Veterinary Parasitology 218 (2016) 43–45

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Proteomics analysis of *Echinococcus granulosus* protoscolex stage

Christian Hidalgo[a], María Pía García[a], Caroll Stoore[a], Juan Pablo Ramírez[a], Karina Mariante Monteiro[b], Ulf Hellman[c], Arnaldo Zaha[b], Henrique Bunselmeyer Ferreira[b], Norbel Galanti[d], Eduardo Landerer[e], Rodolfo Paredes[a,*]

[a] *Escuela de Medicina Veterinaria, Facultad de Ecologia y Recursos Naturales, Universidad Andres Bello, Santiago, Chile*
[b] *Laboratório de Genômica Estrutural e Funcional and Laboratório de Biologia Molecular de Cestódeos, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil*
[c] *Ludwig Institute for Cancer Research Ltd., Uppsala University, Biomedical Center, Uppsala, Sweden*
[d] *Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile*
[e] *Escuela de Medicina, Facultad de Medicina, Universidad Andres Bello, Santiago, Chile*

ARTICLE INFO

Article history:
Received 24 February 2015
Received in revised form
24 December 2015
Accepted 27 December 2015

Keywords:
Echinococcus granulosus
Proteome
Protoscolex
Bovine
Hydatid cyst fertility

ABSTRACT

Echinococcus granulosus protoscolex proteins were separated using two-dimensional electrophoresis and then identified using mass spectrometry; we identified 61 proteins, 28 which are newly described of which 4 could be involved in hydatid cyst fertility molecular mechanisms.

Hydatidosis, also known as Cystic Echinococcosis (CE), is a parasitic disease caused by the metacestode of the flatworm *Echinococcus granulosus*. This parasite has a complex life cycle involving a definitive carnivore host (most commonly dogs) and an intermediate mammalian host (cattle, sheep, pigs, among others). The adult worm resides in the small intestine of the definitive host, and shed both eggs and proglottids in the feces, contaminating fields, watercourses, produce and the fur of the host. While grazing, the intermediate hosts ingest eggs, and develop the metacestode known as hydatid cyst in the viscera (liver and lungs). Humans are infected when drinking contaminated water, eating raw produce or by having a close relation with the definitive host (Mandal and Mandal, 2012). The metacestode is described as a unilocular fluid filled cyst comprised of 3 layers: the innermost layer is called the germinal layer where the cellular component of the parasite resides, the middle layer is the laminated layer an extracellular matrix unique to the *Echinococcus* genus, and the outermost layer is the adventitial layer, formed by the host reaction to the parasite. The germinal layer cells are responsible for secreting the laminated layer, the hydatid fluid that fills the cyst and also differentiate to the infective stage, a structure called protoscolex (PSC). When the PSC is ingested by the definitive host, it evaginates and attaches to the small intestine, completing the cycle (Siracusano et al., 2012). The PSC is an ideal sample to analyze because it has three characteristics: it is not directly infective to humans; it can be studied with minimal host protein contamination and it can generate both the adult worm and new hydatid cysts under appropriate conditions. When studying the metacestode biology, the hydatid cysts can be classified into two groups: fertile and infertile cysts; the most important difference being that the latter is unable to produce PSC. The molecular mechanisms that explain the cause of cyst infertility remain elusive (Riesle et al., 2014). We propose that studying the PSC proteome using both two-dimensional electrophoresis (2D-E) and mass spectrometry (MS) could generate valuable information of proteins that have not previously been reported at this stage. By routinely visiting local abattoirs, we obtained bovine lung hydatid cysts with a diameter > 3 cm and in the laboratory. Under sterile conditions, the cysts were processed as follows: using a disposable surgical blade, the cyst was opened and all the hydatid fluid was

* Corresponding author at: Escuela de Medicina Veterinaria, Universidad Andres Bello, República 440, 2° piso, Santiago, Chile.
E-mail address: rparedes@unab.cl (R. Paredes).

http://dx.doi.org/10.1016/j.vetpar.2015.12.026
0304-4017/

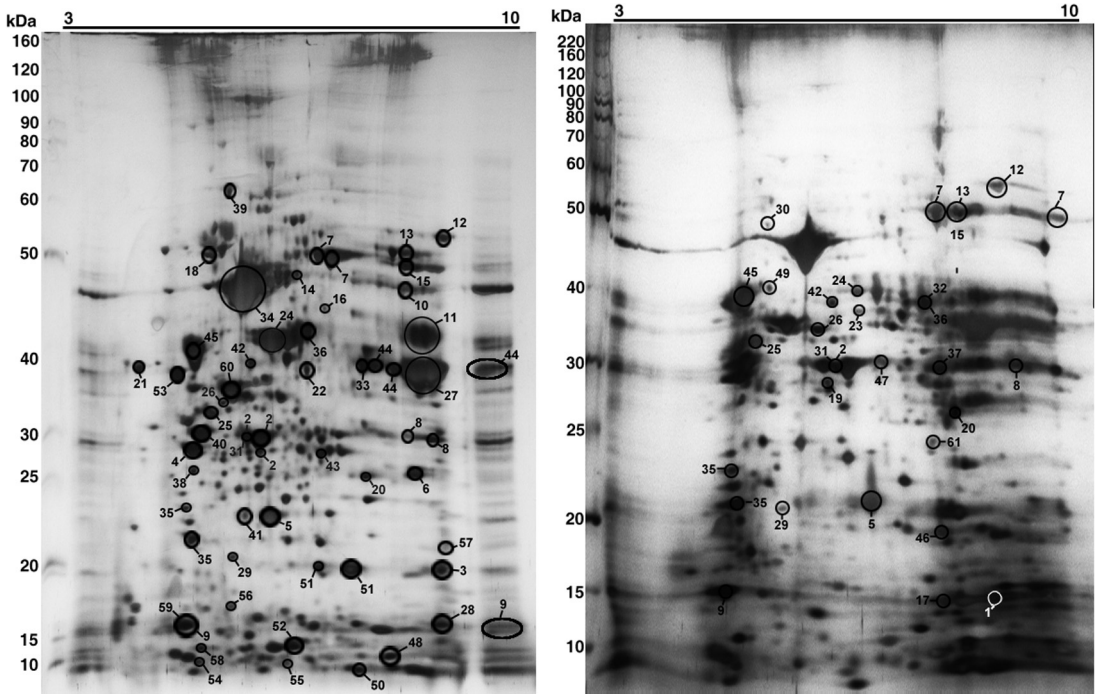

Fig. 1. 2D-E of PSC protein extracts run on 13 cm pH 3–10 IPG strips in the first dimension followed by the second dimension on a 12% SDS-PAGE maxigel and stained with silver nitrate. Both gels represent 2 different experiments with the same protein sample. A total of 473 spots were excised, from these, 90 spots were successfully matched to 61 proteins via MASCOT analysis. Proteins are identified with the correlative number in Supplementary Table 1.

collected. Cyst fertility was determined by both visual inspection of the innermost layer of the cyst and by microscopic examination of the germinal layer. Cysts were considered fertile when PSC were present at microscopic examination. The germinal layer of fertile cysts was washed with PBS to detach all the PSC; then were then transferred to a 50 mL tube, where they were washed three times with PBS to remove dead PSC and germinal layer remnants. The remaining PSC were transferred to a 1,5 mL tube and solubilized in 3 times the volume of a 2D-E lysis buffer, centrifuged at 17,000 × *g*, saving the supernatant and discarding the pellet. Protein quantity was measured with the Bradford assay. Isoelectric focusing (IEF) was performed on 13 cm immobilized pH gradient strips, at ranges 3–10 and 4–7, loading 300 μg of total protein. Second dimension was done in 20 × 20 cm SDS-PAGE and stained with silver nitrate. Once standardized, we manually excised 473 visible spots from silver nitrate stained gels to perform MS. Excised gel spots were placed in labeled tubes and processed at the Ludwig Institute for Cancer Research, Uppsala, Sweden, and at the Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil. MALDI-TOF–TOF analyses were performed on a Bruker Ultraflex III TOF/TOF mass spectrometer, with Flex Control v3.3. Data analyses were made with Flex Analysis v3.3 (Bruker Daltonik GmbH); MS/MS analyses were made with Biotools v3.0 (BrukerDaltonik GmbH). PMF data were evaluated against databases using MASCOT software v. 2.3. Ninety spots were matched with a total of 61 unique proteins; all of them from the genus *Echinococcus*; 59 specific to *E. granulosus*, whereas 2 proteins were matched to *Echinococcus multilocularis*. Experimental pI and molecular masses were paired with the *in silico* data provided by the MASCOT search; both excised and identified proteins were marked with circles. From these spots, 41 proteins were identified in a single spot each, and the other 20 proteins appeared in more than one spot. Proteins identified this way were grouped using Blast2Go Software according to molecular function, cellular component or biological process; most molecular functions being protein binding (14 proteins) and heterocyclic compound binding (12 proteins). In the cellular component analysis, most proteins identified were classified as being structural proteins (19 proteins). The spot analyzed are shown in Fig. 1 and protein identification is provided as a Supplementary Table 1. The use of 2D-E associated with mass spectrometry has been widely used to identify both host and parasite proteins. Monteiro et al. (2010) identified 215 PSC protein spots from 2-DE gels in two diferente pH ranges. More recently, Cui et al. (2013) used in tandem with liquid chromatography mass spectrometry and identified 1588 PSC proteins. Li and Zhao (2012) have also published data of *E. granulosus* PSC proteins and have identified 233 new proteins, using the same approach. During the course of this research, the genome of *E. granulosus* was published by two groups (Tsai et al., 2013; Zheng et al., 2013), and with the new data added to public databases, many PMF data that matched other helminthes now matches *E. granulosus* instead, greatly refining our results. However, there is still mass spectrometry data from 412 spots that has no match with proteins in different databases. Four of these proteins were already described in the *Echinococcus* genus but not at this particular stage, like SOD in the cyst wall (Feng et al., 1995) and 14-3-3 proteins as excretory secretory products (Virginio et al., 2012). Nevertheless, the protein identified in PSC is a different isoform. Recently, four 14-3-3 protein isoforms have been previously detected in different *E. granulosus* metacestode components, including in protoscoleces (Teichmann et al., 2015) and ubiquitin was previously described in another cestode (Zhang et al., 2010). Two proteins are found in *Schistosoma mansoni*, the major egg antigen p40 is very well described in this trematode (Stadecker and Hernandez, 1998), and nucleoside diphosphate kinase is also described, but to a lesser extent (Marques Ide et al., 2012). Two proteins are described in nematodes; Prostaglandin H2 D isomerase (PTGDS) has been described in *Onchocerca volvulus* (Perbandt et al., 2008), and inorganic pyrophosphatase in ascaris (Islam et al., 2003). Eight proteins are described in parasitic protozoa, Ndr (Hergovich et al., 2006), Tubulin beta 2C chain (Kumar et al., 2010), 6 phosphogluconolactonase (Jortzik et al., 2011), GDP L fucose synthase (Sanz et al., 2013), protein DJ 1 (Hall et al., 2011), eukaryotic translation

initiation factor 5A (Carvajal-Gamez et al., 2011), annexin a7 (Lang et al., 2009) and FK506-binding protein-like protein, which has a promising role as a therapeutical target (Bell et al., 2006). There are 11 proteins that are not described in parasites of any kind. Of these proteins, some could be potentially involved either in the evasion of the immune system or in the mechanisms underlying the hydatid cyst fertility. Special attention should be directed towards proteins identified in the 30, 40, 57, 62 and 69 kDa molecular weights. Indeed, we previously described that PSC proteins of these molecular weights could be associated with cyst fertility mechanisms (Paredes et al., 2011); at the 30 kDa mark, we identified the prohibitin protein WPH, which is prevalent in the mitochondria and highly conserved in evolution. However, its biological function is unclear (Chen et al., 2015). At the 40 kDa mark we identified a Nuclear DBF2-related kinase (Ndr), which has been described in parasites as an essential enzyme, its depletion disrupts cytokinesis, leading to cell cycle deregulation and cell death (Ma et al., 2010); this could be a potential candidate for the host immune response in infertile cysts. We were unable to identify proteins at the precise molecular weight between the 57 and 69 kDa range. In summary, the method used in this work allows for the study of highly purified *E. granulosus* proteins with minimal contamination from host proteins. The use of 2D-E + Mass Spectrometry enabled us to identify 61 PSC proteins, 28 of them were not described before at this stage and generates new data in the characterization of the proteins expressed in PSC, providing further insight into the physiology of this parasite. Further studies in both the function and molecular pathways of these proteins could generate the information for new therapeutic targets and understanding the mechanisms involved in cyst fertility. The approach presented in this work could also be used to study the proteins expressed in other compartments of the hydatid cyst, such as the germinal and laminated layers.

Acknowledgments

Grant sponsor: FONDECYT-Chile, grant numbers: N° 1130717; Grant sponsor: Cooperación Internacional Conicyt (Chile)/CNPq (Brazil); Grant sponsor: K.M.M. is a former recipient of post-doctoral fellowships from CNPq and CAPES (Brazil). Part of the *in silico* analyses of MS data was performed at the UNIPROTE-MS facilities of the Biotechnology Center, UFRGS (Brazil).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2015.12.026.

References

Bell, A., Monaghan, P., Page, A.P., 2006. Peptidyl-prolyl cis–trans isomerases (immunophilins) and their roles in parasite biochemistry, host–parasite interaction and antiparasitic drug action. Int. J. Parasitol. 36, 261–276.

Carvajal-Gamez, B.I., Arroyo, R., Camacho-Nuez, M., Lira, R., Martínez-Benitez, M., Alvarez-Sánchez, M.E., 2011. Putrescine is required for the expression of eif-5a in *Trichomonas vaginalis*. Mol. Biochem. Parasitol. 180, 8–16.

Chen, L., Chen, H., Yao, C., Chang, C., Xia, H., Zhang, C., Zhou, Y., Yao, Q., Chen, K., 2015. The toxicity of NaF on BmN cells and a comparative proteomics approach to identify protein expression changes in cells under NaF-stress: impact of NaF on BmN cells. J. Hazard. Mater. 286, 624–631.

Cui, S.-J., Xu, L.-L., Zhang, T., Xu, M., Yao, J., Fang, C.-Y., Feng, Z., Yang, P.-Y., Hu, W., Liu, F., 2013. Proteomic characterization of larval and adult developmental stages in *Echinococcus granulosus* reveals novel insight into host–parasite interactions. J. Proteom. 84, 158–175.

Feng, J.J., Guo, H.F., Yao, M.Y., Xiao, S.H., 1995. Effects of mebendazole, albendazole, and praziquantel on glutathione *S*-transferase and superoxide dismutase of *Echinococcus granulosus* cyst wall harbored in mice. Zhongguo Yao Li Xue Bao 16, 297–300.

Hall, C.I., Reese, M.L., Weerapana, E., Child, M.A., Bowyer, P.W., Albrow, V.E., Haraldsen, J.D., Phillips, M.R., Sandoval, E.D., Ward, G.E., Cravatt, B.F., Boothroyd, J.C., Bogyo, M., 2011. Chemical genetic screen identifies toxoplasma DJ-1 as a regulator of parasite secretion, attachment, and invasion. Proc. Natl. Acad. Sci. U. S. A. 108, 10568–10573.

Hergovich, A., Stegert, M.R., Schmitz, D., Hemmings, B.A., 2006. NDR kinases regulate essential cell processes from yeast to humans. Nat. Rev. Mol. Cell Biol. 7, 253–264.

Islam, M.K., Miyoshi, T., Kasuga-Aoki, H., Isobe, T., Arakawa, T., Matsumoto, Y., Tsuji, N., 2003. Inorganic pyrophosphatase in the roundworm *Ascaris* and its role in the development and molting process of the larval stage parasites. Eur. J. Biochem. 270, 2814–2826.

Jortzik, E., Mailu, B.M., Preuss, J., Fischer, M., Bode, L., Rahlfs, S., Becker, K., 2011. Glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase: a unique bifunctional enzyme from *Plasmodium falciparum*. Biochem. J. 436, 641–650.

Kumar, A., Sisodia, B., Misra, P., Sundar, S., Shasany, A.K., Dube, A., 2010. Proteome mapping of overexpressed membrane-enriched and cytosolic proteins in sodium antimony gluconate (SAG) resistant clinical isolate of *Leishmania donovani*. Br. J. Clin. Pharmacol. 70, 609–617.

Lang, P.A., Kasinathan, R.S., Brand, V.B., Duranton, C., Lang, C., Koka, S., Shumilina, E., Kempe, D.S., Tanneur, V., Akel, A., Lang, K.S., Foller, M., Kun, J.F., Kremsner, P.G., Wesselborg, S., Laufer, S., Clemen, C.S., Herr, C., Noegel, A.A., Wieder, T., Gulbins, E., Lang, F., Huber, S.M., 2009. Accelerated clearance of plasmodium-infected erythrocytes in sickle cell trait and annexin-A7 deficiency. Cell. Physiol. Biochem. 24, 415–428.

Li, Z.J., Zhao, W., 2012. Analysis of protoscoleces-specific antigens from *Echinococcus Granulosus* with proteomics combined with Western blot. Biomed. Environ. Sci. 25, 718–723.

Ma, J., Benz, C., Grimaldi, R., Stockdale, C., Wyatt, P., Frearson, J., Hammarton, T.C., 2010. Nuclear DBF-2-related kinases are essential regulators of cytokinesis in bloodstream stage *Trypanosoma brucei*. J. Biol. Chem. 285, 15356–15368.

Mandal, S., Mandal, M.D., 2012. Human cystic echinococcosis: epidemiologic, zoonotic, clinical, diagnostic and therapeutic aspects. Asian Pac. J. Trop. Med. 5, 253–260.

Marques Ide, A., Romanello, L., DeMarco, R., Pereira, H.D., 2012. Structural and kinetic studies of *Schistosoma mansoni* adenylate kinases. Mol. Biochem. Parasitol. 185, 157–160.

Monteiro, K.M., de Carvalho, M.O., Zaha, A., Ferreira, H.B., 2010. Proteomic analysis of the *Echinococcus granulosus* metacestode during infection of its intermediate host. Proteomics 10, 1985–1999.

Paredes, R., Godoy, P., Rodriguez, B., Garcia, M.P., Cabezon, C., Cabrera, G., Jimenez, V., Hellman, U., Saenz, L., Ferreira, A., Galanti, N., 2011. Bovine (*Bos taurus*) humoral immune response against *Echinococcus granulosus* and hydatid cyst infertility. J. Cell. Biochem. 112, 189–199.

Perbandt, M., Hoppner, J., Burmeister, C., Luersen, K., Betzel, C., Liebau, E., 2008. Structure of the extracellular glutathione *S*-transferase OvGST1 from the human pathogenic parasite *Onchocerca volvulus*. J. Mol. Biol. 377, 501–511.

Riesle, S., Garcia, M.P., Hidalgo, C., Galanti, N., Saenz, L., Paredes, R., 2014. Bovine IgG subclasses and fertility of *Echinococcus granulosus* hydatid cysts. Vet. Parasitol. 205, 125–133.

Sanz, S., Bandini, G., Ospina, D., Bernabeu, M., Marino, K., Fernandez-Becerra, C., Izquierdo, L., 2013. Biosynthesis of GDP-fucose and other sugar nucleotides in the blood stages of *Plasmodium falciparum*. J. Biol. Chem. 288, 16506–16517.

Siracusano, A., Delunardo, F., Teggi, A., Ortona, E., 2012. Host-parasite relationship in cystic echinococcosis: an evolving story. Clin. Dev. Immunol. 2012, http://dx.doi.org/10.1155/2012/639362, Article ID 639362, 12 pages.

Stadecker, M.J., Hernandez, H.J., 1998. The immune response and immunopathology in infection with *Schistosoma mansoni*: a key role of major egg antigen Sm-p40. Parasite Immunol. 20, 217–221.

Tsai, I.J., Zarowiecki, M., Holroyd, N., Garciarrubio, A., Sanchez-Flores, A., Brooks, K.L., Tracey, A., Bobes, R.J., Fragoso, G., Sciutto, E., 2013. The genomes of four tapeworm species reveal adaptations to parasitism. Nature 496, 57–63.

Teichmann, A., Vargas, D.M., Monteiro, K.M., Meneghetti, B.V., Dutra, C.S., Paredes, R., Galanti, N., Zaha, A., Ferreira, H.B., 2015. Characterization of 14-3-3 isoforms expressed in the *Echinococcus granulosus* pathogenic larval stage. J. Proteome. Res. 14, 1700–1715.

Virginio, V.G., Monteiro, K.M., Drumond, F., de Carvalho, M.O., Vargas, D.M., Zaha, A., Ferreira, H.B., 2012. Excretory/secretory products from in vitro-cultured *Echinococcus granulosus* protoscoleces. Mol. Biochem. Parasitol. 183, 15–22.

Zhang, H., Zhao, W.J., Kang, L.C., Wang, X.H., Bo, X.W., 2010. Characterization of a *Moniezia expansa* ubiquitin-conjugating enzyme E2 cDNA. Mol. Biol. Rep. 37, 1585–1590.

Zheng, H., Zhang, W., Zhang, L., Zhang, Z., Li, J., Lu, G., Zhu, Y., Wang, Y., Huang, Y., Liu, J., Kang, H., Chen, J., Wang, L., Chen, A., Yu, S., Gao, Z., Jin, L., Gu, W., Wang, Z., Zhao, L., Shi, B., Wen, H., Lin, R., Jones, M.K., Brejova, B., Vinar, T., Zhao, G., McManus, D.P., Chen, Z., Zhou, Y., Wang, S., 2013. The genome of the hydatid tapeworm *Echinococcus granulosus*. Nat. Genet. 45, 1168–1175.