical protein BN1106_s378B000167 and BN1106_s2053B000154 contained more than 10 serine phosphosites and with 6 threonine phosphosites and one tyrosine phosphosite, respectively. In addition, metabolic enzymes like glycogen synthase (BN1106_s2333B000155) and phosphatidylinositol-4-phosphate 3-kinase (BN1106_s403B000429) were rich in these phosphorylation modifications. The phosphoserine, phosphothreonine, or phosphotyrosine modification ratios found in this work were similar to those previously described in other organisms (Wang et al. 2020; Marchini et al. 2011; Hu et al. 2021) and most (90.0%, 1120/1244) phosphosites located at the second amino acid of the peptides or after the position (Fig. 1A, Supplementary Table 1). Out of the identified phosphopeptides, 249 peptides (24.2%) were found phosphorylated at ≥ 2 phosphosites, and most peptides (82.5%) contain ≥ 10 amino acids (Fig. 1A, Supplementary Table 2). The phosphoproteins account for 3% of *F. gigantica* proteins (Pandey et al. 2020). Therein, 196 (30.9%) phosphoproteins contained ≥ 2 phosphopeptides, while BN1106_s323B000258 (myosin heavy chain 6/7) and BN1106_s378B000167 (hypothetical protein) contained more than 10 phosphopeptides (Fig. 1A, Supplementary Table 3). Furthermore, a number ($n = 25$) of phosphorylated protein kinases that covered 5.5% of total *F. gigantica* kinases (Das et al. 2020) were identified including mitogen-activated protein kinase (MAPK) and calcium/calmodulin-dependent protein kinase (CAMK/CDPK) as well as serine/threonine/tyrosine-protein kinase, etc.

GO enrichment analysis

Using the automatic annotation tool Blast2GO, functional annotations were conducted to characterize the identified phosphoproteins in this work. The GO enrichment analysis showed that these phosphoproteins were involved in a wide variety of biological processes (BP) including cellular,

A

Categories (Phosphorylation)	Total number	Remarks
Proteins	635	30.9 % ≥ 2 peptides
Peptides	1030	24.2 % ≥ 2 sites
Sites	1244	90.0 % ≥ 2nd position of peptide N-terminal

B

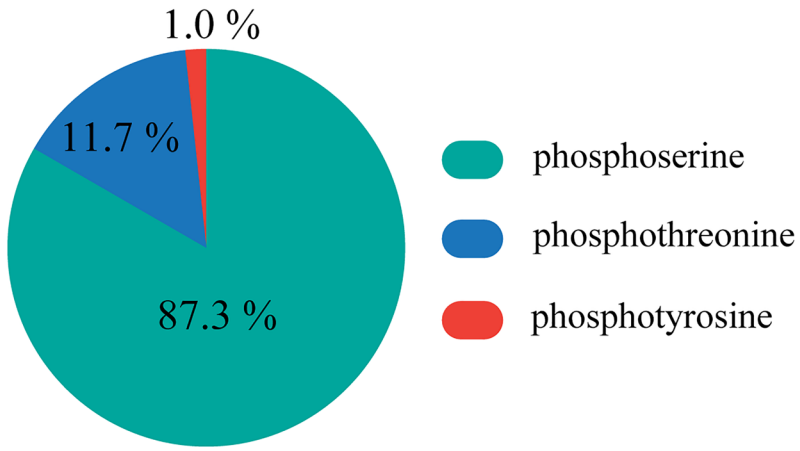

Fig. 1 Overall characteristics of the phosphoproteomics of *F. gigantica*. **A** General description about remarks of *F. gigantica* phosphoproteins, phosphopeptides, and phosphosites. **B** Distribution of identified phosphosites on serine, threonine, and tyrosine

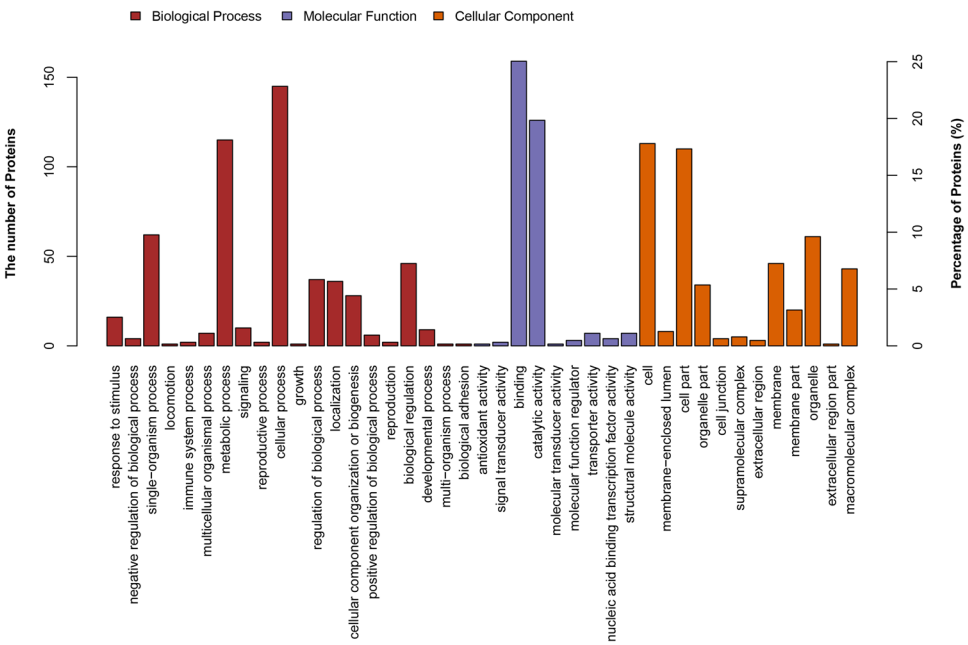

Fig. 2 Gene ontology (GO) term distribution of *F. gigantica* phosphoproteins in three categories (biological process, molecular function, and cellular component). GO annotation and categorization were performed using Blast2GO

metabolic, or single-organism processes and biological regulation, localization, cellular component organization or biogenesis, as well as in response to stimulus (Fig. 2, Supplementary Table 4). Regarding the subcellular localization of the phosphoproteins, the GO annotations based on level 2 and level 5 inferred that these proteins were found predominantly in cellular components (CC) like membranes, organelles, and nuclei. Interestingly, only a small portion of them were predicted to be located in extracellular regions (Fig. 2, Supplementary Table 5), which made the finding reliable since extracellular components were not typically regulated by phosphorylation. According to the localization, the molecular functions (MF) of phosphoproteins was predominantly (51.3%) associated with term "binding" based on level 2, followed by different activities such as catalytic activity (40.6%) and both transporter activity and structural molecule activity that occupied a small proportion (Fig. 2, Supplementary Table 6). In total, we found that half (317/635) of the phosphoproteins were annotated in at least one GO category (BP, CC, or MF), and one-third of them were annotated in at least two GO categories (Supplementary Table 7).

KEGG pathway annotation

In order to determine whether these phosphoproteins act independently in related signaling pathways or work in concert with other proteins, the KEGG database was used to annotate these phosphoproteins to help us understand their potential functions. The KEGG annotation inferred that the most enriched pathways of the phosphoproteins included tight junction, spliceosome, and RNA transport, and each one contained 15 identified proteins (Fig. 3, Supplementary Table 8). PI3K-akt signaling pathway, oxytocin signaling pathway, cAMP and cGMP-PKG signaling pathway, as well as hippo and insulin signaling pathway were ranked in the top 20 of KEGG pathway annotation. Additionally, parts of phosphoproteins were also predicted to participate in the focal adhesion and protein processing as well as carbon metabolism (Supplementary Table 8). Furthermore, we found that 30.4% (193/635) of phosphoproteins were annotated in at least one KEGG pathway and 16.9% was annotated in at least two KEGG pathways (Supplementary Table 8). Therein, metabolic enzymes, such as adenylate cyclase 9 (ADCY9) and hexokinase (HK), and protein kinases including p21-activated kinase 1 (PAK1), Rho-associated protein kinase 2 (ROCK2), mitogen-activated protein kinase 1/3 (MAPK1/3), and serine/threonine-protein kinase mTOR, as well as effectors like calmodulin (CaM) and heat shock protein 90 (HSP90) were predicted to be annotated in more than 10 KEGG pathways (Supplementary Table 9).

Discussion

As a food-borne trematode, *F. gigantica* is responsible for the hepatobiliary disease fascioliasis (Good and Scherbak 2021). In terms of parasite growth and signal transduction, protein phosphorylation or dephosphorylation catalyzed by specialized protein kinases or phosphatases of *F. gigantica* played essential roles in corresponding cellular processes, which helps maintain the cell homeostasis of parasites (Das et al. 2020). Thus, to study the phosphoproteomics profile is of great significance for exploring the roles of these proteins

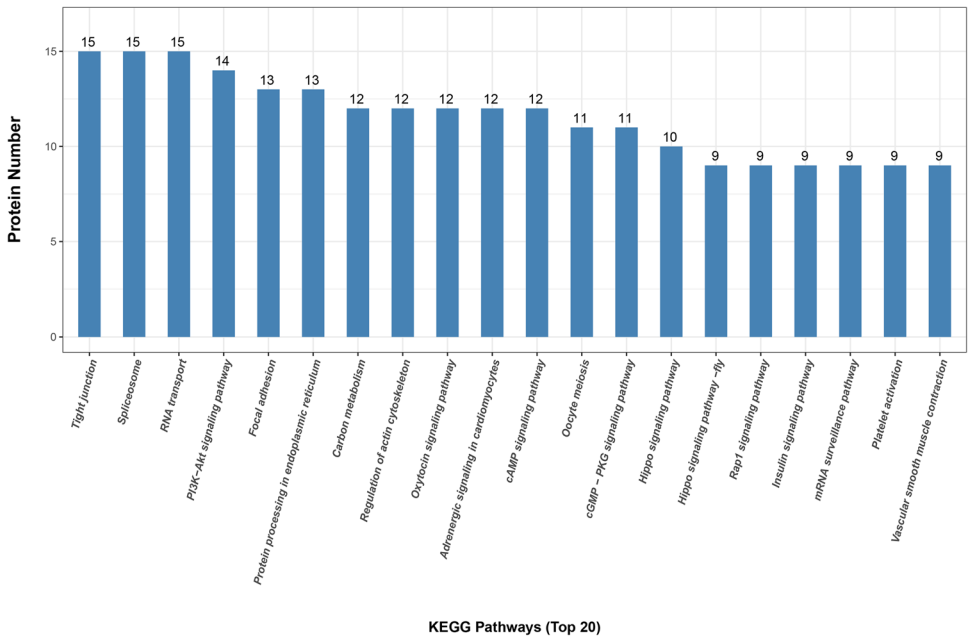

Fig. 3 Enrichment analysis of top 20 KEGG pathways of *F. gigantica* phosphoproteins

in the growth and development of parasite and for developing intervention strategies for fascioliasis.

In this work, a global phosphoproteomics analysis of *F. gigantica* was characterized, although it is probably not yet exhaustive. The majority of the detected phosphorylation events (> 99%) occurred on serine and threonine residues (Supplementary Table 1), and glycogen synthase was rich in serine and threonine phosphorylation in particular. Glycogen synthase was phosphorylated by a conserved glycogen synthase kinase 3 (GSK3) in eukaryotes, and GSK3 acted as a downstream component of the Wnt pathway during embryogenesis (Vital et al. 2010). A study demonstrated that GSK3 was involved in regulation of oogenesis and embryogenesis of *Rhodnius prolixus* (a blood-feeding insect as a media of *Trypanosoma*), and the activity and transcription levels of GSK3 were related to glycogen content (Mury et al. 2016). In *Schistosoma japonicum*, GSK3β (one GSK3 isoform) was highly expressed in the eggs (Liu et al. 2020a). GSK3β RNAi led to a significant decrease in kinase activity and cell viability, showing GSK3β-dependent phosphorylation played an important role in worm survival (Liu et al. 2020a). In that case, future developmental phosphoproteomics analyses supplementing with knockdown experiments are needed to confirm whether the hyper-phosphorylated glycogen synthase correlates with the development and survival of *F. gigantica* or not in a certain stage. On the other hand, previous studies had found that the mutations in important phosphosites might result in the termination of the phosphorylation, leading to disorders of biological processes (Liu et al. 2020b). So an additional point mutation in glycogen synthase will be used to test the essentiality of the serine or threonine phosphorylation and further explore its functional domains.

For phosphoproteins identified in this work, cathepsin L1 (CL1) and glutathione S-transferase (GST) were regarded as lead vaccine candidates against liver flukes (McManus 2020), and in *Fasciola* spp., they could be efficiently inhibited by chalcones and two anti-parasitic agents (thymoquinone and curcumin), respectively (Ferraro et al. 2016; Ullah et al. 2017). Given that CL1 and GST showed good effects on the prevention and treatment of fascioliasis, the use of multistage-specific cocktails with better antigen-delivery systems and more small-molecule chemicals to elicit lethal or sub-lethal phenotypes would be considerable merit in vaccine development and drug target predictions in the future. Moreover, the phosphorylated effectors such as 14–3-3 and thioredoxin were also identified, and previous studies found that they could be recognized by sera from goats or mice experimentally infected with *F. gigantica* (Tian et al. 2018; Changklungmoa et al. 2020), making them considerable for immunodiagnostic candidates for fascioliasis.

According to KEGG annotation, nearly 7% of phosphoproteins indicating multiple roles in different biological processes were predicted to be annotated in more than 5 KEGG pathways (Supplementary Table 9). Therein, malate dehydrogenase (MDH) of *F. gigantica* was a single cytosolic enzyme to catalyze the reversible oxidation of malate to oxaloacetate using NAD^+ (Chetri et al. 2020). Been as an isoenzyme, the pattern of FgMDH was the same as FhMDH due to similar relative mobilities (Sarkari et al. 2016). The superimposition structure model of FgMDH and human MDH showed overall structural similarity in the active site loop region, while the conformation of the residues was different (Chetri et al. 2020). Combining the structure model and our annotations in FgMDH in metabolic pathways such as pyruvate metabolism, cysteine and methionine metabolism, and glyoxylate and dicarboxylate metabolism, the roles of FgMDH can be used to better understand the biochemistry of *F. gigantica*. P21-activated protein kinase (PAKs) had long been established to play important roles in vital cellular functions such as proliferation, survival, and motility (Arias-Romero and Chernoff 2008). Emerging evidence showed host PAK1 increased cell survival during the stage of virus infection (Van den Broeke et al. 2010), and recent study found PAK1 enhanced macrophage activation, resulting in promoting of Th17 cell response during *Schistosoma japonicum* infection (Chang et al. 2020), both of which guide the future research on the immunomodulation roles of host PAK1 and parasitic PAK1 in *Fasciola* infection. To further understand the functions of *F. gigantica* phosphoproteins annotated in more than 10 KEGG pathways, we took them in comparison with protozoa and helminths to identify possible connections between these phosphoproteins and biological processes of the parasites. Based on recent reports (Table 1), calcium/calmodulin-dependent protein kinase (CAMK4/CDPK4) and 14–3-3 protein of *Plasmodium falciparum* participated in controlling the parasite invasion and regulating the assembly of signaling complexes, respectively (Fang et al. 2018; More et al. 2020). In *Leishmania*, mitogen-activated protein kinase 1 (MAPK1) and heat shock protein 90 (HSP90) played important roles in stress regulation and nascent protein synthesis (Kaur et al. 2021; Kalesh et al. 2021). Additionally, the metabolic enzymes acted as sensors to regulate the metabolites and metabolic flux, contributing to parasite growth (Liang et al. 2020; Rodriguez-Saavedra et al. 2021). Camodulins (CaMs) were involved in fundamental processes including the phosphorylation of protein kinases, gene transcription, and calcium transport (Hoeflich and Ikura 2002). In *Schistosoma mansoni*, calmodulin had been implicated in egg hatching, miracidial transformation, and larval development (Guidi et al. 2015). While in *F. hepatica*, the FhCaMs functioned as Ca^{2+} modulators were proved to be important for the growth and movement of juvenile fluke (McCammick et al. 2016). Recent research clarified calmodulin of *Caenorhabditis elegans* was implicated in the plasticity impairment of high-activity neurons with age,

Table 1 Recent researches on the functions of phosphoproteins in other protozoa and helminths

Phosphoproteins		Functions	Parasites	References
Metabolic enzymes	Hexokinase (HK)	A glucose sensor to regulate the glycolysis flux and ATP production	*Trypanosoma brucei*	Rodriguez-Saavedra et al. 2021
	Pyruvate dehydrogenase (PDH E1α)	Contributes to parasite growth via enhanced fatty acid synthesis	*Toxoplasma gondii*	Liang et al. 2020
Protein kinases	Calcium/calmodulin-dependent protein kinase 4 (CAMK4/CDPK4)	Controls parasite motility and host cell invasion	*Plasmodium falciparum*	Fang et al. 2018
	Mitogen-activated protein kinase 1 (MAPK1)	Acts in regulation of stress machinery through post-translational modifications	*Leishmania donovani*	Kaur et al. 2021
Effectors	Calmodulin	Declines the plasticity of high-activity neurons	*Caenorhabditis elegans*	Li et al. 2020
	14–3-3	Binds phosphorylated PKAr and CDPK1 to mediate the assembly of signaling complexes	*Plasmodium falciparum*	More et al. 2020
	Heat shock protein 90 (HSP90)	Regulates nascent protein synthesis	*Leishmania mexicana*	Kalesh et al. 2021

indicating a different but novel role in neuronal activity (Li et al. 2020). In short, the relationship between these proteins of different parasites will help us better understand the phosphoproteomics profile of *F. gigantica.*

Conclusions

In this work, we defined the global phosphoproteomics of a tropical liver fluke by a phosphorylation-specific enrichment technique. From the mass spectrometry and data analysis, we identified several phosphoproteins and predicted their functions, which enhanced our knowledge of key biological processes in *F. gigantica*. With a view towards future exploration into the phosphoproteomics analyses of this parasite at different life-cycle stages, new effective intervention strategies will be developed for the control of fascioliasis.

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Author contribution SYH and MP conceived and designed the study. MP, SYB, JZG, and DDL performed the laboratory analyses. QWJ and JPT analyzed the data. All authors critically appraised and interpreted the results. MP drafted the first version of the manuscript. All authors provided feedback on the manuscript and read and approved the final version.

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Declarations

Ethics approval and consent to participate The experiments were performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. All experimental animal procedures and protocols (Approval No. 14567) were approved by the Institutional Animal Care and Use Committee of Yangzhou University.

Competing interests The authors declare no competing interests.

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