UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Identificação e caracterização de proteínas expressas pelo metacestódeo de *Echinococcus granulosus* durante a infecção do seu hospedeiro intermediário

Karina Mariante Monteiro

Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor em Ciências

Orientador: Dr. Henrique Bunselmeyer Ferreira
Co-orientador: Dr. Arnaldo Zaha

Porto Alegre, maio de 2010

Este trabalho foi realizado no Laboratório de Biologia Molecular de Cestódeos e Laboratório de Genômica Estrutural e Funcional do Centro de Biotecnologia da UFRGS, sendo financiado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Agradecimentos

Aos meus orientadores, Dr. Henrique B. Ferreira e Dr. Arnaldo Zaha, pela oportunidade de realização deste trabalho e pela dedicação, auxílio, incentivo e amizade durante este período.

Ao Laboratório Nacional de Luz Síncrotron (LNLS) pelo suporte financeiro e técnico nos experimentos de espectrometria de massas (propostas MAS-5116 e MAS-6409), especialmente ao Dr. Fábio César Gozzo e Thais Dombroski do Laboratório de Espectrometria de Massas.

Ao Uniprote-MS (CBIOT), especialmente ao Dr. Paulo Marcos Pinto, pelo suporte com as análises de LC-MS/MS.

À Dra. Nádya Pesce da Silveira, do Laboratório de Instrumentação e Dinâmica Molecular do Instituto de Química da UFRGS, pela colaboração com as medidas de espalhamento de luz.

Ao Dr. Mateus Borba Cardoso, do Grupo de Espalhamento de Raios X do LNLS, pelo auxílio com os experimentos de espalhamento de luz e microscopia de força atômica.

Ao Dr. Cristian Follmer, do Laboratório de Investigação de Neuroproteção do Instituto de Química da UFRJ, responsável pelas análises de dissociação induzida por pressão.

Ao Dr. Elliot Watanabe Kitajima, do Núcleo de Apoio à Pesquisa em Microscopia Eletrônica Aplicada a Agricultura (NAP/MEPA) da Escola Superior de Agricultura Luiz de Queiroz (ESALQ), Universidade de São Paulo, pelo auxílio com as análises de miscroscopia eletrônica de transmissão.

A todos os colegas dos laboratórios 204/206 (LBMC), Karina L., Lu, Helier, Martín, Edi, Taís e Ana, pela constante colaboração e amizade, e por terem tornado esses anos muito mais agradáveis. Em especial aos meus “orientados” e “ex-orientados”, Daiani, Aline, Carol, André e Bárbara, por me deixarem brincar de ensinar e me retribuírem com amizade e colaboração.

Às amigas, colegas e ex-colegas, Veri, Mel e Alice, pela constante amizade, incentivo e apoio.

Aos colegas do laboratório 210 (LAGEF), Lucas, Cláudio, Carol, Jéssica e Thiely pela colaboração, incentivo e amizade.

Aos colegas de outros laboratórios do Centro de Biotecnologia, em especial aos labs 217, 220 e 222.

Aos funcionários do Centro de Biotecnologia, em especial à Sílvia Centeno e Luciano Saucedo, pelo auxílio e colaboração.

À minha família, por todo amor, apoio, compreensão, incentivo e dedicação.

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Lista de abreviaturas, símbolos e unidades

ACN	acetonitrila
AFM	microscopia de força atômica
AgB	antígeno B
AgB8/1	subunidade 1 do antígeno B
AgB8/2	subunidade 2 do antígeno B
AgB8/3	subunidade 3 do antígeno B
CD	dicroísmo circular
cDNA	DNA complementar
CHD	hidatidose cística
DBI	inibidor da ligação do diazepam
DLS	espalhamento de luz dinâmico
DNA	ácido desoxirribonucleico
DTT	ditiotreitol
EgTeg	proteína de tegumento de *E. granulosus*
EgTPx	tiorredoxina-peroxidase de *E. granulosus*
ELISA	ensaio de imunoadsorção ligado a enzima
ES	excreção/secreção
ESI	ionização por *electrospray*
EST	marca de sequência expressa
GL	camada germinativa
GST	glutationa-S-transferase
HCF	líquido hidático
HLBP	proteína que se liga a compostos hidrofóbicos
HSP	proteína de choque térmico
IEF	focalização isoelétrica
kDa	quilodalton (1000 daltons)
LC	cromatografia líquida
LC-MS/MS	cromatografia líquida acoplada a espectrometria de massas em tandem
LDL	lipoproteína de baixa densidade

LS	espalhamento de luz
MALDI	ionização/dessorção a laser assistida por matriz
MMP	metaloproteinase de matriz
MS	espectrometria de massas
MS/MS	espectrometria de massas em tandem
PAGE	eletroforese em gel de poliacrilamida
pb	pares de bases
PBMC	células mononucleares de sangue periférico
PBS	tampão fosfato-salina
pH	potencial hidrogeniônico
pI	ponto isoelétrico
PSC	protoescólex, protoescólices
PVDF	fluoreto de polivinilideno
Q	quadrupolo
R_h	raio hidrodinâmico
RNA	ácido ribonucleico
RT-PCR	transcrição reversa-reação em cadeia da polimerase
SDS	dodecilsulfato de sódio
TCA	ácido tricloroacético
TEM	microscopia eletrônica de transmissão
TFA	ácido trifluoroacético
TOF	tempo de vôo
Vh	volts-hora
2-DE	eletroforese bidimensional

Resumo

A hidatidose cística é uma zoonose helmíntica endêmica causada pela infecção com a forma larval ou metacestódeo de *Echinococcus granulosus*. Neste trabalho, foi realizada uma análise proteômica do metacestódeo de *E. granulosus* durante a infecção do seu hospedeiro intermediário bovino. A identificação de proteínas do parasito e do hospedeiro presentes nos diferentes componentes da larva do parasito (protoescólices, camada germinativa e líquido hidático) gerou informações valiosas sobre a relação parasito-hospedeiro. Os resultados obtidos permitiram a caracterização da resposta imune montada pelo hospedeiro contra a infecção por *E. granulosus*, bem como de potenciais estratégias moleculares adotadas pelo parasito para evadir essa resposta e promover a sua sobrevivência dentro do corpo do hospedeiro. Além disso, novas proteínas-alvo foram identificadas para o desenvolvimento ou melhora de ferramentas de diagnóstico, tratamento e controle da hidatidose. Nesse trabalho, foi também estudada a estrutura do antígeno B (AgB) de *E. granulosus*, uma proteína implicada em múltiplas interações parasito-hospedeiro durante a infecção. Medidas de espalhamento de luz dinâmico utilizando subunidades recombinantes (AgB8/1, AgB8/2 e AgB8/3) demonstraram que o AgB forma diferentes estruturas moleculares sob condições fisiológicas, incluindo oligômeros e agregados de alta massa molecular. As subunidades recombinantes do AgB mostraram diferentes tendências agregativas, sendo os agregados de alta massa molecular formados por AgB8/3 os mais similares, em morfologia e tamanho, àqueles do AgB produzido pelo parasito. Experimentos de dissociação induzida por pressão revelaram que a formação de pontes dissulfeto confere maior estabilidade aos homo-oligômeros de AgB8/2 e AgB8/3. A composição de subunidades do AgB foi avaliada por espectrometria de massas (MS), identificando as subunidades AgB8/1, AgB8/3 e AgB8/4, o que indica uma contribuição principal dessas subunidades nas propriedades estruturais, biológicas e imunológicas do AgB de *E. granulosus*. A análise de MS indicou também uma associação entre AgB e Ag5, os principais antígenos secretados pelo metacestódeo de *E. granulosus*.

Abstract

Cystic hydatid disease (CHD) is an endemic helminthic zoonosis caused by infection with the larval stage or metacestode of the tapeworm *Echinococcus granulosus*. Here, we performed a proteomic analysis of the *E. granulosus* metacestode during infection of its intermediate bovine host. Identification of parasite and host proteins present in different components of parasite larvae (protoscoleces, germinal layer and hydatid cyst fluid) provided valuable information on host-parasite interplay. Our results allowed the characterization of host immune response mounted against *E. granulosus* infection, as well as potential molecular strategies adopted by the parasite to avoid this response and promote its survival inside the host body. Moreover, new protein targets were identified for the development or improvement of CHD diagnosis, treatment, and control tools. In this work, we also study the structure of *E. granulosus* antigen B (AgB), a protein implicated in multiple host-parasite interactions during infection. Dynamic light scattering experiments with recombinant subunits (AgB8/1, AgB8/2 and AgB8/3) demonstrated that AgB form different molecular assemblies under physiological conditions, including oligomers and high-molecular-weight aggregates. AgB recombinant subunits showed different aggregative tendencies, being AgB8/3 high-molecular-weight aggregates most similar, both in morphology and size, to those of parasite-produced AgB. Pressure-induced dissociation experiments revealed that disulfide bonds formation confers greater stability to AgB8/2 and AgB8/3 homo-oligomers. AgB subunit composition was evaluated by mass spectrometry (MS), identifying AgB8/1, AgB8/3 and AgB8/4 subunits, which indicates a major contribution for these subunits on *E. granulosus* AgB structural, biological, and immunological properties. The MS analysis also indicated an association between AgB and Ag5, the major antigens secreted by *E. granulosus* metacestode.

1. Introdução

1.1. Espécies do gênero *Echinococcus* e a hidatidose

A hidatidose (ou equinococose) é a infecção pela fase larval de cestódeos do gênero *Echinococcus* (família Taeniidae), sendo uma zoonose emergente/re-emergente em nível mundial (Budke *et al*., 2006; Moro & Schantz, 2009). São reconhecidas seis espécies do gênero *Echinococcus*, sendo quatro delas infectivas para seres humanos: *Echinococcus granulosus*, a qual causa a hidatidose cística; *Echinococcus multilocularis*, a qual causa a hidatidose alveolar; *Echinococcus vogeli e Echinococcus oligarthrus*, as quais causam a hidatidose policística. Duas novas espécies foram recentemente identificadas, *Echinococcus shiquicus* em pequenos mamíferos do platô tibetano (Xiao *et al*., 2005), e *Echinococcus felidis* em leões africanos (Hüttner *et al*., 2008), mas seu potencial zoonótico de transmissão é ainda desconhecido. As espécies *E. granulosus* e *E. multilocularis* apresentam especial importância pela sua ampla distribuição geográfica e seu impacto médico e econômico, enquanto a hidatidose policística é menos frequente e está restrita às Américas do Sul e Central (Jenkins *et al*., 2005; Moro & Schantz, 2009). *E. granulosus* é considerada a espécie de maior relevância epidemiológica, devido a sua distribuição cosmopolita e seu importante impacto na saúde pública humana e animal (Budke *et al*., 2006). A hidatidose cística é a forma mais comum da hidatidose em seres humanos, representando cerca de mais de 90% dos 3 milhões de casos estimados em todo o mundo (Budke *et al*., 2006; Craig *et al*., 2007).

1.2. *Echinococcus granulosus*

Como outros membros da família Taeniidae, *E. granulosus* necessita de dois hospedeiros mamíferos para completar o seu ciclo vital (Fig. 1.1). *E. granulosus* utiliza

cães e outros canídeos como hospedeiros definitivos, os quais abrigam a forma adulta intestinal do parasito; e ungulados domésticos e selvagens como hospedeiros intermediários, nos quais a forma larval (metacestódeo ou cisto hidático) patogênica do verme se desenvolve usualmente nas vísceras. Seres humanos são considerados hospedeiros intermediários aberrantes ou acidentais, pois não desempenham papel na

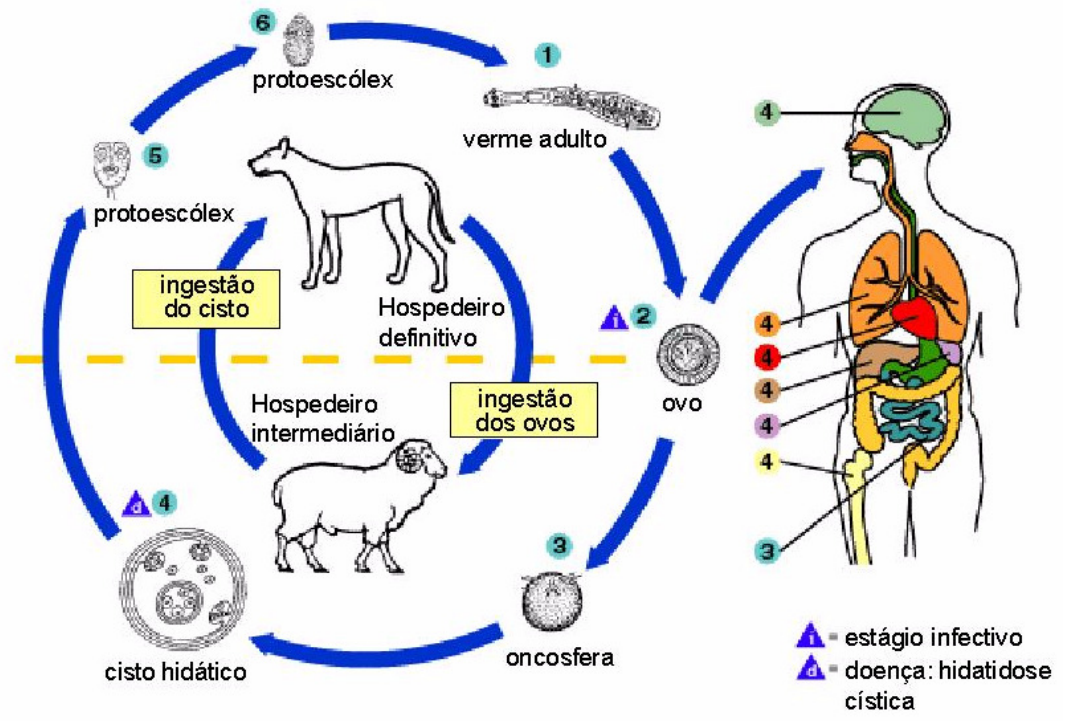

Figura 1.1. Ciclo vital de *E. granulosus*. O verme adulto (1) vive no intestino delgado do hospedeiro definitivo (canídeos). Os ovos (2) são liberados no ambiente juntamente com as fezes do hospedeiro, sendo infectivos para os hospedeiros intermediários (ovinos, bovinos, suínos, cavalos, camelos, humanos, etc.). Ao serem ingeridos pelo hospedeiro intermediário, os ovos liberam a oncosfera (3), que penetra na parede intestinal e é transportada pelo sistema circulatório ou linfático até atingir um órgão, principalmente fígado e pulmões. Neste órgão, a oncosfera diferencia-se num cisto hidático (4), que aumenta gradualmente de tamanho e produz protoescólices (5) assexualmente. O hospedeiro definitivo contamina-se através da ingestão de vísceras do hospedeiro intermediário contendo cistos do parasito. Após a ingestão, os protoescólices evaginam (6), fixam-se na mucosa intestinal e transformam-se em vermes adultos (1), fechando o ciclo vital do parasito. Modificado do Centro para o Controle e Prevenção de Doenças Infecciosas (http://www.dpd.cdc.gov).

perpetuação do ciclo de transmissão do parasito (Thompson & McManus, 2001). O ciclo selvagem de transmissão se dá através da relação presa/predador, enquanto o ciclo doméstico é perpetuado através de práticas rurais condenáveis, como a alimentação de cães com vísceras de ungulados domésticos (principalmente bovinos e ovinos) contaminadas com cistos hidáticos (Moro & Schantz, 2009).

O verme adulto de ***E. granulosus*** é uma pequena tênia, com 2 a 7 mm de comprimento (Fig. 1.2), que vive no intestino delgado do hospedeiro definitivo, aderido às criptas de Lieberkühn. Na sua porção anterior, o adulto apresenta um escólex com quatro ventosas musculares localizadas lateralmente e um rostelo móvel e extensível na sua porção apical, armado com duas fileiras de ganchos envolvidos na fixação do parasito ao hospedeiro. O estróbilo é segmentado e consiste de três unidades reprodutivas (proglótides), raramente apresentam até 6 proglótides, que diferem no seu estágio de maturação sexual. A primeira proglótide é dita jovem ou imatura; a segunda é a proglótide sexualmente madura; e a última, a proglótide grávida que contém os ovos do parasito.

A reprodução do verme adulto pode se dar por autofecundação, uma vez que ***E. granulosus*** é hermafrodita, ou por fecundação cruzada. A autofecundação é a forma de reprodução predominante, mas a fecundação cruzada também ocorre (Haag ***et al.***, 1998), sendo possível devido ao comportamento agregativo dos vermes adultos no intestino do hospedeiro (Lymbery ***et al.***, 1989). Após a fecundação, ocorre a produção dos ovos, podendo a proglótide grávida conter até 1500 ovos (Thompson, 1995). A proglótide grávida se destaca do estróbilo por um fenômeno denominado apólise, e se desintegra no intestino grosso, liberando os ovos juntamente com as fezes do hospedeiro definitivo.

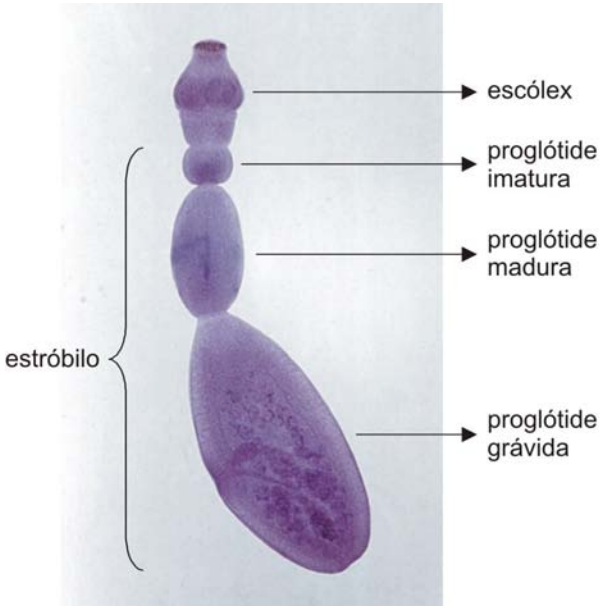

Figura 1.2. Adulto de *E. granulosus*. O parasito adulto é constituído de um escólex globular, onde encontram-se estruturas de adesão e fixação, e um estróbilo composto por três segmentos (proglótides). O primeiro segmento do estróbilo é a proglótide imatura, o segundo a proglótide madura e o último segmento é a proglótide grávida, que contém os ovos do parasito. Modificado de http://www.cmpt.ca/photo_album_parasitology/parasitology_photos_4_ces.htm.

Quando liberados no ambiente, os ovos do parasito são presumivelmente maduros e, portanto, possuem capacidade infectiva (Thompson, 1995). Morfologicamente, o ovo de *E. granulosus* é semelhante ao de outros membros da família Taeniidae, apresentando forma esférica a elipsóide, e medindo entre 30 e 60 μm e 22 e 44 μm, nos seus dois diâmetros. Os ovos contêm um único embrião ou oncosfera, a qual se encontra circundada por várias camadas e membranas, sendo o embrióforo a principal camada protetora, espessa e queratinizada, conferindo proteção física para a oncosfera. Essa estrutura confere grande resistência ao ovo de *E. granulosus*, o que permite que ele permaneça viável no ambiente mesmo em condições extremas de temperatura e umidade, sendo infectivo por ingestão a várias espécies de hospedeiros intermediários herbívoros ou onívoros, inclusive o homem (Thompson & McManus, 2001). Após a liberação no ambiente, os ovos são

dispersos pelo vento, pela água e por insetos, e o homem é infectado acidentalmente pela ingestão de ovos do parasito através das mãos, comida, água ou outros materiais contaminados (Eckert & Deplazes, 2004).

Quando ingerido pelo hospedeiro intermediário, o ovo sofre a ação de enzimas digestivas que desintegram o embrióforo, levando à liberação da oncosfera. A oncosfera ativada penetra na mucosa intestinal com o auxílio de secreções glandulares e é transportada passivamente via venosa ou linfática, atingindo diferentes órgãos do hospedeiro, principalmente pulmões e fígado, onde fica retida em vasos de pequeno calibre. Uma vez fixada, a oncosfera inicia um complexo processo de diferenciação, dando origem ao metacestódeo ou cisto hidático. O tropismo da oncosfera para diferentes órgãos em distintos hospedeiros intermediários é provavelmente influenciado por sinais do hospedeiro (Brehm ***et al***., 2006) e em humanos o metacestódeo de ***E. granulosus*** se desenvolve principalmente no fígado (>65%) e pulmões (25%), sendo menos frequente no baço, rins, coração, ossos, musculatura e sistema nervoso central (Jenkins ***et al***., 2005; Moro & Schantz, 2009). A maioria das infecções primárias em humanos consiste de um único cisto, porém, 20-40% dos indivíduos apresentam cistos múltiplos ou o envolvimento de múltiplos órgãos (Kammerer & Schantz, 1993).

O cisto hidático possui uma estrutura unilocular, subesférica, com uma cavidade interna preenchida por um fluido denominado líquido hidático (Fig. 1.3) (Thompson, 1995). A parede do cisto consiste de duas camadas: uma camada interna de tecido vivo, a camada germinativa, responsável pela proliferação assexual do parasito e pela síntese de uma camada externa acelular e de espessura variável, a camada laminar. O cisto hidático totalmente desenvolvido é tipicamente circundado por uma camada adventícia produzida

pelo hospedeiro, a qual é o produto da reação granulomatosa do hospedeiro em resposta à presença do parasito (Moro & Schantz, 2009).

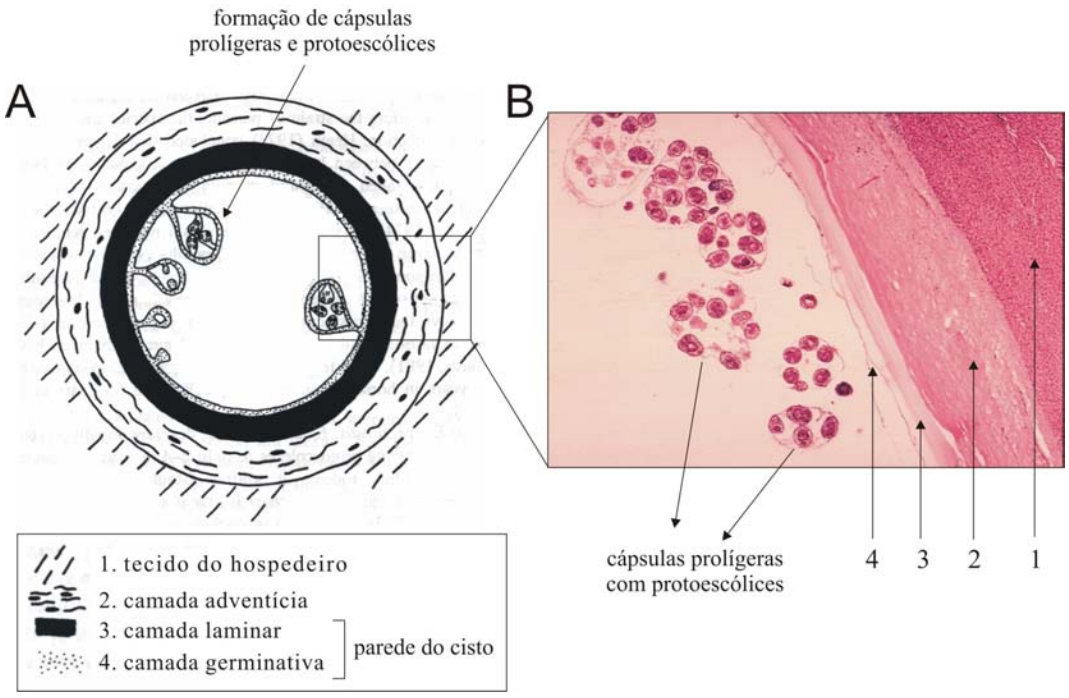

Figura 1.3. Estrutura do metacestódeo de *E. granulosus*. Desenho esquemático (A) e corte histológico (B) mostrando os diferentes componentes do estágio larval do parasito. Modificado de Thompson, 1995 (A) e http://phil.cdc.gov/phil/quicksearch.asp (B).

A camada germinativa é o tecido vivo que recobre toda a superfície do metacestódeo de *E. granulosus* e é formada por diferentes tipos celulares, incluindo células tegumentares, musculares, armazenadoras de glicogênio e células indiferenciadas. A parte mais externa da camada germinativa é o tegumento, o qual é similar em estrutura ao tegumento do verme adulto, consistindo de um sincício citoplasmático do qual se projetam microtríquias que penetram na camada laminar. O tegumento desempenha papel importante na sobrevivência do parasito através da captação de nutrientes, excreção e imunomodulação (Thompson, 1995; Ortona *et al.*, 2005; Camicia *et al.*, 2008). As células

indiferenciadas, chamadas ‘neoblastos’ no caso de platelmintos de vida-livre ou ‘células germinativas’ no caso dos organismos parasitas obrigatórios, são consideradas o único tipo celular mitoticamente ativo em platelmintos (Reuter & Kreshchenko, 2004; Spiliotis *et al.*, 2008). Essas células-tronco totipotentes são responsáveis pelo crescimento do metacestódeo de ***E. granulosus*** através da fusão com o tegumento, e pela reprodução assexual do parasito a partir da formação das cápsulas prolígeras, as quais se originam como pequenas massas nucleadas que proliferam em direção à cavidade do cisto (Mehlhorn *et al.*, 1983; Thompson, 1995). As cápsulas prolígeras aumentam, vacuolizam e podem ficar presas por um pedúnculo curto à camada germinativa ou soltar-se no interior da cavidade cística. No lúmen das cápsulas prolígeras, novos processos de proliferação assexual e diferenciação dão origem aos protoescólices.

Os protoescólices são formas pré-adultas nas quais as principais regiões presentes na porção anterior do verme adulto já se apresentam diferenciadas. Um único cisto de ***E. granulosus*** pode produzir milhares de protoescólices, porém, nem todos os metacestódeos são capazes de produzir protoescólices, sendo então chamados cistos estéreis ou inférteis. A infertilidade observada em alguns cistos hidáticos de ***E. granulosus*** parece estar relacionada com possíveis falhas nos mecanismos de reparação de danos ao DNA, o que acaba levando a apoptose (Paredes *et al.*, 2007; Cabrera *et al.*, 2008). Quando estão no interior do cisto, os protoescólices permanecem num estado dormente, até que a integridade do cisto seja afetada (Thompson & Lymbery, 1990). A partir daí, os protoescólices possuem capacidade dualística, diferenciando-se em vermes adultos e completando o ciclo vital do parasito quando ingeridos por um hospedeiro definitivo adequado, ou se desdiferenciando em novos cistos hidáticos, chamados cistos secundários,

quando liberados no interior do hospedeiro intermediário por ruptura do cisto (Thompson, 1995).

A camada laminar é uma matriz extracelular especializada presente exclusivamente nos parasitos do gênero *Echinococcus*. Ela é sintetizada e secretada pela camada germinativa, tendo como principal componente uma trama de fibrilas ricas em carboidratos, constituídas predominantemente de glicoproteínas do tipo mucina (Díaz *et al*., 2009). Além disso, a camada laminar contém também abundantes depósitos de mio-inositol-hexaquisfosfato (InsP_6) (Casaravilla *et al*., 2006). A camada laminar confere uma importante proteção ao parasito, pois além de constituir uma barreira mecânica evitando o contato direto com as células de defesa do hospedeiro, ela é também capaz de inibir a ativação do sistema complemento (Irigoín *et al.*, 2008; Ferreira *et al.*, 2000) e regular negativamente a produção de óxido nítrico por macrófagos, *in vitro* e *in vivo* (Steers *et al*., 2001). Embora seja uma barreira para as células inflamatórias do hospedeiro, a camada germinativa é altamente permeável a macromoléculas e a regulação do trânsito de moléculas entre parasito e hospedeiro parece ser função da camada germinativa (Coltorti & Varela-Díaz, 1974).

O líquido hidático contém os produtos de excreção/secreção (ES) da camada germinativa e dos protoescólices, juntamente com proteínas do hospedeiro que penetram no metacestódeo (Thompson, 1995). O trânsito de moléculas entre parasito e hospedeiro se dá por mecanismos não totalmente conhecidos, mas pode envolver difusão passiva, através de fissuras nas camadas do cisto, endocitose ou outros mecanismos de transporte mais específicos (Shapiro *et al.*, 1992; Thompson, 1995). As proteínas secretadas/excretadas pelo parasito são capazes de interagir com o sistema imune do hospedeiro, de forma a estimular e/ou modular a resposta imune (Siracusano *et al.*, 2008b). O líquido hidático

contém proteínas capazes de ativar o sistema complemento e induzir uma resposta celular e humoral (Zhang *et al*., 2003; Zhang & McManus, 2006; Siracusano *et al*., 2008b), além de possuir efeitos imunomoduladores em diversos tipos celulares, como macrófagos (Janssen *et al*., 1997), neutrófilos (Virginio *et al*., 2007), células dendríticas (Kanan & Chain, 2006; Riganò *et al*., 2007) e linfócitos T e B (Macintyre *et al*., 2001; Riganò *et al*., 2001, 2004).

O metacestódeo totalmente desenvolvido de ***E. granulosus*** é circundado pela camada adventícia, a qual é o produto da reação granulomatosa do hospedeiro iniciada nos primeiros estágios de diferenciação da oncosfera. Essa camada é caracterizada por uma massiva infiltração celular, que pode, posteriormente, se tornar fibrosada ou calcificada (Thompson, 1995; Zhang *et al*., 2003; Peng *et al*., 2006). A intensidade inicial dessa reação varia entre diferentes hospedeiros, e se muito intensa, pode causar a degeneração e eventual morte do parasito, enquanto em uma relação parasito-hospedeiro estável a reação inflamatória inicial se resolve, deixando uma espessa cápsula fibrosa (Thompson, 1995). O granuloma que envolve o cisto possui tipicamente três camadas: a mais interna é composta por células epitelióides e células gigantes multinucleadas arranjadas radialmente em relação ao cisto, em contato íntimo com a camada laminar; externamente a essa camada existe um chamado “infiltrado microcelular”, composto por linfócitos, um pequeno número de eosinófilos, e possivelmente monócitos; a camada mais externa do granuloma é colagenosa com a presença de fibroblastos, que quando da resolução da inflamação se torna a cápsula de tecido conjuntivo fibroso (Slais & Vanek, 1980; Marco *et al*., 2006; Peng *et al*., 2006). O parênquima do órgão em torno do granuloma é geralmente minimamente afetado.

1.3. A hidatidose cística

A hidatidose cística é uma zoonose causada pela infecção com o metacestódeo de *E. granulosus*, afetando animais domésticos e seres humanos. A hidatidose cística apresenta considerável impacto tanto para a agricultura como para a saúde pública, causando importantes consequências sócio-econômicas em áreas endêmicas (Carabin *et al*., 2005; Budke *et al*., 2006; Battelli, 2009; Moro & Schantz, 2009). A doença acarreta perdas estimadas em 1 milhão de DALYs (*disability adjusted life years*) devido a sua morbidade e prejuízos anuais da ordem de US$ 760 milhões em gastos no tratamento de pacientes humanos. Já as perdas associadas à redução na produção pecuária somam mais de US$ 2 bilhões em todo o mundo.

1.3.1. Distribuição geográfica, prevalência e epidemiologia

A distribuição de *E. granulosus* é considerada cosmopolita (Fig. 1.4), com apenas alguns poucos países, como a Islândia, Irlanda e a Groenlândia, sendo considerados livres de hidatidose cística humana autóctone (Budke *et al*., 2006). A maior prevalência da hidatidose cística em seres humanos e animais é encontrada em países de zonas temperadas, incluindo o sul da América do Sul, o litoral mediterrâneo, partes sul e central da antiga União Soviética, Ásia Central, China, Austrália e partes da África (Moro & Schantz, 2009). Além disso, a infecção por *E. granulosus* tem status re-emergente em certas áreas onde era considerada controlada, como, por exemplo, na Bulgária e no País de Gales (Romig *et al*., 2006). Na América do Sul, a infecção causada por *E. granulosus* tem caráter endêmico ou hiperendêmico no Cone Sul (incluindo o sul do Brasil) e na região andina (Arambulo III, 1997; Farias *et al*., 2004; Moro & Schantz, 2006, 2009; de la Rue, 2008; Moro *et al*., 2009; Zanini *et al*., 2009).

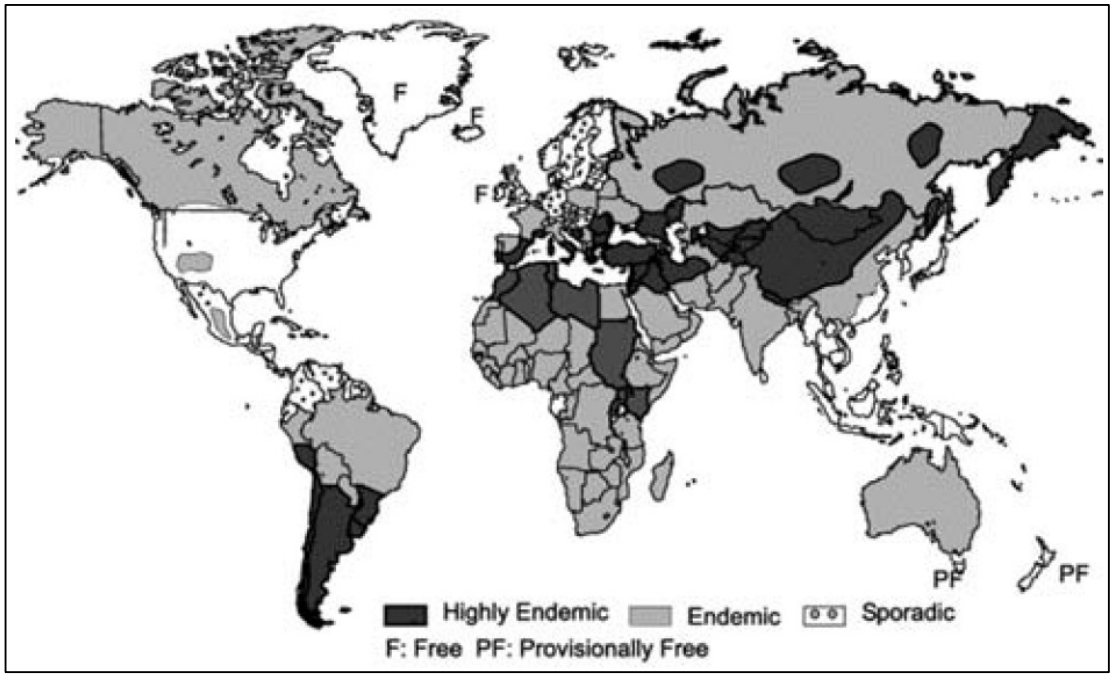

Figura 1.4. Distribuição mundial de *E. granulosus*. (Budke *et al*., 2006)

O Rio Grande do Sul é considerado zona altamente endêmica da hidatidose, constituindo um grave problema de saúde pública, especialmente nos municípios que fazem fronteira com Uruguai e Argentina (de la Rue, 2008). A alta incidência da doença nestas regiões é resultado da pecuária, atividade econômica expressiva nestes municípios, com a criação de bovinos e ovinos. Nestas localidades, o ciclo doméstico do parasito se perpetua principalmente pela alimentação dos cães com vísceras contaminadas, em abates clandestinos ou domésticos. Em Santana do Livramento, por exemplo, um estudo de 2004 revelou que o abate domiciliar ocorre em 83,3% das propriedades rurais, sendo que a prática de alimentar os cães com vísceras contaminadas é comum em 58,3% das propriedades e 47,4% dos cães estavam infectados com o verme adulto de *E. granulosus* (Farias *et al*., 2004).

No Rio Grande do Sul, a prevalência da hidatidose cística em bovinos vem se mantendo estável em torno dos 12% há mais de uma década, enquanto a prevalência da

doença em ovinos tem aumentado nos últimos 5 anos, subindo de 7 para 18% (de la Rue, 2008). Em seres humanos, a média anual de casos diagnosticados no Rio Grande do Sul variou entre 13 e 43, de 1995 a 2001, de acordo com o Programa Estadual de Controle da Hidatidose. Entretanto, sabe-se que a hidatidose cística humana apresenta prevalências muito mais elevadas em certas áreas, sendo subestimadas pelas estatísticas oficiais (Arambulo III, 1997; Torgerson *et al*., 2000; Farias *et al*., 2004; Carabin *et al*., 2005).

1.3.2. Manifestações clínicas

As manifestações clínicas da hidatidose são variáveis, pois dependem do local, tamanho e condição do cisto hidático (Pawlowski *et al*., 2001). Como o cisto possui crescimento lento e localização variável, a doença apresenta sintomas tardios e inespecíficos (McManus *et al*., 2003). Sendo assim, mesmo quando a infecção por ***E. granulosus*** é adquirida na infância, a maioria dos casos só se tornam sintomáticos e são diagnosticados nos pacientes já adultos (Moro & Schantz, 2009). Desta forma, o cisto hidático é frequentemente tolerado até que comprometa a função do órgão afetado e cause compressão em órgãos e tecidos vizinhos devido ao seu tamanho. Por outro lado, o rompimento eventual do cisto, com a súbita liberação do seu conteúdo, pode precipitar reações alérgicas, as quais podem variar em severidade desde média até uma anafilaxia fatal (McManus *et al*., 2003; Moro & Schantz, 2009). Além disso, as complicações relacionadas à liberação do conteúdo cístico incluem ainda infecções bacterianas ou fúngicas e a disseminação de protoescólices, a qual pode resultar em múltiplas infecções secundárias (cistos secundários) (Pawlowski *et al*., 2001; Moro & Schantz, 2009).

1.3.3. Diagnóstico e tratamento

O diagnóstico da hidatidose cística é baseado na identificação do cisto através de técnicas de imagem, principalmente ultra-sonografia, tomografia computadorizada, radiologia e ressonância magnética (Pawlowski *et al*., 2001; Teggi & DiVico, 2002). Entretanto, estes métodos são inespecíficos, uma vez que detectam apenas uma lesão cística, que pode corresponder ou não a um cisto hidático. Portanto, a confirmação do diagnóstico é realizada por testes imunodiagnósticos, mais sensíveis e específicos, predominantemente ELISA e *immunoblotting* (Eckert & Deplazes, 2004; Moro & Schantz, 2009). Estes testes, baseados na detecção de anticorpos específicos contra antígenos do parasito no soro de pacientes, são úteis não apenas no diagnóstico primário, mas também no monitoramento de pacientes após o tratamento cirúrgico ou farmacêutico (Pawlowski *et al*., 2001). O atual status do diagnóstico da hidatidose cística em humanos e animais foi recentemente revisado por Carmena *et al.* (2006), Zhang & McManus (2006) e Torgerson & Deplazes (2009).

Até a década de 1980, a cirurgia era a única opção para o tratamendo da hidatidose cística. Desde então, a quimioterapia com benzimidazoles e, mais recentemente, o tratamento com PAIR (Punção, Aspiração, Injeção e Re-aspiração), têm complementado ou até mesmo substituído a cirurgia como tratamento preferencial (Moro & Schantz, 2009). A remoção cirúrgica de cistos hidáticos intactos, quando possível, continua sendo a terapia mais eficaz no tratamento de indivíduos com hidatidose cística, levando imediatamente a cura completa da doença. Todavia, o procedimento é delicado, pois a remoção do cisto intacto é difícil, podendo ocorrer consequências adversas decorrentes da liberação do conteúdo cístico. Também a cirurgia pode ser impraticável em certos casos, principalmente em pacientes com múltiplos cistos em vários órgãos, ou em regiões ou órgãos inoperáveis,

e em pacientes com alto risco cirúrgico (Eckert & Deplazes, 2004). Para esses casos é indicada a PAIR, a qual tem demonstrado maior eficácia clínica e parasitológica, menores taxas de morbidade, mortalidade e recorrência da doença, e menor tempo de permanência no hospital, quando comparada com o tratamento cirúrgico (Smego *et al*., 2003).

A quimioterapia tem sido amplamente utilizada no tratamento da hidatidose cística (Pawlowski *et al.*, 2001), e aproximadamente um terço dos pacientes tratados com benzimidazoles (albendazol ou mebendazol) apresentaram cura da doença (desaparecimento completo e permanente dos cistos), com proporções ainda mais altas (30-50%) demonstrando significante redução no tamanho dos cistos e diminuição dos sintomas (El-On, 2003; Moro & Schantz, 2009). A quimioterapia é também muito utilizada em conjunto com a cirurgia e a PAIR, para evitar o surgimento de cistos secundários após a intervenção cirúrgica (Pawlowski *et al*., 2001).

1.3.4. Prevenção e controle

Os programas de controle da hidatidose são baseados predominantemente em medidas preventivas como educação sanitária, eliminação/proibição dos abates domésticos e a conscientização da população para mudança do hábito associado à prática rural de alimentar os cães com vísceras contaminadas, além do tratamento de cães com anti-helmínticos (Eckert *et al.*, 2000; Craig *et al*., 2007; Moro & Schantz, 2009). Também a vacinação de um ou de ambos hospedeiros pode diminuir, ou até mesmo eliminar, a infecção em humanos e animais domésticos (Zhang & McManus, 2006). Nesse sentido, o desenvolvimento recente da vacina recombinante EG95 representa um avanço promissor, sendo capaz de conferir altos níveis de proteção contra a infecção por *E. granulosus* (Lightowlers *et al*., 1999; Heath *et al*., 2003; Gauci *et al*., 2005). Vacinas contra o estágio

adulto do parasito foram também desenvolvidas recentemente, conferindo de 97-100% de proteção, afetando o crescimento do verme e a produção de ovos (Zhang *et al*., 2006; Petavy *et al*., 2008). Embora a vacinação de hospedeiros intermediários e definitivos seja uma realidade como opção de controle da hidatidose cística, o alto custo ainda impede a sua utilização em larga-escala (Zhang & McManus, 2006). Estudos baseados em modelos matemáticos propõem que a estratégia mais eficaz e de menor custo para o controle da hidatidose cística é uma combinação da vacinação do hospedeiro intermediário e do tratamento do hospedeiro definitivo com anti-helmínticos (Torgerson, 2006).

Contudo, apesar do desenvolvimento de ferramentas preventivas eficazes e de eventuais resultados positivos obtidos em programas locais de controle, não houve mudança acerca da distribuição global e do impacto na saúde pública humana e animal causado por *E. granulosus* (Jenkins *et al*., 2005; Craig *et al*., 2007; Moro & Schantz, 2009). A hidatidose cística continua sendo uma doença negligenciada, para a qual, na grande maioria das áreas, um controle efetivo não foi alcançado ou mesmo pretendido, apresentando status re-emergente em diversas áreas (Jenkins *et al*., 2005; Budke *et al*., 2006, 2009; Moro & Schantz, 2009).

1.4. Proteínas potencialmente envolvidas no estabelecimento da infecção crônica pelo metacestódeo de *E. granulosus*

Parasitos helmintos são capazes de estabelecer infecções crônicas, vivendo longos períodos de tempo dentro do corpo do hospedeiro. Para tanto, desenvolveram mecanismos altamente sofisticados de interação com o hospedeiro, os quais estão envolvidos na evasão da resposta imune, nutrição, metabolismo e desenvolvimento destes parasitos (Maizels & Yazdanbakhsh, 2003; Brindley *et al*., 2009). Sendo assim, o metacestódeo de *E.*

granulosus expressa e secreta nas suas estruturas císticas proteínas envolvidas nos mais diversos aspectos da relação parasito-hospedeiro, como invasão dos tecidos, incorporação de metabólitos do hospedeiro e evasão da resposta imune, de forma a promover o seu estabelecimento e manutenção dentro do corpo do hospedeiro (Rosenzvit *et al*., 2006; Siracusano *et al*., 2008b). As proteínas envolvidas nessas estratégias de sobrevivência não são totalmente conhecidas, pois os diferentes componentes do metacestódeo de *E. granulosus* (camada germinativa, protoescólices e líquido hidático) são ainda pouco caracterizados. Mas, assim como ocorre em outros helmintos, acredita-se que estes mecanismos de interação com o hospedeiro sejam mediados por proteínas presentes no tegumento e produtos de ES do parasito (Rosenzvit *et al*., 2006; Van Hellemond *et al*., 2006; Siracusano *et al*., 2008b; Hewitson *et al*., 2009).

Nesse sentido, destacam-se o antígeno B (AgB) e o antígeno 5 (Ag5), principais proteínas secretadas no líquido hidático de *E. granulosus* (Oriol *et al*., 1971; Lightowlers *et al*., 1989). A sua expressão e secreção em grande quantidade pelo metacestódeo de *E. granulosus* sugere um papel importante para essas proteínas na sobrevivência da forma larval patogênica do parasito (Lorenzo *et al*., 2003; Mamuti *et al*., 2006a). Além disso, estes antígenos são importantes biomarcadores para a hidatidose cística humana (Carmena *et al*., 2006; Siracusano *et al*., 2008a).

1.4.1. O antígeno B

O papel do AgB na biologia de *E. granulosus* não está totalmente elucidado, mas ele está envolvido em diferentes mecanismos de interação com o hospedeiro, relacionados com o estabelecimento e manutenção da infecção crônica (Mamuti *et al*., 2006a; Siracusano *et al*., 2008a, 2008b). O AgB é capaz de evadir a resposta imune inata e

adaptativa do hospedeiro através de efeitos moduladores sobre diversos tipos celulares. Experimentos *in vitro* demonstraram que o AgB inibe proteases do hospedeiro e a quimiotaxia de neutrófilos (Shepherd *et al*., 1991), sendo também capaz de diminuir a produção de H_2O_2 por essas células (Virginio *et al*., 2007). Adicionalmente, o AgB inibe o recrutamento de células polimorfonucleares e estimula a secreção de citocinas Th2 pelas PBMC (Riganò *et al*., 2001). Em células dendríticas, o AgB exerce efeitos em duas estratégias distintas: interferindo na diferenciação destas células a partir de monócitos precursores e modulando a secreção de citocinas pelas células dendríticas já diferenciadas, estimulando-as a polarizar linfócitos em uma resposta do tipo Th2 (Riganò *et al*., 2007). Uma resposta celular Th2 é menos eficiente na eliminação do parasito, e portanto o microambiente Th2 criado pelo AgB é extremamente favorável para o desenvolvimento da infecção crônica pelo metacestódeo de *E. granulosus* (Siracusano *et al*., 2008b).

O AgB é homólogo a um grupo recentemente identificado de proteínas ricas em alfa-hélice que se ligam a compostos hidrofóbicos (HLBPs, de *hydrophobic ligand binding proteins*), uma família de proteínas exclusiva de cestódeos (Saghir *et al*., 2000, 2001; Chemale *et al*., 2005). Essas proteínas desempenham papéis importantes para o desenvolvimento e sobrevivência destes parasitos através da captação de ácidos graxos do hospedeiro, uma vez que cestódeos são incapazes de sintetizar estes compostos *de novo* (Barrett, 1981). As HLBPs têm também função homeostática, mantendo a concentração de ácidos graxos livres abaixo de níveis tóxicos (Glatz & van der Vusse, 1996). A HLBP de *Taenia solium*, que assim como o AgB é uma proteína extracelular, estaria envolvida na captação e transporte de lipídeos da reação granulomatosa do hospedeiro que circunda o parasito para dentro do cisto (Lee *et al*., 2007). Chemale *et al*. (2005) demonstraram que o AgB possui diferentes propriedades de ligação a compostos hidrofóbicos quando

comparado com HLBPs de outros cestódeos, não apresentando capacidade de trocar ácidos graxos, indicando que o AgB não está relacionado com o metabolismo e transporte de moléculas hidrofóbicas. O AgB poderia, portanto, estar envolvido em diferentes papéis para a sobrevivência do parasito, como homeostase ou detoxificação, sequestrando compostos tóxicos (compostos xenobióticos, como anti-helmínticos, por exemplo) presentes no líquido hidático.

O AgB é altamente imunogênico em infecções humanas, sendo amplamente proposto para o imunodiagnóstico da hidatidose humana, devido a sua alta sensibilidade e especificidade (Virginio *et al.*, 2003; Lorenzo *et al.*, 2005a). Apesar de resultados variáveis, o AgB é atualmente considerado o antígeno mais específico de *E. granulosus* para utilização em ensaios imunodiagnósticos (Carmena *et al.*, 2006; Mamuti *et al.*, 2006a; Siracusano *et al.*, 2008a). Portanto, nos últimos anos, muitas pesquisas têm sido realizadas para caracterizar proteínas recombinantes e peptídeos sintéticos derivados do AgB que possam ser utilizadas no imunodiagnóstico da hidatidose cística (González-Sapienza *et al.*, 2000; Virginio *et al.*, 2003; Lorenzo *et al.*, 2005a; Carmena *et al.*, 2006).

Estruturalmente, o AgB é uma proteína oligomérica de 120-160 kDa, formada por subunidades de 8 kDa (Oriol *et al.*, 1971; Lightowlers *et al.*, 1989; Monteiro *et al.*, 2007). Essas subunidades possuem comportamento altamente agregativo, formado populações com diferentes estados de agregação, incluindo agregados de alta massa molecular (Oriol *et al.*, 1971). As subunidades do AgB são codificadas por uma família multigênica, incluindo pelo menos 5 genes (*EgAgB8/1-EgAgB8/5*), os quais são altamente polimórficos (Shepherd *et al.*, 1991; Frosch *et al.*, 1994; Fernández *et al.*, 1996; Chemale *et al.*, 2001; Arend *et al.*, 2004; Haag *et al.*, 2004; Kamenetzky *et al.*, 2005; Mamuti *et al.*, 2007). As sequências genômicas dos genes que codificam subunidades do AgB possuem a mesma

estrutura característica, apresentando dois éxons separados por um pequeno íntron (Ferreira *et al.*, 2004). O éxon 1 codifica uma região hidrofóbica N-terminal, seguida de potenciais sítios de clivagem para proteases, indicando um potencial peptídeo sinal responsável pela secreção do peptídeo maduro codificado pelo éxon 2. Haag *et al.* (2004) propuseram que os genes que codificam subunidades do AgB podem representar uma família de genes de contingência, com expressão diferencial dentro e/ou entre indivíduos. Estudos recentes demonstraram que os genes que codificam o AgB de *E. granulosus* apresentam expressão diferencial entre os tecidos do metacestódeo, sendo *EgAgB8/1* e *EgAgB8/3* os genes mais expressos em camada germinativa e protoescólices, respectivamente, enquanto o gene *EgAgB8/2* é o menos expresso em ambos (Arend *et al.*, submetido para publicação). Em *E. multilocularis*, os ortólogos dos genes que codificam o AgB (*EmAgB8/1-EmAgB8/5*) são regulados ao longo do desenvolvimento, tendo expressão diferencial em vesículas, protoescólices e vermes adultos imaturos (Mamuti *et al.*, 2006b). Uma vez que o grau de identidade em nível de aminoácidos (considerando os polipeptídeos maduros putativos) entre as cinco subfamílias que codificam o AgB é de 25 a 69%, qualquer variação na composição de subunidades do AgB deve ter efeito nas relações adaptativas entre parasito e hospedeiro, bem como nos resultados dos métodos imunodiagnósticos baseados no AgB (Monteiro *et al*., 2008).

Em SDS-PAGE, o AgB purificado de líquido hidático se dissocia em multímeros regularmente espaçados em 8 kDa (8, 16, 24 e 32 kDa), os quais correspondem a monômeros, dímeros, trímeros e tetrâmeros de subunidades de 8 kDa (Lightowlers *et al*., 1989). O seqüenciamento de peptídeos trípticos derivados das bandas do AgB resolvidas em SDS-PAGE mostrou que diferentes subunidades fazem parte dos multímeros de 8, 16 e 24 kDa (González *et al*., 1996). Estes resultados demonstraram que o AgB é formado por

diferentes subunidades de 8 kDa, as quais estão envolvidas na formação da estrutura oligomérica da proteína e dos multímeros observados em condições desnaturantes. Porém, não foi possível determinar se estes múltimeros possuem natureza homo ou hetero-oligomérica, nem a força envolvida na associação covalente entre as subunidades de 8 kDa. Recentemente, nosso grupo demonstrou que subunidades recombinantes do AgB (AgB8/1, AgB8/2 e AgB8/3) se auto-associam em homo-oligômeros com propriedades semelhantes às do AgB purificado de líquido hidático, validando-os como bons modelos para o estudo estrutural do AgB (Monteiro *et al*., 2007). Além disso, devido a sua composição de subunidades conhecida e homogênea, os homo-oligômeros recombinantes podem superar alguns problemas associados à caracterização do AgB produzido pelo parasito, o qual possui composição de subunidades e conteúdo de modificações pós-traducionais desconhecidos, bem como auxiliar no estudo de propriedades estruturais e funcionais de diferentes subunidades do AgB (Monteiro *et al*., 2008).

1.4.2. O antígeno 5

Embora avanços recentes tenham sido feitos na caracterização molecular do Ag5, sua relevância biológica para o metacestódeo de ***E. granulosus*** é ainda desconhecida. O Ag5 é uma glicoproteína de 67 kDa, composta por duas subunidades de 38 e 22 kDa (Lorenzo *et al*., 2003). O Ag5 é codificado como uma única cadeia polipeptídica que é posteriormente processada nas subunidades de 22 e 38 kDa, as quais encontram-se ligadas por uma única ponte dissulfeto. O sequenciamento N-terminal da subunidade de 38 kDa do Ag5 revelou a presença de resíduos alternativos em algumas posições, sugerindo que a proteína está presente em diferentes isoformas (Zhang & McManus, 1996). A subunidade de 38 kDa apresenta alta similaridade com serino proteases da família da tripsina, mas o

resíduo de serina catalítico está substituído por uma treonina e nenhuma atividade foi demonstrada até o momento (Lorenzo *et al*., 2003). A subunidade menor do Ag5 contém um motivo de ligação a glicosaminoglicanos altamente conservado, o qual pode proporcionar a interação do Ag5 com a superfície celular e a matriz extracelular, confinando o antígeno no tecido do hospedeiro que circunda o cisto. O Ag5 contém também grupos fosforilcolina (Lightowlers *et al*., 1989; Lorenzo *et al*., 2005b), e estudos de glicoproteínas contendo fosforilcolina de outros parasitos têm demonstrado que esses epitopos podem apresentar diversas atividades imunomoduladoras (Harnett & Harnett, 2001; Grabitzki & Lochnit, 2009).

O Ag5 é altamente imunogênico em infecções humanas, sendo considerado desde os primeiros estudos como um dos mais relevantes antígenos de *E. granulosus* (Lorenzo *et al*., 2005a). Embora alguns estudos revelem problemas de sensibilidade e especificidade associados ao Ag5 (Di Felice *et al*., 1986; Lightowlers *et al*., 1989; Barbieri *et al*., 1998), esta proteína é ainda amplamente utilizada na rotina de diagnóstico sorológico da hidatidose, particularmente como uma técnica confirmatória, através da identificação de uma linha de precipitação (arco 5) em ensaios de imunoeletroforese (Carmena *et al*., 2006). Lorenzo *et al*. (2005b), demonstraram que a antigenicidade do Ag5 é determinada por modificações pós-traducionais presentes na proteína, sendo os epitopos sacarídicos imunodominantes. Além disso, os epitopos fosforilcolina presentes no Ag5 possuem um papel considerável na reatividade cruzada com soros de indivíduos com outras parasitoses. Esse conjunto de dados demonstra que o Ag5 é menos útil que o AgB para fins diagnósticos (Carmena *et al*., 2006).

1.4.3. Outras proteínas expressas e secretadas pelo metacestódeo de *E. granulosus*

A caracterização molecular do metacestódeo de *E. granulosus* é ainda limitada, sendo o seu estudo historicamente focado na caracterização de proteínas individuais, principalmente antígenos (Brehm *et al*., 2006; Siracusano *et al*., 2008b). O estudo dos produtos de ES do parasito, por exemplo, é limitado a caracterizações imunológicas e enzimáticas (Carmena *et al*., 2004, 2005). Recentemente, esforços significativos nas áreas de genômica e transcriptômica têm sido realizados na tentativa de caracterizar o repertório de genes expressos pelo metacestódeo de *E. granulosus*, ampliando o conhecimento sobre a biologia da fase larval patogênica do parasito, bem como da suas interações com as espécies hospedeiras. O projeto de sequenciamento do genoma de *E. granulosus* está em andamento, enquanto o de sequenciamento do genoma da espécie relacionada *E. multilocularis* está em fase de montagem (Sanger Institute: http://www.sanger.ac.uk/Projects/Echinococcus/). Além disso, cerca de 20.000 ESTs foram geradas a partir do sequenciamento de bibliotecas de cDNA de diferentes componentes dos metacestódeos de *E. granulosus* e *E. multilocularis* (Fernández *et al*., 2002; Sanger Institute: http://www.sanger.ac.uk/Projects/Echinococcus/; LophDB: http://xyala.cap.ed.ac.uk/NeglectedGenomes/Lopho/LophDB.php). Rosenzvit *et al*. (2006), construíram bibliotecas de cDNA utilizando a técnica de *signal sequence trap* (SST), identificando especificamente proteínas de membrana e secretadas por *E. granulosus*.

A disponibilização de um maior número de seqüências gênicas de espécies do gênero *Echinococcus* possibilita a utilização de técnicas proteômicas para o estudo dos parasitos deste gênero. Porém, a identificação das proteínas expressas nas formas larvais patogênicas desses parasitos está ainda restrita a análises proteômicas preliminares de

protoescólices de *E. granulosus* e *E. multilocularis* (Chemale *et al.*, 2003; Wang *et al.*, 2009).

1.4.4. Estudos proteômicos de parasitos

A análise proteômica, pela sua alta resolução e sensibilidade, tem grande potencial para a identificação de proteínas parasitárias, principalmente de fluidos biológicos, tipicamente limitados em quantidade e concentração de proteínas. Por isso, abordagens proteômicas vêm sendo utilizadas com sucesso na identificação de proteínas somáticas e de ES de diferentes parasitos (Knudsen *et al.*, 2005; Braschi & Wilson, 2006; Guillou *et al.*, 2007; Pérez-Sánchez *et al.*, 2008; Bennuru *et al.*, 2009). Essas análises têm revelado a diversidade de proteínas expressas e secretadas por diferentes espécies, formas de vida ou sexo de parasitos helmintos, auxiliando na elucidação dos mecanismos moleculares utilizados por cada um deles na interação com as espécies hospedeiras e na manutenção da infecção.

Diferentes técnicas proteômicas têm sido utilizadas na investigação de diversos aspectos da biologia, bioquímica e fisiologia de espécies parasitas, como a invasão dos tecidos do hospedeiro, nutrição e imunomodulação (Knudsen *et al.*, 2005; Braschi & Wilson, 2006; Liu *et al.*, 2009; Mulvenna *et al.*, 2009; Santivañez *et al.*, 2010). Além disso, análises proteômicas de amostras obtidas durante a infecção podem fornecer informações importantes sobre a interação parasito-hospedeiro, destacando os mecanismos de defesa empregados pelo hospedeiro contra o parasito e as estratégias utilizadas pelo parasito para promover a sua sobrevivência dentro do corpo do hospedeiro (Liu *et al.*, 2007; Morphew *et al.*, 2007; Hansell *et al.*, 2008). Também estudos imunoproteômicos, utilizando técnicas proteômicas associadas a análises imunológicas, têm auxiliado na

identificação de proteínas antigênicas, com potencial imunodiagnóstico e/ou vacinal contra diferentes parasitos (Sotillo *et al.*, 2008; Ju *et al*., 2009).

Técnicas como eletroforese bidimensional (2-DE) e LC-MS/MS podem também ser utilizadas na análise e comparação de diferentes formas de vida parasitárias, identificando diferenças na expressão e modificação pós-traducional de proteínas durante o desenvolvimento de diferentes parasitos (Lasonder *et al.*, 2008; Rosenzweig *et al.*, 2008; Lal *et al.*, 2009). Da mesma forma, diferenças na expressão de proteínas em respostas a estímulos, como estresse ou tratamento com drogas, podem ser identificados utilizando-se abordagens proteômicas (Prieto *et al*., 2008; Cooper & Carucci, 2004).

Desta forma, a proteômica tem se mostrado uma abordagem importante na identificação de proteínas envolvidas em diferentes interações físicas e bioquímicas entre parasito e hospedeiro, destacando proteínas essenciais para a sobrevivência e manutenção de diferentes parasitos. Sendo assim, essas análises podem auxiliar na descoberta de proteínas com potencial para o desenvolvimento de novas drogas, vacinas e abordagens diagnósticas para utilização no controle, tratamento e prevenção de diferentes parasitoses (Barrett *et al*., 2000; Biron *et al*., 2005; De Marco & Verjovski-Almeida, 2009).

2. Justificativas e objetivos

E. granulosus, assim como outros helmintos, é capaz de manter a infecção por longos períodos apesar das defesas apresentadas pelo seu hospedeiro. Os mecanismos de interação parasito-hospedeiro responsáveis pelo estabelecimento e manutenção da infecção crônica pelo metacestódeo de *E. granulosus* não são totalmente compreendidos, principalmente do ponto de vista molecular, mas eles são em grande parte mediados por moléculas expostas e secretadas pelo cisto hidático na sua interface com o hospedeiro. Sendo assim, a identificação de proteínas expressas e secretadas por parasito e hospedeiro durante a infecção pelo metacestódeo de *E. granulosus* pode levar a um melhor entendimento da resposta montada pelo hospedeiro contra o parasito, bem como dos mecanismos moleculares utilizados pelo parasito para evadir essa resposta e promover o estabelecimento e desenvolvimento de sua fase larval patogênica. Além disso, a caracterização de proteínas expressas durante a infecção leva a um aumento no número de proteínas candidatas para abordagens imunodiagnósticas e terapêuticas para a hidatidose cística.

Da mesma forma, o estudo de proteínas envolvidas em diferentes interações com o hospedeiro, como o AgB, pode auxiliar sobremaneira no melhor entendimento da biologia de *E. granulosus*. O estudo da estrutura e composição de subunidades do AgB possui tanto implicações funcionais, com relação aos papéis desempenhados por essa proteína nas relações adaptativas entre parasito e hospedeiro, quanto práticas, do ponto de vista da sua utilização em estratégias de diagnóstico e tratamento. O melhor entendimento da estrutura e mecanismo de oligomerização do AgB podem auxiliar no futuro desenvolvimento de drogas capazes de inibir e/ou interferir com as interações entre subunidades, levando à

perturbação da estabilidade do oligômero e interferindo com as funções biológicas de *E. granulosus* mediadas pelo AgB.

Sendo assim, este trabalho tem como objetivos:

1. Identificar as proteínas do parasito e do hospedeiro presentes nos diferentes componentes do metacestódeo de *E. granulosus* durante a infecção do hospedeiro intermediário bovino;
2. Estabelecer um mapa proteômico de referência de protoescólices de *E. granulosus* baseado em 2-DE;
3. Identificar proteínas antigênicas em extratos do parasito;
4. Estudar a estrutura de oligômeros e agregados de alta massa molecular do AgB de *E. granulosus* utilizando subunidades recombinantes como modelo;
5. Estabelecer a composição de subunidades do AgB secretado no líquido hidático do metacestódeo de *E. granulosus*;

CAPÍTULO I

Análise proteômica do metacestódeo de *Echinococcus granulosus* durante a infecção do seu hospedeiro intermediário

Artigo publicado na revista Proteomics

Proteomic analysis of the *Echinococcus granulosus* metacestode during infection of its intermediate host

Monteiro, K. M., de Carvalho, M. O., Zaha, A., Ferreira, H. B.

Proteomics, 10(10): 1985-1999, 2010.

Proteomics 2010, *10*, 1985–1999 DOI 10.1002/pmic.200900506

RESEARCH ARTICLE

Proteomic analysis of the *Echinococcus granulosus* metacestode during infection of its intermediate host

Karina M. Monteiro[1,2], *Marcos O. de Carvalho*[1*], *Arnaldo Zaha*[1,2,3] *and Henrique B. Ferreira*[1,2,3]

[1] Laboratório de Biologia Molecular de Cestódeos e Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil
[2] Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil
[3] Departamento de Biologia Molecular e Biotecnologia, Instituto de Biociências, UFRGS, Porto Alegre, RS, Brazil

Cystic hydatid disease (CHD) is caused by infection with the *Echinococcus granulosus* metacestode and affects both humans and livestock. In this work, we performed a proteomic analysis of the *E. granulosus* metacestode during infection of its intermediate bovine host. Parasite proteins were identified in different metacestode components (94 from protoscolex, 25 from germinal layer and 20 from hydatid cyst fluid), along with host proteins (58) that permeate into the hydatid cyst, providing new insights into host-parasite interplay. *E. granulosus* and platyhelminth EST data allowed successful identification of proteins potentially involved in downregulation of host defenses, highlighting possible evasion mechanisms adopted by the parasite to establish infection. Several intracellular proteins were found in hydatid cyst fluid, revealing a set of newly identified proteins that were previously thought to be inaccessible for inducing or modulating the host immune response. Host proteins identified in association with the hydatid cyst suggest that the parasite may bind/adsorb host molecules with nutritional and/or immune evasion purposes, masking surface antigens or inhibiting important effector molecules of host immunity, such as complement components and calgranulin. Overall, our results provide valuable information on parasite survival strategies in the adverse host environment and on the molecular mechanisms underpinning CHD immunopathology.

Received: July 14, 2009
Revised: January 19, 2010
Accepted: February 16, 2010

Keywords:
Animal proteomics / Cestode / Host-parasite interaction / Immune evasion / Mass spectrometry

Correspondence: Dr. Henrique B. Ferreira, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Caixa Postal 15005, Porto Alegre, 91501-970, RS, Brazil
E-mail: henrique@cbiot.ufrgs.br
Fax: +55-51-33087309.

Abbreviations: AgB, antigen B; **Ag5,** antigen 5; **CHD,** cystic hydatid disease; **DBI,** Diazepam-binding inhibitor; **EgAFFP,** *E. granulosus* actin filament-fragmenting protein; **EgcMDH,** *E. granulosus* cytosolic malate dehydrogenase; **EgTeg,** *E. granulosus* tegumental protein; **EgTPx,** *E. granulosus* thioredoxin peroxidase; **ES,** excretory/secretory; **GL,** germinal layer; **HCF,** hydatid cyst fluid; **LDL,** low-density lipoprotein; **MMP-9,** matrix metalloproteinase-9; **MMP-12,** matrix metalloproteinase-12; **MVP,** major vault protein; **PSC,** protoscolex, protoscoleces; **TRx,** thioredoxin

1 Introduction

Cystic hydatid disease (CHD), caused by infection with the larval stage, or metacestode, of the tapeworm *Echinococcus granulosus*, is a worldwide zoonosis of medical and economic impact, with an emerging/re-emerging status in several countries [1, 2]. The *E. granulosus* metacestode (Fig. 1) develops into a large fluid-filled hydatid cyst in the internal organs (mostly liver and lungs) of livestock or human intermediate hosts. The innermost layer of the cyst wall (germinal layer, GL) is responsible for both pre-adult

*Current address: Programa de Pós-Graduação em Genética e Biologia Molecular, Instituto de Biociências, UFRGS, Porto Alegre, RS, Brazil.

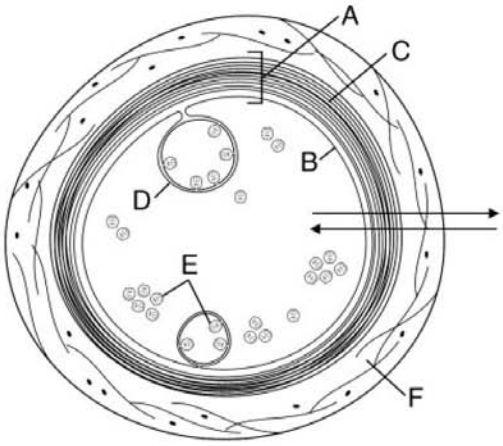

Figure 1. Diagrammatic representation of the *E. granulosus* metacestode. The parasite bladder-like larva is bounded by the hydatid cyst wall (A), which comprises two layers: an innermost germinal layer (B) of parasite live tissue and an outer thick acellular laminated layer (C), which is synthesized by the germinal layer and protects the cyst from direct attack of host cells. The germinal layer is also responsible for the formation of brood capsules (D), which proliferate towards the cyst cavity and give rise to protoscoleces (E), also found free in the hydatid cyst fluid. The hydatid fluid fills the cyst and contains the ES products from the germinal layer and protoscoleces, along with host proteins. The fully developed hydatid cyst is typically surrounded by a host-produced adventitial layer (F), which is the product of a host granulomatous reaction. The two cyst wall layers are, in different extents, permeable to macromolecules, allowing the host-parasite molecular cross-talk (arrows).

(protoscolex, PSC) formation and synthesis of an outer acellular carbohydrate-rich laminated layer. The hydatid cyst is surrounded externally by the adventitial layer, a fibrous capsule of host origin, resultant from the initial inflammatory reaction. PSC mature into adult worms when ingested by a definitive canid host, but they are also able to dedifferentiate into new, fully developed cysts when released into the intermediate host body cavity upon cyst rupture [3].

Parasite helminths, as *E. granulosus*, must be able to maintain infection for long time periods despite the defense mechanisms displayed by their hosts. They have therefore developed a wide range of highly elaborate survival strategies, such as immunomodulation, antioxidant defenses, and resistance to host proteolytic enzymes [4, 5]. Most of the mechanisms underpinning downregulation of host responses are not fully understood, especially at the molecular level, but they are likely mediated by proteins found in the parasites' tegument and excretory-secretory (ES) products [6, 7]. In *E. granulosus*, the outermost part of the GL is a syncytial tegument, which surrounds the entire metacestode and plays an important role in parasite survival by nutrient uptake, excretion and immune evasion [3, 4, 8]. The hydatid cyst fluid (HCF) contains the *E. granulosus* metacestode ES products, which, despite being able to induce strong host cellular and humoral responses, mediate evasion mechanisms through cytotoxic and modulatory effects on host immune cells [3, 4].

Advances in the sensitivity and resolution of proteomic technology enable the exploitation of parasite material, mainly biological fluids, whose analysis is typically limited by the amount of material and the low representativity of protein content. Proteomic approaches have been successfully used for the identification of helminth somatic and ES proteins [9–11]. *In vivo* proteomic analyses performed with parasite material sampled during infection allow real-time analyses and have provided valuable insights into host-parasite interplay and the immunopathology of parasitic diseases [12, 13]. Furthermore, the identification of a wide range of proteins expressed during infection conditions are expected to improve the repertoire of candidate proteins for use in immunodiagnosis, therapy, and vaccination approaches.

Though cestode species are etiological agents of major parasitic diseases in both humans and livestock [14], proteomic analyses of such species have been restricted to preliminary proteomic surveys of *E. granulosus* and *Echinococcus multilocularis* PSC [15, 16]. With the recent availability of extensive *E. granulosus* EST data [17], we have extended the proteomic analysis to all primary metacestode components, aiming to identify parasite and host proteins expressed during infection of the bovine intermediate host. The repertoire of identified proteins provides insight into parasite survival mechanisms as well as into the nature of host immune responses mounted against *E. granulosus* infection.

2 Materials and methods

2.1 Parasite material

E. granulosus hydatid cysts were obtained from the livers and lungs of cattle (2–3 years old animals) slaughtered at abattoirs in Rio Grande do Sul, Brazil. HCF was aseptically aspirated from individual fertile cysts, clarified by centrifugation at $10000 \times g$ for 15 min at 4°C, and concentrated and dialyzed against PBS using an Amicon Ultra-15 5000 MWCO centrifugal filter device (Millipore, USA). PSC were collected by aspiration, washed extensively in PBS, and then washed with 40 mM Tris to remove salts. Cyst walls (GL and the underlying laminated layer) were carefully separated from surrounding host tissue with forceps and washed with PBS until all PSC were removed. GLs were then separated from the laminated layer using a tissue scraper and washed as described for PSC. Parasite tissues were homogenized separately in a glass Dounce tissue grinder, and homogenates were centrifuged at

20 000 × *g* for 30 min at 4°C to separate soluble and insoluble protein fractions. Soluble proteins were quantified using a Qubit™ quantitation fluorometer and Quant-it™ reagents (Invitrogen, USA). Individual HCF and GL samples were analyzed by SDS-PAGE to select those with the lowest content of host abundant serum proteins (albumin and immunoglobulins) for downstream applications. PSC samples analyzed were pools from liver and lung cysts, while all GL samples were from liver cysts, which were individually processed and analyzed. HCF samples were either individually analyzed (lung cysts) by LC-MS/MS or pooled for parasite protein immunopurification (see section 2.2).

2.2 Purification of metacestode excretory/secretory proteins from the hydatid cyst fluid

Parasite ES proteins present in the HCF were immunopurified using anti-PSC rabbit polyclonal antibodies immobilized on a cyanogen bromide-activated Sepharose 4B matrix (GE Healthcare, UK). Control immunopurifications were performed using a column with rabbit non-related antibodies. HCF from liver and lung cysts were collected as described in section 2.1 and pooled in order to obtain a minimum of 250 ml of sample (~50 ml of HCF from each individual cyst) with relatively low contamination with host abundant serum proteins, as monitored by SDS-PAGE. Each HCF pool was passed through the column three times. After extensive washing with PBS, bound proteins were eluted with 100 mM glycine, pH 2.7, dialyzed against PBS, and concentrated ten-fold with Amicon Ultra-4 5000 MWCO (Millipore).

2.3 2-DE, immunoblot and image software analysis

PSC protein samples (2 mg) were precipitated overnight at −20°C with two volumes of ice cold 20% (w/v) trichloroacetic acid/acetone. Protein precipitates were recovered by centrifugation (10 min at 14 000 rpm) and washed five times with ice cold acetone. The pellet was air-dried and solubilized into sample buffer containing 7 M urea, 2 M thio-urea, 4% (w/v) CHAPS, 1% (w/v) DTT, and 0.2% (v/v) ampholytes (pH 3–10) (Bio-Rad, USA). The linear 17-cm IPG strips (pH 3–10 and 4–7, Bio-Rad) were passively rehydrated with the samples for 16 h, and IEF was run for a total of 60 000 Vh using a Protean IEF cell (Bio-Rad). Following IEF, the strips were reduced in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, and 0.375 M Tris, pH 8.8) containing 1% DTT over 15 min and alkylated in equilibration buffer containing 4% iodoacetamide for an additional 15 min. In the second dimension, the strips were run onto 20 × 20-cm 12% SDS-PAGE gels using a Protean II xi 2D Cell (Bio-Rad). Gels were stained with Coomassie blue and scanned with an ImageScanner (GE Healthcare). Two analytical replicates were produced for each of two independent biological samples.

For immunoblot analysis, proteins were electrotransferred from 2-DE gels to PVDF membranes (Hybond™-P, GE Healthcare) at 20 V for 16–18 h. Membranes were blocked for 1 h with 5% nonfat dry milk in PBS-T (PBS containing 0.1% v/v Tween-20) and then incubated with a pool of six sera from CHD patients (confirmed by surgery) at a 1:1000 dilution for 1 h and 30 min. After three washes with PBS-T, blots were incubated with HRP-labelled anti-human IgG (Sigma-Aldrich, USA) at a 1:10 000 dilution for 1 h. The 2-DE blots were revealed with ECL detection reagent (GE Healthcare) and imaged using the VersaDoc imaging system (Bio-Rad). A pool of six sera from healthy individuals was used as negative control.

The 2-DE gel and blot images were analyzed using PDQuest 8.0 software (Bio-Rad) for spot detection and matching. Antigenic spots of 2-DE blots were identified based on matches with a 2-DE proteomic map.

2.4 Mass spectrometry analyses

2.4.1 MALDI-Q-TOF MS/MS

Protein spots were manually excised, digested with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, USA), and eluted from the gel as previously described [18]. Peptides were resuspended in 10 µl of Milli-Q water, and 1.5 µl of the resuspension was mixed with an equal volume of CHCA matrix (5 mg/ml in 50% ACN/0.1% TFA aqueous solution), spotted onto a 96-well target plate, and allowed to air-dry at room temperature. MALDI-Q-TOF MS/MS analyses were performed on a Waters Micromass Q-TOF Premier mass spectrometer equipped with a standard MALDI source (Waters, UK). MS spectra were recorded from 800 to 3000 m/z range, and the automatic scan rate was 1 s, with an interscan delay of 0.1 s. In data dependent acquisition (DDA) mode, MS/MS spectra for the five most intense ions were automatically acquired when the peak intensity rose above a threshold of 30 counts. The CID collision energy was automatically set according to the m/z ratio of the precursor ion. Sequence information from the MS/MS data was processed using Mascot Distiller 2.2.1 to form a peak list (.mgf file).

2.4.2 LC-ESI-Q-TOF MS/MS

Protein samples were diluted in denaturing buffer (25 mM NH_4HCO_3/8 M urea, pH 8.9), reduced by adding DTT (0.02 µg/µg protein), and carboxyamidomethylated with iodoacetamide (0.1 µg/µg protein). Samples were then diluted to 1 M urea with 25 mM NH_4HCO_3 (pH 8.9), and trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) was added at a ratio of 0.01 µg/µg protein. After digestion for

4 h at 37°C, an additional aliquot of enzyme was added, and samples were further incubated for 16–20 h at 37°C. The resulting peptides were desalted using OASIS® HLB Cartridge (Waters, USA) and analyzed by on-line liquid chromatography/mass spectrometry (LC-MS/MS) using a Waters nanoACQUITY UPLC system coupled to a Waters Micromass Q-TOF Micro or Q-TOF Ultima API mass spectrometer (Waters MS Technologies, UK). The peptides were eluted from the reverse-phase column toward the mass spectrometer at a flow rate of 200 nl/min with a 10–50% water/ACN 0.1% formic acid linear gradient over 90 min. The MS survey scan was set to 1 s (0.1 s interscan delay) and recorded from 200 to 2000 m/z. MS/MS scans were acquired from 50 to 2000 m/z, and scan and interscan rates were set as for MS. The samples were run in DDA mode where each full MS scan was followed by three consecutive MS/MS scans. For each survey scan, the three most intense multiple charged ions over a threshold of 8 counts were selected for MS/MS analysis. The collision energies for peptide fragmentation were set using the charge state recognition files for +2, +3, and +4 peptide ions provided by MassLynx (Waters). MS/MS raw data were processed using ProteinLynx Global Server 2.0 software (Waters), and peak lists were exported in the micromass (.pkl) format. For each protein sample, at least two independent LC-MS/MS runs were performed.

2.5 Database searching and bioinformatics

All MS/MS data were searched against databases using MASCOT software 2.0 (http://www.matrixscience.com, Matrix Science) with the following parameters: maximum of one missed cleavage, fixed carbamidomethyl alkylation of cysteines, variable oxidation of methionine, and 0.1 mass unit tolerance on parent and fragment ions. The significance threshold was set at $p<0.05$, and only peptides with individual ion scores above this significance threshold were considered for protein identification. The MS/MS spectra of protein identifications based on a single peptide and on borderline scores were manually inspected for acceptance. In addition, a decoy database search was used to estimate false discovery rates for PSC analyses (larger data sets), resulting in average probabilities of 3,01% and 2,03% in searches against *E. granulosus* and platyhelminth decoy sequences, respectively.

Searches were performed in local *E. granulosus* and platyhelminthes EST databases, in a local *Bos taurus* database and in the public NCBInr database. The local *E. granulosus* database was constructed using ESTs grouped in the LophDB public database (http://www.nematodes.org/NeglectedGenomes/Lopho/LophDB.php), which contains 2799 clusters (2733 EST clusters and 66 cDNA clusters) corresponding to *E. granulosus* genes expressed in PSC and/or GL [17]. Sequence traces were initially analyzed by PHRED [19], with regions possessing quality scores lower than 20 being masked, along with vector sequences, by the use of Lucy2 software [20]. Cluster assembly was performed using the PHRAP package [21], and manual inspection of clusters was carried out using the CONSED program [22]. Sequence consensuses were generated for all clusters larger than 30 nucleotides, and a search for ORFs was performed using the getorf software from the EMBOSS package [23]. The resulting ORFs were subjected to a six-frame translation and grouped in a single fasta file for data analysis. Correspondence with LophDB clusters was examined by running a local BLAST against this database. The local platyhelminthes database was constructed based on the platyhelminth EST sequences available from GenBank dbEST (http://www.ncbi.nlm.nih.gov/dbEST/) followed by six-frame translation. All annotation was done using the BLAST algorithm. For protein identification purposes, each *E. granulosus* cluster or platyhelminth EST retrieved from MASCOT searches was considered to be one protein identification. The *B. taurus* local database was constructed with public protein entries deposited in GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein) for this species.

Eukaryotic Orthologous Group (KOG) annotations [24] were assigned based on sequence similarity searches against the KOG annotated proteins (http://www.ncbi.nlm.nih.gov/COG/grace/kognitor.html). Signal sequence and non-classical secretion predictions were obtained using SignalP 3.0 [25] and SecretomeP 2.0 [26]. The Compute pI/MW tool from the ExPASy Proteomics Server (http://ca.expasy.org/) was used to estimate the pI and M_w of 2-DE protein spots.

3 Results

3.1 Proteomic analysis of *E. granulosus* metacestode components

3.1.1 Protoscoleces

Two complementary proteomic approaches, 2-DE/MALDI-TOF MS/MS and LC-MS/MS, were used to analyze proteins expressed in *E. granulosus* PSC. PSC total proteins were electrofocused on pH 3–10 or 4–7 IPG strips, and approximately 400 and 350 spots were resolved on Coomassie-stained 2-DE gels, respectively (Fig. 2A and B). The 2-DE profiles were reproducible (~90% matching between replicates), and the prominent matched spots, in a total of 340 for pH 3–10 gels and 280 for pH 4–7 gels, were submitted in replicate to MALDI-TOF MS/MS analysis. Protein identification was obtained for 215 of the 620 analyzed spots, 104 from pH 3-10 and 111 from pH 4–7, corresponding to a total of 57 different proteins (Supporting Information Table 1). Several proteins were present in multiple spots, probably corresponding to protein isoforms or post-translational modifications. More than one *E. granulosus* cluster was

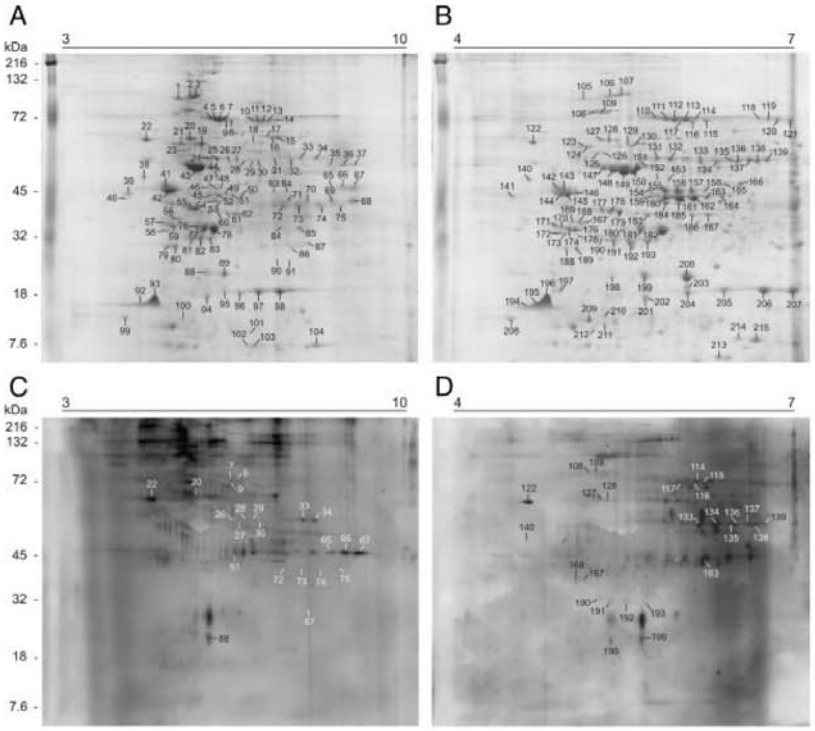

Figure 2. Representative 2-DE gels and immunoblots of *E. granulosus* protoscolex proteins. Proteins (2 mg) were separated on a linear pH range of 3–10 (A and C) or 4–7 (B and D) using IEF in the first dimension and 12% SDS-PAGE in the second dimension. Proteins were Coomassie Blue-stained (A and B) or electrotransferred to PVDF membranes and probed with a pool of sera from CHD patients (C and D). Molecular weight markers are shown on the left. Detailed protein identifications are available in Supporting Information Table 1.

assigned to some individual protein spots, such as those of enolase, phosphoenolpyruvate carboxykinase, and β-tubulin, and although these sequences have been grouped into different clusters, they likely correspond to different segments of single proteins.

In order to identify PSC antigenic proteins, we performed 2-DE immunoblots using a pool of sera from CHD patients confirmed by surgery. Complex profiles were observed, comprising at least 180 different antigenic spots (Fig. 2C and D). Regions of high reactivity with no detectable spots on corresponding Coomassie-stained 2-DE gels were observed (see Fig. 2A and B), suggesting that some antigenic proteins are expressed at very low levels. In contrast, some high density spots (spot 43, for example) displayed no immunological reaction at all, reflecting the specificity of immune recognition of antigenic spots. Moreover, no significant reactivity was observed in 2-DE immunoblots with sera from uninfected individuals (data not shown). We were able to identify 48 antigenic spots, corresponding to fourteen proteins: calreticulin, enolase, P-29, 14–3–3ε, EgTPx, GST, tropomyosin, HSP20, HSP70, grp78, putative MVP protein, fructose-bisphosphate aldolase, EgcMDH, and citrate synthase.

The analysis of the PSC soluble proteins by LC-MS/MS allowed the identification of 65 parasite proteins (Supporting Information Table 2A). Overall, the 2-DE/MALDI-TOF MS/MS and LC-MS/MS complementary proteomic approaches allowed the identification of 94 non-redundant PSC proteins, and 28 of these proteins were identified by both techniques. According to KOG functional classification (Fig. 3A and Supporting Information Table 2A), most of the identified PSC proteins are related to the Z (cytoskeleton–24%) and O (post-translational modification, protein turnover, and chaperones–14%) categories, but we also identified several proteins related to metabolism (C, G, E, and P), signal transduction (T) and translation (J). Eight proteins were not classified by KOG, including a Kunitz-type proteinase inhibitor, a putative MVP protein, and two hypothetical proteins. Several identified proteins were previously characterized as antigenic or involved in mechanisms of host immune evasion, such as P-29, EpC1, and EgTeg. These proteins were arbitrarily grouped into a category of antigens or immunomodulatory proteins.

3.1.2 Hydatid cyst fluid

HCF usually presents large amounts of host serum proteins, which impairs the identification of less represented proteins, such as those of parasite origin. LC-MS/MS of different crude HCF samples resulted in the identification of only five parasite proteins (Table 1), and affinity chromatography and centrifugal ultrafiltration techniques failed

Table 1. Parasite and host proteins identified in the host-interacting *E. granulosus* metacestode components

LophDB cluster ID or accession no.	Protein[a]	Organism	Sequence coverage (%)	MASCOT score[b]
Hydatid cyst fluid (HCF)				
Proteins identified in crude HCF samples				
1 EGC00006 (contig2)	**Actin**	*E. granulosus*	7	79
2 EGC00287 (contig1)	**Citrate synthase**	*E. granulosus*	16	96
3 EGC04146 (contig1)	**Citrate synthase**	*E. granulosus*	26	128
4 EGC00481 (contig1), EGC00094 (contig1), EGC02671 (contig1) or EGC02744 (CN649084)	**Polyubiquitin**	*E. granulosus*	19	81
5 EGC00084 (contig1), EGC02722 (contig1) or EGC01022 (BQ173439)	**Thioredoxin peroxidase (EgTPx)**	*E. granulosus*	5	77
6 NP_001030182	Adenylyl cyclase-associated protein 1	*B. taurus*	3	123
7 NP_851335	Albumin	*B. taurus*	12	371
8 P11116	Beta-galactoside-binding lectin (Galectin-1)	*B. taurus*	11	82
9 CAA48828	Beta-2-microglobulin precursor	*B. taurus*	11	63
10 P25417	Cystatin B	*B. taurus*	45	117
11 P07107	Diazepam-binding inhibitor (DBI)	*B. taurus*	51	158
12 NP_776342	Hemoglobin beta chain	*B. taurus*	40	254
13 AAI03002	Lymphocyte cytosolic protein 1 (L-plastin)	*B. taurus*	3	74
14 AAA30462	Osteopontin	*B. taurus*	9	90
15 AAI02049	SH3 domain binding glutamic acid-rich protein like 3	*B. taurus*	20	290
16 NP_776393	Thioredoxin	*B. taurus*	24	151
17 XP_001789311	Thymosin beta-4	*B. taurus*	44	139
18 AAG03074	Thymosin beta-10	*B. taurus*	30	67
Proteins identified in immunopurified HCF samples				
1 EGC00006 (contig2)	**Actin**	*E. granulosus*	12	143
2 CAA81235	**Antigen B subunit 1 (AgB8/1)**	*E. granulosus*	33	200
3 EGC03443	**Antigen 5 (Ag5)**	*E. granulosus*	5	64
4 2704 (our clustering) or DR748735	**Antigen 5 (Ag5)**	*E. granulosus* or *E. multilocularis*	23 or 5	62
5 EGC00287 (contig1)	**Citrate synthase**	*E. granulosus*	12	83
6 EGC04146 (contig1)	**Citrate synthase**	*E. granulosus*	18	123
7 EGC00521 (contig1)	**Dynein light chain**	*E. granulosus*	19	156
8 EGC03031 (contig1)	**Dynein light chain**	*E. granulosus*	26	85
9 EGC00369 (contig1)	**Fructose-bisphosphate aldolase**	*E. granulosus*	34	521
10 EGC03425 (contig1) or EGC03590 (CN650779)	**Histone H2A**	*E. granulosus*	6	37
11 EGC00502 (contig1) or EGC01157 (BQ173683)	**Histone H2B**	*E. granulosus*	6	43

Table 1. Continued

LophDB cluster ID	or accession no.	Protein[a]	Organism	Sequence coverage (%)	MASCOT score[b]
12	EGC00939 (contig1) or EGC04177 (contig1)	**HSP20 related protein**	*E. granulosus*	3	56
13	EL757670	**LDL-receptor**	*T. solium*	5	91
14	EGC00125 (contig1)	**Myosin essential light chain**	*E. granulosus*	6	50
15	P35417	**Paramyosin**	*E. granulosus*	6	228
16	Q56JA3	**Putative glucose phosphate isomerase**	*E. granulosus*	3	73
17	EGC00319 (contig1)	**Tegumental protein (EgTeg)**	*E. granulosus*	18	94
18	EGC00129 (contig1) or EGC03207 (CN649957)	**Tetraspanin family member**	*E. granulosus*	17	47
19	EGC00084 (contig1), EGC02722 (contig1) or EGC01022 (BQ173439)	**Thioredoxin peroxidase (EgTPx)**	*E. granulosus*	12	111
20	NP_001107197	Calgranulin A (S100A8)	*B. taurus*	13	59
21	NP_001070018	Complement component 1, s subcomponent	*B. taurus*	2	69
22	NP_776963	Fc gamma receptor I (CD64)	*B. taurus*	9	127
23	CAB56828	Hemoglobin alpha chain	*B. taurus*	17	144
24	NP_776342	Hemoglobin beta chain	*B. taurus*	40	254
25	AAB37381	IgG1 heavy chain constant region	*B. taurus*	8	128
26	AAB37380	IgG2a heavy chain constant region	*B. taurus*	9	44
27	AAB62251	IgM heavy chain constant region	*B. taurus*	19	374
28	NP_786967	Immunoglobulin J chain	*B. taurus*	9	77
29	B30554	Immunoglobulin lambda chain C region	*O. aries*	18	58
Germinal layer (GL)					
1	EGC00006 (contig1)	**Actin**	*E. granulosus*	47	402
2	EGC00006 (contig2)	**Actin**	*E. granulosus*	36	504
3	CAA81235	**Antigen B subunit 1 (AgB8/1)**	*E. granulosus*	20	53
4	EGC03147 (contig1)	**Calcium-binding protein**	*E. granulosus*	11	44
5	EGC00287 (contig1)	**Citrate synthase**	*E. granulosus*	49	251
6	EGC04146 (contig1)	**Citrate synthase**	*E. granulosus*	54	265
7	EGC02757 (contig1), EGC02229 (contig1) or EGC00123 (BF643012)	**14-3-3 protein**	*E. granulosus*	3	31
8	AAK39122	**EF-hand calcium-binding protein (EgCaBPII)**	*E. granulosus*	31	83
9	EGC00020 (contig1)	**Ferritin**	*E. granulosus*	7	42
10	EGC00369 (contig1)	**Fructose-bisphosphate aldolase**	*E. granulosus*	25	115
11	EGC00080 (contig1)	**Glutathione-S-transferase**	*E. granulosus*	7	32
12	BP187957, EE283416, DN290221, DN294018, DN304358, EG350980, EC386157, EC386168, EC386565, EG404969, EG406839, EG414978, EL619196, BW635094, BW636689,	**Isocitrate dehydrogenase 2 (NADP+), mitochondrial**	*D. japonica*, *S. mediterranea*, *O. viverrini*, *D. ryukyuensis* or *S. japonicum*	6	47

Table 1. Continued

LophDB cluster ID or accession no.		Protein[a)]	Organism	Sequence coverage (%)	MASCOT score[b)]
	BW638802, EE667132, EE669215, EE672236, EE673373 or BU720808				
13	EGC00284 (contig2)	**Lactate dehydrogenase**	*E. granulosus*	29	174
14	O704F5	**Malate dehydrogenase precursor (mitochondrial)**	*E. granulosus*	30	405
15	EGC00294 (BI244007)	**Ornithine aminotransferase**	*E. granulosus*	40	283
16	EL752974, EL753252, EL754566, EL756558, EL757797, EL761379 or EL763277	**Ornithine aminotransferase**	*T. solium*	23	179
17	P35417	**Paramyosin**	*E. granulosus*	6	232
18	EGC04068 (contig1)	**Phosphoenolpyruvate carboxykinase**	*E. granulosus*	33	184
19	EGC03250 (contig1)	**Phosphoenolpyruvate carboxykinase**	*E. granulosus*	18	97
20	EGC04111 (contig1)	**Phosphoenolpyruvate carboxykinase**	*E. granulosus*	6	31
21	EGC05022 (CN653636)	**Pyruvate dehydrogenase E1 component**	*E. granulosus*	6	56
22	EGC04361 (CN652347)	**Serine hydrolase similar to OVCA2**	*E. granulosus*	7	26
23	EGC00319 (contig1)	**Tegumental protein (EgTeg)**	*E. granulosus*	18	268
24	EGC00084 (contig1), EGC02722 (contig1) or EGC01022 (BQ173439)	**Thioredoxin peroxidase (EgTPx)**	*E. granulosus*	18	112
25	EGC00902 (contig1)	**UDP-glucose 4-epimerase**	*E. granulosus*	4	32
26	NP_851335	Albumin	*B. taurus*	35	1210
27	AAI02741	Alpha-1 acid glycoprotein (Orosomucoid)	*B. taurus*	7	61
28	NP_776409	Alpha-2-HS-glycoprotein (Fetuin)	*B. taurus*	2	71
29	NP_001103265	Alpha-2-macroglobulin	*B. taurus*	1	59
30	AAI02236	Annexin A5 (Annexin V)	*B. taurus*	9	183
31	NP_001032557	Apolipoprotein A-IV	*B. taurus*	13	207
32	NP_001029607	Cathepsin K	*B. taurus*	8	134
33	AAI12453	Complement component 3	*B. taurus*	3	240
34	XP_589483	Complement component 5	*B. taurus*	2	107
35	AAI14015	Complement component 6	*B. taurus*	1	45
36	NP_001030441	Complement component 9	*B. taurus*	14	336
37	AAI02334	Creatine kinase	*B. taurus*	4	135
38	P07107	Diazepam-binding inhibitor (DBI)	*B. taurus*	39	50
39	AAI02747	Endopin 2B	*B. taurus*	4	46
40	AAD33073	Enolase 1 (alpha)	*B. taurus*	8	150
41	CAA60506	Fatty acid-binding protein, liver (L-FABP)	*B. taurus*	14	82
42	NP_001029619	Fructose-1,6-bisphosphatase **1**	*B. taurus*	5	50
43	AAI03417	Glucose phosphate isomerase (Neuroleukin)	*B. taurus*	2	94
44	AAB72239	Glutathione S-transferase isoform 1	*B. taurus*	16	106
45	AAI26697	Glutathione S-transferase mu 4	*B. taurus*	7	46
46	CAB56828	Hemoglobin alpha chain	*B. taurus*	50	310

Table 1. Continued

LophDB cluster ID	or accession no.	Protein[a]	Organism	Sequence coverage (%)	MASCOT score[b]
47	NP_776342	Hemoglobin beta chain	*B. taurus*	66	517
48	AAB37381	IgG1 heavy chain constant region	*B. taurus*	13	87
49	AAB62251	IgM heavy chain constant region	*B. taurus*	3	111
50	AAX08719	Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	*B. taurus*	2	61
51	BAA14169	Lactate dehydrogenase-A	*B. taurus*	12	111
52	AAA30608	Lumican	*B. taurus*	7	40
53	NP_777169	Matrix metallopeptidase-9 (MMP-9)	*B. taurus*	2	101
54	XP_605366	Matrix metallopeptidase 12 (MMP-12) (macrophage elastase)	*B. taurus*	4	88
55	Q3SZ62	Phosphoglycerate mutase	*B. taurus*	7	102
56	BAA13690	Prostaglandin F synthase	*B. taurus*	18	65
57	NP_776382	Regucalcin (Senescence marker protein-30)	*B. taurus*	14	137
58	AAI02049	SH3 domain binding glutamic acid-rich protein like 3	*B. taurus*	10	43
59	NP_776393	Thioredoxin	*B. taurus*	12	59
60	NP_001030222	Vitronectin	*B. taurus*	3	64

a) Identified parasite proteins are in bold.
b) MASCOT score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Scores >24 for *E. granulosus*, >30 for *B. taurus* and >42 for other database searched, indicate identity or extensive homology ($p<0.05$).

to eliminate host serum protein contamination (data not shown). As an alternative approach, we immunopurified HCF parasite proteins using immobilized anti-PSC antibodies, and the LC-MS/MS analysis of immunopurified samples of two independent HCF pools allowed the identification of an additional 15 *E. granulosus* ES proteins, which are listed in Table 1.

From the parasite ES products, we identified the major proteins reported to be secreted by the *E. granulosus* metacestode, AgB and Ag5, along with several intracellular proteins. The peptides obtained of AgB are related to the AgB8/1 subunit (Supporting Information Table 2B), which was recently identified as the most abundant AgB subunit in HCF (our unpublished data). A novel variant of Ag5 was identified for the first time in HCF, with the peptide *VDSPFDVALLR* corresponding to an Ag5 isoform coded by sequences represented in both the platyhelminthes database (by an *E. multilocularis* EST) and in our *E. granulosus* clustering (Supporting Information Fig. 1). The sequences were absent from the LophDB.

The intracellular proteins found in the HCF include cytoskeletal proteins, chaperones, antioxidant enzymes, histones, and proteins involved in metabolism, in addition to proteins with antigenic and immunomodulatory properties (Fig. 3B). Actin and histones are highly conserved proteins, and it was not possible to determine whether the identified peptides were from parasite or host proteins. However, the presence of other parasite intracellular proteins in the HCF suggest that these proteins may be part of parasite ES products.

Only sequences related to AgB and Ag5 were predicted by SignalP to have an amino-terminal secretion signal peptide and, therefore, are likely secreted through the classical pathway (Supporting Information Table 2B). In

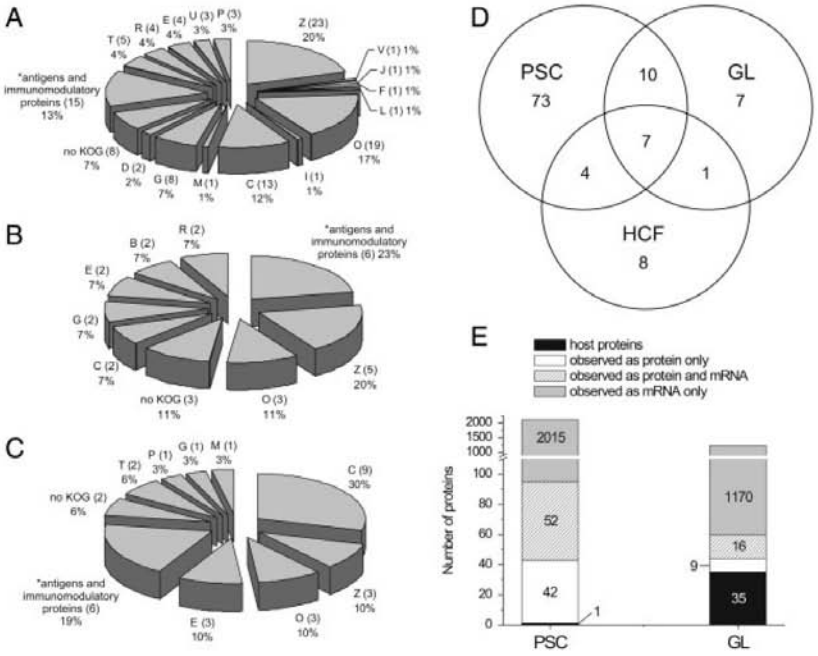

Figure 3. Analysis of *E. granulosus* metacestode proteins. Distribution of KOG functional categories of proteins identified in different components of hydatid cyst, (A) PSC, (B) HCF, and (C) GL. Percentages and the number (in parentheses) of proteins identified in each functional category are indicated in the sectors of circle. The number of proteins in the graphic exceed the total of identified proteins because some of them were grouped in more than one functional category. *(Z) Cytoskeleton; (O) Posttranslational modification, protein turnover, chaperones; (C) Energy production and conversion; (G) Carbohydrate transport and metabolism; (R) General function prediction only; (T) Signal transduction mechanisms; (J) Translation, ribosomal structure and biogenesis; (U) Intracellular trafficking, secretion, and vesicular transport; (P) Inorganic ion transport and metabolism; (E) Amino acid transport and metabolism; (I) Lipid transport and metabolism; (M) Cell wall/membrane/envelope biogenesis; (F) Nucleotide transport and metabolism; (V) Defense mechanisms; (D) Cell cycle control, cell division, chromosome partitioning; (L) Replication, recombination and repair; (B) Chromatin structure and dynamics; (no KOG) protein not related to any KOG category; (*) antigens or immunomodulatory proteins (proteins arbitrarily classified based on their prior immunological characterization according to published literature).* (D) Venn diagram showing the distribution of proteins identified in the components of parasite metacestode. (E) Comparison of transcriptomic and proteomic data from the PSC and GL.

contrast, nine (45%) ES proteins may be secreted through non-classical secretory pathways, as predicted by SecretomeP. Four proteins presented truncated sequences at their *N*-terminus and predictions of their secretion could not be performed.

3.1.3 Germinal layer

Similar to HCF samples, GL samples contain significant contamination of host serum proteins. Moreover, the large amount of immunoglobulins co-purified with GL proteins (data not shown) precluded the use of an immunopurification approach to analyze this metacestode component. Nevertheless, the analysis of crude GL samples by LC-MS/MS resulted in the identification of 25 parasite proteins (Table 1 and Supporting Information Table 2C). Functional categories related to metabolism and energy production and conversion (C, E, G, and P–56%) were the most represented among the identified GL proteins (Fig. 3C). This result fits well with the proliferative character of this layer in PSC generation and cyst growth, which would be expected to be highly metabolically active. Antigenic and/or immunomodulatory proteins, such as AgB8/1, paramyosin, EgTeg, and EgTPx, were also identified in this parasite tissue.

The distribution of identified proteins among the three hydatid cyst components is shown in Fig. 3D. Of the 94 proteins identified from PSC samples, only 17 and 11 proteins, respectively, were also identified in the GL and HCF. This is probably due to major qualitative and quantitative differences in protein composition between samples. Seven proteins (paramyosin, EgTeg, EgTPx, actin, fructose-bisphosphate aldolase, and citrate synthase clusters EGC00287 and EGC04146) were found in all metacestode components.

3.2 Comparison between proteomic and EST expression data

When we compared the available *E. granulosus* EST data and proteins identified from PSC and GL in this study, we found a high degree of concordance. More than half (52 out of 94) of the PSC identified proteins are also represented by corresponding cDNA sequences from this developmental stage (Fig. 3E). This correlation is even greater for the GL, with 16 of the identified proteins (64%) overlapping transcripts assigned for this parasite tissue. Our proteomic analysis apparently does not present preferential sampling, as we were able to identify high abundant clusters as well as clusters represented by only a single EST. A total of 14% of the metacestode identified proteins were matched with sequences from other helminth species (Supporting Information Table 2), primarily to *Taenia solium*. This reveals the importance of extending the search to databases of related organisms to increase the chances of protein identification in proteomic surveys of organisms without sequenced genomes.

3.3 Host proteins present in *E. granulosus* metacestode components

To gain a deeper view on the host-parasite interplay, we identified host proteins adsorbed or co-purified with parasite components (Table 1 and Supporting Information Table 2). Some host molecules, such as immunoglobulins (IgG1, IgG2, and IgM), Fc receptor, complement C1s, calgranulin A, and hemoglobin, co-purified with HCF proteins in our immunopurification approach. In control immunopurification experiments using non-related antibodies, only peptides corresponding to hemoglobin were found (data not shown), confirming the specificity of the other observed interactions between parasite ES proteins and host proteins. This indicates that parasite ES products are able to directly interact with host proteins during infection, including components of the host immune response. Albumin, TRx, DBI, thymosins, galectin-1, osteopontin, and cystatin-B were among the host proteins found to be present in crude HCF samples. Thirty-five host proteins, including serum proteins, complement components, antioxidant enzymes, and proteinases, were found adsorbed to the parasite GL, an important host-interacting parasite surface. Unlike the high host protein content identified from the GL and HCF samples, vimentin was the only identified host protein adsorbed to the PSC stage.

4 Discussion

4.1 Proteomic analysis of *E. granulosus* metacestode

In this work, we performed a proteomic analysis of the pathogenic larval stage of *E. granulosus* during infection of a bovine intermediate host. This analysis provided, for the first time, a general view of both parasite and host proteins expressed during a cestode parasite infection. The hydatid cyst is likely the largest parasite structure that lodges within mammal tissues, and its location and immunogenicity result in a large potential for eliciting inflammation. Nevertheless, the metacestode is able to survive for decades in the intermediate host, secreting and exposing molecules that downregulate host responses. We separately analyzed three different metacestode components (the GL, HCF, and PSC), which provided information on proteins present in the parasite's tegumental interface with the host (GL), in the ES products (HCF), and in the pre-adult forms (PSC) in actual infection conditions. We have also identified host proteins, generating further information on host-parasite cross-talk.

The lack of a sequenced genome and the presence of highly abundant host serum proteins present drawbacks for the *E. granulosus* metacestode proteomic analysis. However, these negative factors were at least in part compensated for the availability of a comprehensive *E. granulosus* EST data-

base and by the use of an immunopurification approach to enrich samples with proteins from parasite origin, respectively. The genome project of the related species *E. multilocularis* is in progress, but the available contigs are not yet annotated. Therefore, we used the reverse strategy of searching not only in the *E. multilocularis* EST database but also in the EST data available from other platyhelminthes. These strategies allowed us to extend the previously restricted overall repertoire of known proteins expressed and released by the *E. granulosus* metacestode.

4.1.1 Protoscolex proteins

The conditions for the 2-DE analysis of the PSC protein extracts had been previously standardized by our group [15]. In the present work, we improved the resolution and protein identification process in order to establish a comprehensive proteomic map that may be useful in future analyses of this parasite life form. The established 2-DE map will be a useful reference for future studies on differential protein expression and/or post-translational modifications during PSC *in vitro* differentiation (into the adult worm) or dedifferentiation (into the hydatid cyst), or in response to external factors, such as anthelmintic treatment or stress.

Using LC-MS/MS analyses, proteins expressed by the PSC within the metacestode during infection were identified. These included immunomodulatory proteins, detoxifying enzymes, chaperones, and protease inhibitors, among others. Upon cyst rupture and PSC release within the host body cavity, these proteins may establish direct contacts with the host and interfere with responses against the parasite, favoring PSC survival and development into secondary cysts.

The search for antigenic proteins by 2-DE immunoblots resulted in the identification of fourteen PSC proteins recognized by CHD patient sera. Five of these proteins (P-29 [27], EgTPx [28], EgcMDH [29], HSP70 [30], and grp78 [31]) were previously identified as antigens in *E. granulosus* human infections. Some of the detected antigenic proteins, were also identified in *E. multilocularis* PSC 2-DE immunoblots, including calreticulin, tropomyosin, grp78, HSP70, HSP20, 14–3–3, and P29 [16]. The other five detected antigens, enolase, GST, putative MVP protein, fructose-bisphosphate aldolase, and citrate synthase, are described here for the first time. Enolase has been described on the surface and in the ES products of other parasites as presenting antigenic and host-interacting properties [9, 32], making it an interesting target for future studies.

4.1.2 *E. granulosus* proteins expressed in the host-interacting metacestode components (germinal layer and hydatid cyst fluid)

The GL is the outermost cellular tissue of the *E. granulosus* metacestode, which contains the parasite's tegument and covers the entire surface of the hydatid cyst. Therefore, the GL is an important interface between the parasite and its host, specially considering the laminated layer permeability to macromolecules. The HCF contains the ES products from the PSC and GL and is another important host-interacting component, as it is able to interact with and modulate the host immune system [3, 4]. Therefore, we analyzed these metacestode components searching for potential host-interacting proteins that could be involved in parasite survival mechanisms.

We found proteins that may contribute to immunoregulatory events at the host-parasite interface during infection, some of them with potential for vaccine development, such as paramyosin and tetraspanin [33–35]. Helminth paramyosins are multifunctional modulators of the host immune response, binding complement components, immunoglobulins and secreted components of the cellular immune response [33]. Tetraspanins are known to interact with a variety of cell surface molecules, including the major histocompatibility complex (MHC) and Fc receptor, and may regulate their function or signaling [36], thus constituting a potential immunomodulatory role by acting as receptor for host ligands [35]. AgB and EgTeg are responsible for eliciting and sustaining a Th2-polarized microenvironment, contributing to the establishment of an *E. granulosus* chronic infection [4]. Ag5 contains a highly conserved glycosaminoglycan-binding motif that targets this antigen to the host-parasite interface [37], but the Ag5 biological significance remains to be elucidated.

Other mechanisms of survival during parasite infection could involve EgTPx and the LDL-receptor. In PSC, a greater number of proteins involved in redox homeostasis was found, but in GL and HCF only EgTPx was identified, and therefore, this protein may play a major role in protecting the *E. granulosus* metacestode from oxidative damage. EgTPx is also an antigenic protein that has been proposed to improve CHD immunodiagnosis [28]. Most of the helminths lack the ability to synthesize sterols or fatty acids *de novo* and instead acquire them from their hosts by expressing binding proteins in a mechanism with nutritional and immune evasion purposes [7]. AgB was previously reported as a HCF lipid-binding protein [38], and, along with it, we now identified a putative *E. granulosus* LDL-receptor.

The intracellular and membrane proteins identified in the *E. granulosus* HCF have been previously described in the ES products of other helminth species [10, 11, 39] and their presence in these fluids could be explained by parasite damage or death and tegument turnover. More likely, these proteins may represent products of parasite tegument shedding, since the identified proteins have been described as tegumental proteins [9, 40]. The continuous shedding of the parasite tegument is thought to release components to evade the host immune system [7]. Regardless of their exact source, these proteins are in fact released by the parasite during infection and, therefore, deserve future investigations

regarding their potential use for diagnosis and vaccination strategies. These cytosolic proteins were previously regarded as "hidden" antigens, but their detection in HCF indicates that they are accessible to potentially induce or modulate the host immune response. Indeed, some of the identified HCF proteins were characterized, in this and previous works [4], as antigenic and/or immunomodulatory.

4.1.3 Host proteins in association with parasite components

A number of host proteins were identified in the GL and HCF samples, suggesting that *E. granulosus*, like other helminths [7, 13], may adsorb and/or specifically bind host proteins for nutrient requirements, for general antigen disguise, or to actively interfere with host responses against the parasite. Parasites can interfere with host responses by directly binding and inhibiting effector molecules of host immunity [33, 41], such as complement components, immunoglobulins and calgranulins. Alternatively, host proteins coated on the cyst surface, such as endopin and GST, might be used by the parasite to counteract or attenuate enzymes and toxic compounds released by the host immune cells. Paramyosin and tetraspanin have been identified and proposed to act as helminth receptors for some host proteins, as described above (see section 4.1.2).

While several host proteins were identified in the GL and HCF, only one was found in the PSC samples. This is suggestive of a molecular mechanism in which the GL and HCF proteins would "trap" host proteins, protecting the PSC from at least some of the anti-parasite host responses. In this way, pre-adults would remain viable for longer periods of time, increasing the probability of life cycle completion upon PSC ingestion by a suitable definitive host.

4.2 CHD immunopathology

The immunopathology of CHD is typically characterized by a granulomatous inflammation, a protective response against chronic infections with persistent pathogens [42]. In addition to acute-phase proteins (complement components, alpha-2-macroglobulin, alpha-1 acid glycoprotein, alpha-2-HS-glycoprotein, and inter-alpha-trypsin inhibitor heavy chain 4), we have found in HCF and GL samples, host proteins potentially involved in a granulomatous response against *E. granulosus* infection, such as DBI, TRx, osteopontin, and proteases. DBI-derived peptides and TRx are able to recruit and modulate diverse immune cell types and stimulate the production of proinflammatory cytokines [43, 44], essential activities for granuloma formation [42]. Osteopontin is a key cytokine of granulomatous inflammation and is involved in granulomatous responses of diverse etiology. It is a powerful proinflammatory molecule, supporting adhesion, inducing migration and modulating the function of a large set of immune cells [45]. Osteopontin exists both as a cytokine in body fluids and as an immobilized molecule in mineralized tissues, such as previously described in the granulomatous reaction surrounding the *E. granulosus* hydatid cyst [46], where it may be acting in the inhibition of tissue calcification [45]. The host proteases MMP-9, MMP-12 and cathepsin K, may be involved in matrix remodeling for proper granuloma formation and maintenance, and some of them were previously described to be secreted by the host granuloma in response to hydatid cyst [47, 48]. The high enzymatic activity of MMP-9 in hydatid cysts was also found to be correlated with cyst infertility [47].

4.3 Conclusions

Our proteomic analysis identified, for the first time, proteins that are actually expressed and released by the *E. granulosus* pathogenic larval stage during intermediate host infection. Host proteins involved in the response against the parasite were also identified, providing new insights into some of the intriguing host-parasite interactions. Some parasite survival strategies were highlighted, with at least some of the involved proteins identified, helping to understand how chronic infection is established and the molecular mechanisms underpinning CHD immunopathology. We have identified new protein targets and have validated other previously proposed targets for the development or improvement of CHD diagnosis, treatment, and control strategies.

We thank Dr. Fábio Gozzo and Dr. Paulo M. Pinto for their help with the MS and 2-DE analyses. We thank the Uniprote-MS (CBiot, UFRGS) for the support for some of the LC-MS/MS analyses. K.M.M. is a recipient of a CNPq fellowship and M.O.C. was a recipient of a CAPES MSc fellowship. This work was supported by CNPq, CAPES and the Brazilian Synchrotron Light Laboratory (LNLS) under proposals MAS-5116 and MAS-6409.

The author have declared no conflict of interest.

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PROTEOMICS

Supporting Information for Proteomics

DOI 10.1002/pmic.200900506

Karina M. Monteiro, Marcos O. de Carvalho, Arnaldo Zaha and Henrique B. Ferreira

Proteomic analysis of the *Echinococcus granulosus* metacestode during infection of its intermediate host

Supplementary Table 1: Protoscolex proteins identified by 2-DE/MALDI-Q-Tof MS/MS.

Spot	Protein	LophDB Cluster ID or accession n°	Organism	M_w (kDa) (theo/obs)[a]	pI (theo/obs)[a]	Sequence coverage (%)	MASCOT score[b]	Number of matched peptides	Peptide sequence[c]
1	HSP90	EGC00627 (contig1)	*E. granulosus*	- / 82.0	- / 5.3	10	114	4	**R.APFDLFENR.K** K.RAPFDLFENR.K **K.GVVDSEDLPLNISR.E** **R.NPEDISQEEYGEFYK.S**
2	Paramyosin	P35417	*E. granulosus*	98.7 / 83.1	5.2 / 5.6	5	214	4	**K.LEEAEAFATR.E** **R.LAAENFELVR.V** **R.YEEEAEAAGNLR.N** **R.ITELEDIAEHER.T**
3	Paramyosin	P35417	*E. granulosus*	98.7 / 83.8	5.2 / 5.7	7	229	5	**R.LAAENFELVR.V** **R.YEEEAEAAGNLR.N** **R.ITELEDIAEHER.T** R.VQELQTALEDERR.A **K.DLQAENEALAAENGELTHR.A**
4	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 70.0	5.7 / 6.0	5	98	2	**R.TTPSYVAFTDTER.L** **K.VSDAVVTVPAYFNDSQR.Q**
5	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 69.4	5.7 / 6.1	5	125	2	**R.TTPSYVAFTDTER.L** **K.VSDAVVTVPAYFNDSQR.Q**
6	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 69.0	5.7 / 6.2	5	142	2	**R.TTPSYVAFTDTER.L** **K.VSDAVVTVPAYFNDSQR.Q**
7	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 69.4	5.7 / 6.2	5	70	2	**R.TTPSYVAFTDTER.L** **K.VSDAVVTVPAYFNDSQR.Q**
8	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 68.5	5.7 / 6.4	5	95	2	**R.TTPSYVAFTDTER.L** **K.VSDAVVTVPAYFNDSQR.Q**
9	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 68.0	5.7 / 6.3	5	79	2	**R.TTPSYVAFTDTER.L** **K.VSDAVVTVPAYFNDSQR.Q**
10	Phosphoenolpyruvate carboxykinase	EGC03250 (contig1)	*E. granulosus*	- / 68.7	- / 6.8	11	69	2	**R.FQTVPHVAEGVK.G** **K.VLDYIEEQNTFVR.C**
		458 (our clustering) /EGC04068 (contig1)	*E. granulosus*			6	40	1	**R.RPEGIPLVYEAR.T**
11	Phosphoenolpyruvate carboxykinase	EGC03250 (contig1)	*E. granulosus*	- / 68.2	- / 7.0	5	40	1	**R.FQTVPHVAEGVK.G**
		458 (our clustering) /EGC04068 (contig1)	*E. granulosus*			6	35	1	**R.RPEGIPLVYEAR.T**
		191 (our clustering)/ EGC04111 (cluster1)	*E. granulosus*			16	90	2	**R.AINPEAGFFGVAPGTNHK.T** **K.TIISHIPDDR.L**
12	Phosphoenolpyruvate carboxykinase	EGC03250 (contig1)	*E. granulosus*	- / 68.0	- / 7.1	11	66	2	**R.FQTVPHVAEGVK.G** **K.VLDYIEEQNTFVR.C**
		458 (our clustering) /EGC04068 (contig1)	*E. granulosus*			6	70	1	**R.RPEGIPLVYEAR.T**
		191 (our clustering)/ EGC04111 (cluster1)	*E. granulosus*			10	46	1	**R.AINPEAGFFGVAPGTNHK.T**
13	Phosphoenolpyruvate carboxykinase	EGC03250 (contig1)	*E. granulosus*	- / 68.1	- / 7.2	11	77	2	R.FQTVPHVAEGVK.G **K.VLDYIEEQNTFVR.C**

		458 (our clustering) /EGC04068 (contig1)	*E. granulosus*			12	56	2	R.FAWPGFGNNIR.V R.RPEGIPLVYEAR.T
		191 (our clustering)/ EGC04111 (cluster1)	*E. granulosus*			10	37	1	R.AINPEAGFFGVAPGTNHK.T
14	Phosphoenolpyruvate carboxykinase	458 (our clustering) /EGC04068 (contig1)	*E. granulosus*	- / 68.7	- / 7.3	12	69	2	R.FAWPGFGNNIR.V R.RPEGIPLVYEAR.T
15	Putative glucose phosphate isomerase	Q56JA3	*E. granulosus*	61.6 / 59.5	6.5 / 7.3	10	161	4	K.ALFDADSQR.F K.AVANHFVALSTNTR.A R.FPAYFQQGDMESNGK.Y + Oxidation (M) R.SNSPILVDGQDVMPEVNK.V + Oxidation (M)
16	Putative glucose phosphate isomerase	Q56JA3	*E. granulosus*	61.6 / 59.8	6.5 / 7.2	6	99	3	K.ALFDADSQR.F K.AVANHFVALSTNTR.A R.FPAYFQQGDMESNGK.Y + Oxidation (M)
17	Putative glucose phosphate isomerase	Q56JA3	*E. granulosus*	61.6 / 60.3	6.5 / 7.1	11	143	5	K.ALFDADSQR.F K.NFKPLDTTIK.A K.AVANHFVALSTNTR.A R.FPAYFQQGDMESNGK.Y + Oxidation (M)
18	UDP-glucose pyrophosphorylase	EL748674	*T. solium*	- / 57.3	- / 6.8	12	96	1	K.VHFVSNIDGTHIAETLK.H K.TLDSGINIIQLEEAAGAAIR.N
19	putative MVP protein	Q56J97	*E. granulosus*	- / 56.8	- / 5.8	11	124	3	R.LVTGPLTFIR.K K.ILPPNEALLLR.A K.CVETYHAYNLDEK.H
20	putative MVP protein	Q56J97	*E. granulosus*	- / 59.2	- / 5.6	7	91	2	R.LVTGPLTFIR.K K.ILPPNEALLLR.A
21	β-tubulin	EGC04051 (contig1)	*E. granulosus*	- / 56.7	- / 5.4	13	99	3	R.FPGQLNADLR.K K.LAVNMVPFPR.L + Oxidation (M) R.VSEQFTAMFR.R + Oxidation (M)
22	Calreticulin	ABQ96270	*E. granulosus*	45.3 / 58.9	4.5 / 4.7	10	71	3	R.YSAPFTKPLSSK.G K.GDFDDREDGGIR.T K.HEQNIDCGGGYVK.L
23	ATP synthase beta subunit	EGC05083 (CN653763)	*E. granulosus*	- / 54.0	- / 5.5	8	72	1	R.LILEVAQHLGESTVR.T
24	EgAFFP	AAK00052	*E. granulosus*	42.2 / 50.6	5.9 / 6.0	6	53	2	R.HVKPDEYRPR.L K.QSVHSGDVFILDLGSR.A
25	EgAFFP	AAK00052	*E. granulosus*	42.2 / 50.3	5.9 / 6.1	9	68	3	K.ELEPPKEVIK.S R.EVEGYESQLFK.S K.HSTADEYGTAAYK.T
26	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 49.0	- / 6.4	17	74	2	K.AVYAGEHFR.N K.AGIQIVGDDLTVTNPER.V
27	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 49.2	- / 6.5	17	83	2	K.AVYAGEHFR.N K.AGIQIVGDDLTVTNPER.V
		EGC03002 (CN649593)	*E. granulosus*			9	34	1	R.AAVPSGASTGVHEAVELR.D
28	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 49.4	- / 6.7	17	61	2	K.AVYAGEHFR.N K.AGIQIVGDDLTVTNPER.V
29	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 50.0	- / 6.8	6	57	1	K.AVYAGEHFR.N
30	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 49.9	- / 6.9	6	41	1	K.AVYAGEHFR.N

31	NADP-dependent isocitrate dehydrogenase	DR748835	*E. multilocularis*	- / 50.3	- / 7.3	8	78	2	K.YFDLGLENR.D R.EPIICSNIPR.L
32	NADP-dependent isocitrate dehydrogenase	DR748835	*E. multilocularis*	- / 50.3	- / 7.6	8	79	2	K.YFDLGLENR.D R.EPIICSNIPR.L
33	Citrate synthase	EGC04146 (contig1)	*E. granulosus*	- / 50.6	- / 7.8	10	65	2	R.ALGLPIERPK.S R.VVPGYGHAVLR.K
34	Citrate synthase	EGC04146 (contig1)	*E. granulosus*	- / 50.4	- / 8.1	16	114	3	R.ALGLPIERPK.S R.DFALTHMPDDPLIK.I + Oxidation (M) R.VVPGYGHAVLR.K
		EGC00287 (contig1)	*E. granulosus*			6	56	1	K.VGEVTVDMMYGGMR.G + 3 Oxidation (M)
35	Aspartate aminotransferase	EL742110, EL753611, EL759324 or EL759714	*T. solium*	- / 47.5	- / 8.4	4	50	1	R.DSGIAVQYYR.Y
36	Aspartate aminotransferase	EL742110, EL753611, EL759324 or EL759714	*T. solium*	- / 47.7	- / 8.7	4	54	1	R.DSGIAVQYYR.Y
37	Aspartate aminotransferase	EL742110, EL753611, EL759324 or EL759714	*T. solium*	- / 47.8	- / 8.9	10	81	2	R.DSGIAVQYYR.Y R.NATVQCLSGTGSLR.I
		EL741119, EL744715 or EL748976	*T. solium*			8	43	1	K.NVAYVAQAIHAVTK.-
38	Tropomyosin	EL755360 or EL758694	*T. solium*	- / 43.4	- / 4.6	16	132	4	R.EEAYEENIR.D K.EFTFMAEDADR.K + Oxidation (M) K.SLEISEQEAAQR.E R.AQEAEAEVAALQR.R
39	PP2A inhibitor I2PP2A/SET protein	EGC00630 (contig1)	*E. granulosus*	30.3 / 37.9	4.3 / 4.3	5	46	1	R.AVEVQEFDDITSGYK.I
40	PP2A inhibitor I2PP2A/SET protein	EGC00630 (contig1)	*E. granulosus*	30.3 / 36.6	4.3 / 4.3	5	54	1	R.AVEVQEFDDITSGYK.I
41	Annexin	EGC00278 (contig1)	*E. granulosus*	- / 40.2	- / 5.1	8	49	1	R.LGTDEEAFITILCTR.S
42	Annexin	EGC00278 (contig1)	*E. granulosus*	- / 38.8	- / 5.1	8	58	1	R.LGTDEEAFITILCTR.S
43	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 46.8	5.3 / 5.7	11	169	3	R.AVFPSIVGRPR.H R.VAPEEHPVLLTEAPLNPK.A K.SYELPDGQVITIGNER.F
44	Sarcoplasmic calcium-binding protein	EGC04217 (contig1)	*E. granulosus*	- / 43.1	- / 6.1	4	42	1	R.CHDFIQIGR.A
45	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 37.6	5.8 / 6.0	9	107	3	R.SIPLPPSVDR.N K.TENASHSEHR.E K.GLAIQPTEAQER.Q
46	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 39.9	5.8 / 5.9	9	133	3	R.SIPLPPSVDR.N K.TENASHSEHR.E K.GLAIQPTEAQER.Q
47	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 39.7	5.8 / 6.1	9	88	3	R.SIPLPPSVDR.N K.TENASHSEHR.E K.GLAIQPTEAQER.Q
48	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 39.6	5.8 / 6.2	13	102	4	R.SIPLPPSVDR.N K.TENASHSEHR.E K.GLAIQPTEAQER.Q R.QSLHDDVTNMHR.N + Oxidation (M)
49	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 37.8	5.8 / 6.3	13	144	4	R.SIPLPPSVDR.N K.TENASHSEHR.E

									K.GLAIQPTEAQER.Q
									R.QSLHDDVTNMHR.N
50	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 37.3	5.8 / 6.6	6	76	2	**K.TENASHSEHR.E**
									K.GLAIQPTEAQER.Q
51	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 37.6	5.8 / 6.4	13	77	4	R.SIPLPPSVDR.N
									K.TENASHSEHR.E
									K.GLAIQPTEAQER.Q
									R.QSLHDDVTNMHR.N + Oxidation (M)
52	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 37.5	5.8 / 6.1	13	149	4	**R.SIPLPPSVDR.N**
									K.TENASHSEHR.E
									K.GLAIQPTEAQER.Q
									R.QSLHDDVTNMHR.N + Oxidation (M)
53	Inorganic pyrophosphatase	EGC00895 (BQ173221)	*E. granulosus*	- / 35.7	- / 6.2	5	41	1	**K.CFGESNSTEYR.M**
54	Inorganic pyrophosphatase	EGC00895 (BQ173221)	*E. granulosus*	- / 35.8	- / 6.1	10	53	2	**R.YVNNVFPHK.G**
									K.CFGESNSTEYR.M
55	Annexin	EGC00272 (contig1)	*E. granulosus*	36.7 / 33.8	5.2 / 5.6	4	75	1	**R.SLEQDVIHDVSGDFER.V**
56	14-3-3 epsilon	EGC02229 (contig1)	*E. granulosus*	- / 30.9	- / 5.3	20	154	3	**R.YLAEIATASER.D**
									R.YDDMAQFMHK.I + 2 Oxidation (M)
									K.IVDLNDESAPELTVEER.N
57	14-3-3 zeta	EGC02546 (contig1)	*E. granulosus*	- / 30.0	- / 5.0	8	55	2	**R.FDKELNNEER.N**
									K.AYEEATEIANEK.L
58	14-3-3 zeta	EGC02757 (contig1)	*E. granulosus*	27.8 / 28.2	4.8 / 5.1	11	125	2	**K.TIAEMGNELNNEER.N + Oxidation (M)**
									K.ATSLAEAELSVTHPIR.L
59	14-3-3 epsilon	EGC00123 (BF643012)	*E. granulosus*	27.9 / 29.2	4.9 / 5.2	12	99	3	**R.YVAEFCTGDER.K**
									R.YVAEFCTGDERK.Q
									K.SATEVAEGDMQTTHPIR.L + Oxidation (M)
60	Methylthioadenosine phosphorylase	1398 (our clustering)/ EGC02514 (contig1)	*E. granulosus*	- / 32.6	- / 6.1	12	67	2	**K.HSIMPGNVNFR.A + Oxidation (M)**
									K.FMPNTYIPGEARPEHDVPR.L + Oxidation (M)
61	Methylthioadenosine phosphorylase	1398 (our clustering)/ EGC02514 (contig1)	*E. granulosus*	- / 32.6	- / 6.3	13	84	2	**K.HSIMPGNVNFR.A + Oxidation (M)**
									K.VGIIGGSGLDDPEIFENQHEVR.V
62	Methylthioadenosine phosphorylase	1398 (our clustering)/ EGC02514 (contig1)	*E. granulosus*	- / 32.3	- / 6.4	21	101	3	**K.HSIMPGNVNFR.A + Oxidation (M)**
									K.FMPNTYIPGEARPEHDVPR.L + Oxidation (M)
									K.VGIIGGSGLDDPEIFENQHEVR.V
63	UDP-glucose 4-epimerase	EGC00902 (contig1)	*E. granulosus*	- / 37.9	- / 7.3	3	50	1	**R.YFNPVGAHK.S**
64	UDP-glucose 4-epimerase	EGC00902 (contig1)	*E. granulosus*	- / 37.9	- / 7.6	3	40	1	**R.YFNPVGAHK.S**
65	Fructose-bisphosphate aldolase	EGC00369 (contig1)	*E. granulosus*	39.6 / 40.0	8.3 / 8.4	12	159	4	**K.DGKPFVELLR.E**
									R.FAAINLENTEENR.R
									K.RFAAINLENTEENR.R
									R.TVPVAVPGIVFLSGGQSELDATR.N
66	Fructose-bisphosphate aldolase	EGC00369 (contig1)	*E. granulosus*	39.6 / 39.8	8.3 / 8.7	12	150	4	**K.DGKPFVELLR.E**
									R.FAAINLENTEENR.R
									R.FAAINLENTEENRR.A
									R.TVPVAVPGIVFLSGGQSELDATR.N
67	Fructose-bisphosphate aldolase	EGC00369 (contig1)	*E. granulosus*	39.6 / 39.5	8.3 / 8.9	12	216	5	**R.FVPYLCAEK.M**

									K.DGKPFVELLR.E R.ELLFTTDPEFAK.H R.FAAINLENTEENR.R K.RFAAINLENTEENR.R
68	Glyceraldehyde-3-phosphate dehydrogenase	EGC00305 (contig1)	*E. granulosus*	36.1 / 35.5	8.4 / 8.8	10	72	3	R.VVDLISYMFK.R + Oxidation (M)
									K.LSKPATYDQIK.A R.VPTPNVSVVDLTCK.L
69	Glyceraldehyde-3-phosphate dehydrogenase	EGC00305 (contig1)	*E. granulosus*	36.1 / 35.3	8.4 / 8.5	10	108	2	K.VIISAPSADAPMFVVGVNHEK.Y + Oxidation (M)
									K.LIAWYDNEFGYSCR.V
70	Glyceraldehyde-3-phosphate dehydrogenase	EGC00305 (contig1)	*E. granulosus*	36.1 / 35.5	8.4 / 7.9	9	123	2	K.VIISAPSADAPMFVVGVNHEK.Y + Oxidation (M)
									R.VVDLISYMFK.R + Oxidation (M)
71	Glyceraldehyde-3-phosphate dehydrogenase	EGC00305 (contig1)	*E. granulosus*	36.1 / 35.8	8.4 / 7.5	3	62	1	-.MKPQVGINGFGR.I + Oxidation (M)
72	Malate dehydrogenase, cytoplasmic	EGC00028 (contig1)	*E. granulosus*	36.6 / 34.4	8.1 / 7.5	12	124	3	K.DIYFSFPVTIK.D R.SLFNLSADELVDER.E K.DQQIILHLLDIPEAK.T
73	Malate dehydrogenase, cytoplasmic	EGC00028 (contig1)	*E. granulosus*	36.6 / 33.9	8.1 / 7.8	7	84	2	K.VVDGLSMDEWSR.S + Oxidation (M) R.SLFNLSADELVDER.E
74	Malate dehydrogenase, cytoplasmic	EGC00028 (contig1)	*E. granulosus*	36.6 / 34.1	8.1 / 8.2	12	150	3	K.VVDGLSMDEWSR.S + Oxidation (M) R.SLFNLSADELVDER.E K.DQQIILHLLDIPEAK.T
75	Malate dehydrogenase, cytoplasmic	EGC00028 (contig1)	*E. granulosus*	36.6 / 34.4	8.1 / 8.6	7	80	2	K.VVDGLSMDEWSR.S + Oxidation (M) R.SLFNLSADELVDER.E
76	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 29.0	5.3 / 5.7	7	85	2	R.GYSFTTTAER.E K.SYELPDGQVITIGNER.F
77	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 28.8	5.3 / 5.8	7	126	2	R.GYSFTTTAER.E K.SYELPDGQVITIGNER.F
78	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 28.4	5.3 / 6.0	7	123	2	R.GYSFTTTAER.E K.SYELPDGQVITIGNER.F
79	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 24.9	5.3 / 5.1	7	60	2	R.GYSFTTTAER.E K.SYELPDGQVITIGNER.F
80	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 24.8	5.3 / 5.3	7	70	2	R.GYSFTTTAER.E K.SYELPDGQVITIGNER.F
81	Hydatid disease diagnostic antigen P-29	EGC00013 (contig1)	*E. granulosus*	27.0 / 27.0	5.6 / 5.6	9	89	2	K.MLTEASDVHQR.M + Oxidation (M) R.VHQESLTIFEK.T
82	Hydatid disease diagnostic antigen P-29	EGC00013 (contig1)	*E. granulosus*	27.0 / 26.5	5.6 / 5.8	15	96	3	K.MLTEASDVHQR.M + Oxidation (M) R.VHQESLTIFEK.T
83	Hydatid disease diagnostic antigen P-29	EGC00013 (contig1)	*E. granulosus*	27.0 / 26.5	5.6 / 6.0	22	113	4	K.IITATEEFVDINIASK.V R.VHQESLTIFEK.T K.NFLNTTLSEAQK.A
84	Phosphoglycerate mutase	EGC03341 (contig1)	*E. granulosus*	26.6 / 28.7	8.3 / 7.4	19	183	4	K.IITATEEFVDINIASK.V K.NFNSEVNTTFIEDLK.N R.MYGGLQGLNK.S + Oxidation (M) R.VLITAHGNSLR.A R.HGESVYNQENR.F

									R.AYDISPPPLEVSDKR.W
85	Phosphoglycerate mutase	EGC03341 (contig1)	*E. granulosus*	26.6 / 28.7	8.3 / 7.8	15	147	3	**R.VLITAHGNSLR.A** **R.HGESVYNQENR.F**
									R.AYDISPPPLEVSDKR.W
86	Glutathione-S-transferase	EGC03317 (CN650164)	*E. granulosus*	24.2 / 24.3	6.9 / 7.6	8	27	1	**K.AQMPFNQLPVLEVTTPR.G + Oxidation (M)**
87	Glutathione-S-transferase	EGC04109 (contig1)	*E. granulosus*	24.1 / 25.3	8.3 / 8.0	12	53	3	K.REEIITFR.V **K.LILEHQNFK.Y**
									R.CLGQQYNLLGR.N
88	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1) or EGC02722 (contig1)	*E. granulosus*	21.4 / 20.1	5.8 / 5.9	17	142	3	**R.LLDAFQFTDK.H** **R.QITINDLPVGR.S**
									R.DYGVLIEDQGIALR.G
89	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1) or EGC02722 (contig1)	*E. granulosus*	21.4 / 19.7	5.8 / 6.3	17	119	3	**R.LLDAFQFTDK.H** **R.QITINDLPVGR.S**
									R.DYGVLIEDQGIALR.G
90	Mn-superoxide dismutase (SOD)	757 (our clustering)/ EGC00326 (contig1)	*E. granulosus*	- / 22.1	- / 7.3	10	60	2	**K.NQRPDYVK.A** **K.HHAAYVNNLNIVEEK.M**
91	Mn-superoxide dismutase (SOD)	757 (our clustering)/ EGC00326 (contig1)	*E. granulosus*	- / 22.1	- / 7.6	16	83	3	**K.NQRPDYVK.A** K.GDASTIISLQPAFK.F
									K.HHAAYVNNLNIVEEK.M
92	Antigen Eg19	EGC00615 (contig1)	*E. granulosus*	20.3 / 15.6	4.5 / 4.5	12	37	1	**R.DDESVGGDEGRPLPHEVSHEGK.Q**
93	Antigen Eg19	EGC00615 (contig1)	*E. granulosus*	20.3 / 16.0	4.5 / 4.8	17	98	2	**R.DDESVGGDEGRPLPHEVSHEGK.Q** **K.GDEVDGEDRDDESVGGDEGRPLPHEVSHEGK.Q**
94	Thioredoxin peroxidase	EGC00918 (contig1)	*E. granulosus*	- / 16.5	- / 5.9	13	102	2	**K.APNFAGTAVVDGQFK.E** **R.EYEVLLEDAGVALR.G**
	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1), EGC02722 (contig1), EGC05011 (CN653605) or EGC01022 (BQ173439)	*E. granulosus*	21.4 / 16.5	5.8 / 5.9	6	44	1	**R.DYGVLIEDQGIALR.G**
95	peptidyl-prolyl cys-trans isomerase (Cyclophilin)	EGC00016 (contig1)	*E. granulosus*	17.3 / 17.3	6.4 / 6.3	29	74	4	**K.FEDENFNHK.H** R.IVFALFDDVPK.T
									K.CFFDISIGGKPAGR.I K.HVVFGEVESGEDVVK.D
96	peptidyl-prolyl cys-trans isomerase (Cyclophilin)	EGC00016 (contig1)	*E. granulosus*	17.3 / 17.1	6.4 / 6.6	26	75	3	**K.FEDENFNHK.H** **K.CFFDISIGGKPAGR.I**
97	peptidyl-prolyl cys-trans isomerase (Cyclophilin)	EGC00016 (contig1)	*E. granulosus*	17.3 / 17.1	6.4 / 6.9	29	97	4	R.IIPGFMCQGGDFTAGNGTGGKS + Oxidation (M) K.FEDENFNHK.H R.IVFALFDDVPK.T
98	peptidyl-prolyl cys-trans isomerase (Cyclophilin)	EGC00016 (contig1)	*E. granulosus*	17.3 / 17.2	6.4 / 7.4	29	160	4	**K.CFFDISIGGKPAGR.I** **K.HVVFGEVESGEDVVK.D** **K.FEDENFNHK.H** R.IVFALFDDVPK.T

99	Ca-binding protein (similar to antigen SM20 de *S. mansoni*)	EL755545 or EL762844	*T. solium*	- / 14.0	- / 4.2	26 or 12	72	2	**K.CFFDISIGGKPAGR.I** **K.HVVFGEVESGEDVVK.D** **R.RGEIDVDDLR.W**
100	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1), EGC02722 (contig1), EGC05011 (CN653605) or EGC01022 (BQ173439)	*E. granulosus*	21.4 / 13.9	5.8 / 5.4	6	39	1	K.ILQNEQDEEDLR.Q **R.DYGVLIEDQGIALR.G**
101	Rab5	2542 (our clustering)/ EGC04099	*E. granulosus*	- / 11.8	- / 6.8	9	57	2	K.YAQENGLLFK.E
102	Cytosolic Cu/Zn-superoxide dismutase (SOD)	BU493162	*E. multilocularis*	15.5 / 11.1	6.2 / 6.7	13	89	2	**R.YHSLAPMYYR.G + Oxidation (M)** **K.NHGAPDAAER.H**
103	Fatty acid binding protein (FABP)	EGC00017 (contig1) or EGC01240 (contig1)	*E. granulosus*	15.0 / 11.1	7.7 / 6.9	6	32	1	K.VISLSGEHSVIGR.S **K.FKEVTPDSR.E**
	Cytosolic Cu/Zn-superoxide dismutase (SOD)	BU493162	*E. multilocularis*	15.5 / 11.1	6.2 / 6.9	13	66	2	**K.NHGAPDAAER.H**
104	Fatty acid binding protein (FABP)	EGC00017 (contig1)	*E. granulosus*	15.0 / 11.1	7.7 / 8.1	16	82	2	K.VISLSGEHSVIGR.S **K.FKEVTPDSR.E**
105	HSP90	EGC00627 (contig1)	*E. granulosus*	- / 83.0	- / 5.1	8	123	3	**R.EVTSLITVENGVMK.H + Oxidation (M)** **R.APFDLFENR.K** **K.GVVDSEDLPLNISR.E**
106	Paramyosin	P35417	*E. granulosus*	98.7 / 85.2	5.2 / 5.4	6	190	5	**R.NPEDISQEEYGEFYK.S** **K.LEEAEAFATR.E** **R.LAAENFELVR.V** R.YEEEAEAAGNLR.N **R.ITELEDIAEHER.T** R.VQELQTALEDERR.A
107	Paramyosin	P35417	*E. granulosus*	98.7 / 86.0	5.2 / 5.5	7	211	5	**K.LEEAEAFATR.E** **R.LAAENFELVR.V** R.VQELQTALEDERR.A **K.DLENANAAFETAEATLR.R** **K.DLQAENEALAAENGELTHR.A**
108	78 kDa glucose regulated protein (grp78)	AAC37259	*E. granulosus*	71.9 / 74.9	5.2 / 5.3	5	58	3	**K.FDLTGIPPAPR.G** R.VEIIANDQGNR.I R.ITPSYVAFSGDGER.L
109	78 kDa glucose regulated protein (grp78)	AAC37259	*E. granulosus*	71.9 / 74.7	5.2 / 5.3	3	54	2	**K.FDLTGIPPAPR.G** R.ITPSYVAFSGDGER.L
110	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 71.3	5.7 / 5.8	5	105	2	**R.TTPSYVAFTDTER.L** **K.VSDAVVTVPAYFNDSQR.Q**
111	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 70.6	5.7 / 5.9	9	210	4	**K.VEIIANDQGNR.T** **R.TTPSYVAFTDTER.L** **K.STAGDTHLGGEDFDSR.L** **K.VSDAVVTVPAYFNDSQR.Q**
112	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 70.1	5.7 / 6.0	9	256	4	**K.VEIIANDQGNR.T**

									R.TTPSYVAFTDTER.L
									K.STAGDTHLGGEDFDSR.L
									K.VSDAVVTVPAYFNDSQR.Q
113	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 70.1	5.7 / 6.1	9	213	4	K.VEIIANDQGNR.T
									R.TTPSYVAFTDTER.L
									K.STAGDTHLGGEDFDSR.L
									K.VSDAVVTVPAYFNDSQR.Q
114	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 71.1	5.7 / 6.1	5	79	2	R.TTPSYVAFTDTER.L
									K.VSDAVVTVPAYFNDSQR.Q
115	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 69.1	5.7 / 6.2	5	65	2	R.TTPSYVAFTDTER.L
									K.VSDAVVTVPAYFNDSQR.Q
116	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 68.8	5.7 / 6.1	5	88	2	R.TTPSYVAFTDTER.L
									K.VSDAVVTVPAYFNDSQR.Q
117	HSP70	EGC00004 (contig1)	*E. granulosus*	72.5 / 68.7	5.7 / 6.0	9	207	4	K.VEIIANDQGNR.T
									R.TTPSYVAFTDTER.L
									K.STAGDTHLGGEDFDSR.L
									K.VSDAVVTVPAYFNDSQR.Q
118	Phosphoenolpyruvate carboxykinase	EGC03250 (contig1)	*E. granulosus*	- / 70.7	- / 6.7	5	42	1	R.FQTVPHVAEGVK.G
		458 (our clustering) /EGC04068 (contig1)	*E. granulosus*			6	34	1	R.RPEGIPLVYEAR.T
119	Phosphoenolpyruvate carboxykinase	EGC03250 (contig1)	*E. granulosus*	- / 70.2	- / 6.8	11	69	2	R.FQTVPHVAEGVK.G
									K.VLDYIEEQNTFVR.C
		458 (our clustering) /EGC04068 (contig1)	*E. granulosus*			6	65	1	R.RPEGIPLVYEAR.T
		191 (our clustering)/ EGC04111 (cluster1)	*E. granulosus*			10	44	1	R.AINPEAGFFGVAPGTNHK.T
120	Phosphoenolpyruvate carboxykinase	EGC03250 (contig1)	*E. granulosus*	- / 70.0	- / 6.9	9	38	2	R.FPGCMNDR.T + Oxidation (M)
									R.FQTVPHVAEGVK.G
		458 (our clustering) /EGC04068 (contig1)	*E. granulosus*			11	78	2	K.AVKEELEEQR.K
									R.RPEGIPLVYEAR.T
		191 (our clustering)/ EGC04111 (cluster1)	*E. granulosus*			10	58	1	R.AINPEAGFFGVAPGTNHK.T
121	Phosphoenolpyruvate carboxykinase	EGC03250 (contig1)	*E. granulosus*	- / 69.4	- / 7.0	5	40	1	R.FQTVPHVAEGVK.G
		458 (our clustering) /EGC04068 (contig1)	*E. granulosus*			11	50	2	K.AVKEELEEQR.K
									R.RPEGIPLVYEAR.T
		191 (our clustering)/ EGC04111 (cluster1)	*E. granulosus*			10	38	1	R.AINPEAGFFGVAPGTNHK.T
122	Calreticulin	ABQ96270	*E. granulosus*	45.3 / 58.2	4.5 / 4.7	10	118	3	R.YSAPFTKPLSSK.G
									K.GDFDDREDGGIR.T
									K.HEQNIDCGGGYVK.L
123	ß-tubulin	EGC04051 (contig1)	*E. granulosus*	- / 56.4	- / 5.1	13	82	3	R.FPGQLNADLR.K
									K.LAVNMVPFPR.L + Oxidation (M)
									R.VSEQFTAMFR.R + Oxidation (M)
		EGC03115 (CN649797)	*E. granulosus*			7	63	1	R.INVYYNEASGGK.Y
124	ß-tubulin	EGC04051 (contig1)	*E. granulosus*	- / 56.1	- / 5.2	9	62	2	K.LAVNMVPFPR.L + Oxidation (M)
									R.VSEQFTAMFR.R + Oxidation (M)

		EGC03115 (CN649797)	*E. granulosus*			7	38	1	R.INVYYNEASGGK.Y
125	ATP synthase beta subunit	EGC05083 (CN653763)	*E. granulosus*	- / 53.3	- / 5.3	6	42	1	R.TIAMDGTEGLVR.G + Oxidation (M)
			D. ryukyuensis			7	139	2	K.VVDLLAPYAR.G
		BW636734, BW640268, BW641311 or BW643972							
									R.AHGGYSVFAGVGER.T
126	ATP synthase beta subunit	EGC05083 (CN653763)	*E. granulosus*	- / 53.0	- / 5.3	8	76	1	R.LILEVAQHLGESTVR.T
127	Putative MVP protein	Q56J97	*E. granulosus*	- / 59.2	- / 5.3	3	51	1	K.ILPPNEALLLR.A
128	Putative MVP protein	Q56J97	*E. granulosus*	- / 59.0	- / 5.4	7	81	2	R.LVTGPLTFIR.K
									K.ILPPNEALLLR.A
129	Putative MVP protein	Q56J97	*E. granulosus*	- / 57.4	- / 5.5	7	65	2	R.LVTGPLTFIR.K
									K.ILPPNEALLLR.A
130	Putative MVP protein	Q56J97	*E. granulosus*	- / 57.7	- / 5.6	7	101	2	R.LVTGPLTFIR.K
									K.ILPPNEALLLR.A
131	EgAFFP	AAK00052	*E. granulosus*	42.2 / 50.9	5.9 / 5.8	6	63	2	R.HVKPDEYRPR.L
									K.HSTADEYGTAAYK.T
132	EgAFFP	AAK00052	*E. granulosus*	42.2 / 50.6	5.9 / 5.9	8	88	3	K.ELEPPKEVIK.S
									R.HVKPDEYRPR.L
									K.HSTADEYGTAAYK.T
133	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 49.2	- / 6.2	17	102	2	K.AVYAGEHFR.N
									K.AGIQIVGDDLTVTNPER.V
		EGC03002 (CN649593)	*E. granulosus*			9	43	1	R.AAVPSGASTGVHEAVELR.D
134	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 49.8	- / 6.3	28	153	3	K.AVYAGEHFR.N
									R.SGETEDSTIADIVVGLR.T
									K.AGIQIVGDDLTVTNPER.V
		EGC03002 (CN649593)	*E. granulosus*			9	30	1	R.AAVPSGASTGVHEAVELR.D
135	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 50.0	- / 6.5	17	105	2	K.AVYAGEHFR.N
									K.AGIQIVGDDLTVTNPER.V
		EGC03002 (CN649593)	*E. granulosus*			16	101	2	K.LAMQEFMILPTGAK.S + 2 Oxidation (M)
									R.AAVPSGASTGVHEAVELR.D
136	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 49.8	- / 6.5	28	222	3	K.AVYAGEHFR.N
									R.SGETEDSTIADIVVGLR.T
									K.AGIQIVGDDLTVTNPER.V
		EGC03002 (CN649593)	*E. granulosus*			16	88	2	K.LAMQEFMILPTGAK.S + 2 Oxidation (M)
									R.AAVPSGASTGVHEAVELR.D
137	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 50.2	- / 6.6	28	195	3	K.AVYAGEHFR.N
									R.SGETEDSTIADIVVGLR.T
									K.AGIQIVGDDLTVTNPER.V
		EGC03002 (CN649593)	*E. granulosus*			16	90	2	K.LAMQEFMILPTGAK.S + 2 Oxidation (M)
									R.AAVPSGASTGVHEAVELR.D
138	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 49.8	- / 6.7	28	191	3	K.AVYAGEHFR.N
									R.SGETEDSTIADIVVGLR.T
									K.AGIQIVGDDLTVTNPER.V
		EGC03002 (CN649593)	*E. granulosus*			16	82	2	K.LAMQEFMILPTGAK.S + 2 Oxidation (M)
									R.AAVPSGASTGVHEAVELR.D
139	Enolase	EGC04828 (CN653186)	*E. granulosus*	- / 50.1	- / 6.8	28	218	3	K.AVYAGEHFR.N
									R.SGETEDSTIADIVVGLR.T

									K.AGIQIVGDDLTVTNPER.V
		EGC03002 (CN649593)	*E. granulosus*			9	37	1	R.AAVPSGASTGVHEAVELR.D
140	Tropomyosin	EL741583, EL746198, EL753698, EL755360, EL758694, EL758886, EL759323, EL759503, EL759561 or EL763451	*T. solium*	– / 42.9	– / 4.7	6	73	1	R.AQEAEAEVAALQR.R
141	PP2A inhibitor I2PP2A/SET protein	EGC00630 (contig1)	*E. granulosus*	30.3 / 37.3	4.3 / 4.5	5	43	1	R.AVEVQEFDDITSGYK.I
142	Annexin	EGC00278 (contig1)	*E. granulosus*	– / 39.6	– / 4.9	8	58	1	R.LGTDEEAFITILCTR.S
143	Annexin	EGC00278 (contig1)	*E. granulosus*	– / 39.6	– / 5.0	8	72	1	R.LGTDEEAFITILCTR.S
144	Annexin	EGC00278 (contig1)	*E. granulosus*	– / 38.4	– / 4.9	8	68	1	R.LGTDEEAFITILCTR.S
145	Annexin	EGC00278 (contig1)	*E. granulosus*	– / 38.4	– / 5.0	8	82	1	R.LGTDEEAFITILCTR.S
146	Annexin	EGC00278 (contig1)	*E. granulosus*	– / 38.6	– / 5.1	8	64	1	R.LGTDEEAFITILCTR.S
147	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 47.4	5.3 / 5.3	10	131	3	R.GYSFTTTAER.E R.AVFPSIVGRPR.H K.SYELPDGQVITIGNER.F
148	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 46.7	5.3 / 5.4	15	202	4	R.GYSFTTTAER.E R.AVFPSIVGRPR.H R.VAPEEHPVLLTEAPLNPK.A K.SYELPDGQVITIGNER.F
149	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 44.9	5.3 / 5.5	15	253	4	R.GYSFTTTAER.E R.AVFPSIVGRPR.H R.VAPEEHPVLLTEAPLNPK.A K.SYELPDGQVITIGNER.F
150	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 45.9	5.3 / 5.6	10	183	3	R.GYSFTTTAER.E R.AVFPSIVGRPR.H K.SYELPDGQVITIGNER.F
151	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 47.7	5.3 / 5.7	7	76	2	R.GYSFTTTAER.E K.SYELPDGQVITIGNER.F
152	Sarcoplasmic calcium-binding protein	EGC04217 (contig1)	*E. granulosus*	– / 42.9	– / 5.8	4	45	1	R.CHDFIQIGR.A
153	Sarcoplasmic calcium-binding protein	EGC04217 (contig1)	*E. granulosus*	– / 42.5	– / 5.9	4	51	1	R.CHDFIQIGR.A
154	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 39.3	5.8 / 5.7	9	94	3	R.SIPLPPSVDR.N K.TENASHSEHR.E K.GLAIQPTEAQER.Q
155	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 38.9	5.8 / 5.9	9	122	3	R.SIPLPPSVDR.N K.TENASHSEHR.E K.GLAIQPTEAQER.Q
156	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 38.7	5.8 / 6.0	13	162	4	R.SIPLPPSVDR.N K.TENASHSEHR.E K.GLAIQPTEAQER.Q R.QSLHDDVTNMHR.N + Oxidation (M)
157	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 38.9	5.8 / 6.1	9	120	3	R.SIPLPPSVDR.N K.TENASHSEHR.E K.GLAIQPTEAQER.Q
158	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 39.0	5.8 / 6.2	9	91	3	R.SIPLPPSVDR.N K.TENASHSEHR.E K.GLAIQPTEAQER.Q

159	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 37.5	5.8 / 5.7	14	147	4	**R.SIPLPPSVDR.N** K.GLAIQPTEAQER.Q **K.TQAEMVNGHMVVEAPLFK.- + 2 Oxidati** **R.EFHPELEYTQPGELDFLK.D**
160	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 37.0	5.8 / 5.9	16	111	4	**K.TENASHSEHR.E** K.GLAIQPTEAQER.Q **R.QSLHDDVTNMHR.N + Oxidation (M)** **R.EFHPELEYTQPGELDFLK.D**
161	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 36.7	5.8 / 6.0	17	167	4	**K.TENASHSEHR.E** K.GLAIQPTEAQER.Q **K.TQAEMVNGHMVVEAPLFK.- + 2 Oxidati** **R.EFHPELEYTQPGELDFLK.D**
162	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 36.8	5.8 / 6.1	17	152	4	**K.TENASHSEHR.E** **K.GLAIQPTEAQER.Q** **K.TQAEMVNGHMVVEAPLFK.- + 2 Oxidati** **R.EFHPELEYTQPGELDFLK.D**
163	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 36.9	5.8 / 6.2	12	106	3	**K.TENASHSEHR.E** **K.GLAIQPTEAQER.Q** **R.EFHPELEYTQPGELDFLK.D**
164	HSP20 related protein	EGC00939 (contig1)	*E. granulosus*	35.8 / 37.2	5.8 / 6.4	9	91	3	**R.SIPLPPSVDR.N** **K.TENASHSEHR.E** K.GLAIQPTEAQER.Q
165	Aldo-keto reductase family member	1577 (our clustering)/ EGC00464 (contig1)	*E. granulosus*	- / 41.3	- / 6.4	17	130	3	**K.ITGSNVITFEYHK.L** **K.DKPLLEEESVLEIAER.H** **R.HIDGAVLYENEK.E** K.EHPEYPFHDEY.-
166	Aldo-keto reductase family member	1577 (our clustering)/ EGC00464 (contig1)	*E. granulosus*	- / 41.3	- / 6.5	12	116	3	**K.DKPLLEEESVLEIAER.H** **R.HIDGAVLYENEK.E** K.EHPEYPFHDEY.-
167	14-3-3 epsilon	EGC02229 (contig1)	*E. granulosus*	- / 30.1	- / 5.1	20	149	3	**R.YLAEIATASER.D** **R.YDDMAQFMHK.I + 2 Oxidation (M)** **K.IVDLNDESAPELTVEER.N**
168	14-3-3 epsilon	EGC02229 (contig1)	*E. granulosus*	- / 30.0	- / 5.1	20	120	3	**R.YLAEIATASER.D** **R.YDDMAQFMHK.I + 2 Oxidation (M)** **K.IVDLNDESAPELTVEER.N**
169	14-3-3 epsilon	EGC02229 (contig1)	*E. granulosus*	- / 30.4	- / 5.0	14	88	2	**R.YDDMAQFMHK.I + 2 Oxidation (M)** **K.IVDLNDESAPELTVEER.N**
170	14-3-3 zeta	EGC02546 (contig1)	*E. granulosus*	- / 29.0	- / 4.9	3	51	1	**R.FDKELNNEER.N**
171	14-3-3 zeta	EGC02546 (contig1)	*E. granulosus*	- / 29.2	- / 4.9	8	57	2	**R.FDKELNNEER.N** K.AYEEATEIANEK.L
172	14-3-3 zeta	EGC02757 (contig1)	*E. granulosus*	27.8 / 27.7	4.8 / 4.9	16	142	3	**R.YLAEVQEGEQNDK.S** **K.TIAEMGNELNNEER.N + Oxidation (M)** **K.ATSLAEAELSVTHPIR.L**
173	14-3-3 zeta	EGC02757 (contig1)	*E. granulosus*	27.8 / 27.5	4.8 / 5.0	11	133	2	**K.TIAEMGNELNNEER.N + Oxidation (M)** **K.ATSLAEAELSVTHPIR.L**

174	14-3-3 epsilon	EGC00123 (BF643012)	*E. granulosus*	27.9 / 28.8	4.9 / 5.0	10	77	2	**R.YVAEFCTGDER.K** **K.SATEVAEGDMQTTHPIR.L + Oxidation (M)**
175	14-3-3 epsilon	EGC00123 (BF643012)	*E. granulosus*	27.9 / 27.3	4.9 / 5.1	7	32	1	**K.SATEVAEGDMQTTHPIR.L + Oxidation (M)**
176	14-3-3 epsilon	EGC00123 (BF643012)	*E. granulosus*	27.9 / 28.4	4.9 / 5.1	25	255	5	**K.REENVYMAK.L + Oxidation (M)** **K.DATLIMQLLR.D + Oxidation (M)** **R.YVAEFCTGDER.K** **K.DVLESGADLSVEER.N** **K.SATEVAEGDMQTTHPIR.L + Oxidation (M)**
177	Annexin	EGC00272 (contig1)	*E. granulosus*	36.7 / 33.3	5.2 / 5.3	6	74	2	**K.AHVNASALYR.A** R.YYAEVLYQSMK.G + Oxidation (M)
178	Annexin	EGC00272 (contig1)	*E. granulosus*	36.7 / 33.0	5.2 / 5.4	4	77	1	**R.SLEQDVIHDVSGDFER.V**
179	Annexin	EGC00272 (contig1)	*E. granulosus*	36.7 / 32.7	5.2 / 5.5	7	118	2	**K.AHVNASALYR.A** **R.SLEQDVIHDVSGDFER.V**
180	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 28.7	5.3 / 5.5	7	82	2	**R.GYSFTTTAER.E** **K.SYELPDGQVITIGNER.F**
181	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 28.2	5.3 / 5.6	7	211	2	**R.GYSFTTTAER.E** **K.SYELPDGQVITIGNER.F**
182	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 28.0	5.3 / 5.8	7	178	2	**R.GYSFTTTAER.E** **K.SYELPDGQVITIGNER.F**
183	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 32.0	5.3 / 5.7	7	130	2	**R.GYSFTTTAER.E** **K.SYELPDGQVITIGNER.F**
184	Inorganic pyrophosphatase	EGC00895 (BQ173221)	*E. granulosus*	- / 35.4	- / 5.9	10	62	2	**R.YVNNVFPHK.G** **K.CFGESNSTEYR.M**
185	Inorganic pyrophosphatase	EGC00895 (BQ173221)	*E. granulosus*	- / 35.4	- / 6.0	10	56	2	R.YVNNVFPHK.G **K.CFGESNSTEYR.M**
186	Methylthioadenosine phosphorylase	1398 (our clustering)/ EGC02514 (contig1)	*E. granulosus*	- / 31.9	- / 6.1	21	138	3	**K.HSIMPGNVNFR.A + Oxidation (M)** **K.FMPNTYIPGEARPEHDVPR.L + Oxidation (M)** **K.VGIIGGSGLDDPEIFENQHEVR.V**
187	Methylthioadenosine phosphorylase	1398 (our clustering)/ EGC02514 (contig1)	*E. granulosus*	- / 32.0	- / 6.2	21	114	3	**K.HSIMPGNVNFR.A + Oxidation (M)** **K.FMPNTYIPGEARPEHDVPR.L + Oxidation (M)** **K.VGIIGGSGLDDPEIFENQHEVR.V**
188	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 24.3	5.3 / 5.0	7	76	2	**R.GYSFTTTAER.E** **K.SYELPDGQVITIGNER.F**
189	Actin	EGC00006 (contig2)	*E. granulosus*	41.8 / 24.3	5.3 / 5.1	7	82	2	**R.GYSFTTTAER.E** **K.SYELPDGQVITIGNER.F**
190	Hydatid disease diagnostic antigen P-29	EGC00013 (contig1)	*E. granulosus*	27.0 / 26.0	5.6 / 5.3	9	90	2	**K.MLTEASDVHQR.M + Oxidation (M)** **R.VHQESLTIFEK.T**
191	Hydatid disease diagnostic antigen P-29	EGC00013 (contig1)	*E. granulosus*	27.0 / 26.2	5.6 / 5.4	14	128	3	**K.MLTEASDVHQR.M + Oxidation (M)** K.NFLNTTLSEAQK.A **R.TSDLIHEIDQMK.A + Oxidation (M)**
192	Hydatid disease diagnostic antigen P-29	EGC00013 (contig1)	*E. granulosus*	27.0 / 25.5	5.6 / 5.6	13	141	3	**K.MLTEASDVHQR.M + Oxidation (M)** **R.VHQESLTIFEK.T** **R.TSDLIHEIDQMK.A + Oxidation (M)**
193	Hydatid disease diagnostic antigen P-29	EGC00013 (contig1)	*E. granulosus*	27.0 / 25.8	5.6 / 5.7	14	128	3	**K.MLTEASDVHQR.M + Oxidation (M)** K.NFLNTTLSEAQK.A

									R.TSDLIHEIDQMK.A + Oxidation (M)
194	Antigen Eg19	EGC00615 (contig1)	*E. granulosus*	20.3 / 15.4	4.5 / 4.6	12	35	1	R.DDESVGGDEGRPLPHEVSHEGK.Q
195	Antigen Eg19	EGC00615 (contig1)	*E. granulosus*	20.3 / 15.6	4.5 / 4.7	12	42	1	R.DDESVGGDEGRPLPHEVSHEGK.Q
196	Antigen Eg19	EGC00615 (contig1)	*E. granulosus*	20.3 / 16.0	4.5 / 4.8	17	115	2	R.DDESVGGDEGRPLPHEVSHEGK.Q
									K.GDEVDGEDRDDESVGGDEGRPLPHEVSHEGK.Q
197	TCTP, Translationally-controlled tumor protein homolog	EGC03178 (contig1)	*E. granulosus*	20.1 / 17.3	4.8 / 4.9	6	63	1	R.DDGVTPYFVFIR.D
198	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1) or EGC02722 (contig1)	*E. granulosus*	21.4 / 19.5	5.8 / 5.4	21	175	4	R.IPMLADTNHK.I + Oxidation (M)
									R.LLDAFQFTDK.H
									R.QITINDLPVGR.S
									R.DYGVLIEDQGIALR.G
199	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1) or EGC02722 (contig1)	*E. granulosus*	21.4 / 19.5	5.8 / 5.7	21	188	4	K.TFKPSAGDLK.S
									R.LLDAFQFTDK.H
									R.QITINDLPVGR.S
									R.DYGVLIEDQGIALR.G
200	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1) or EGC02722 (contig1)	*E. granulosus*	21.4 / 19.1	5.8 / 6.1	17	125	3	R.LLDAFQFTDK.H
									R.QITINDLPVGR.S
									R.DYGVLIEDQGIALR.G
201	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1), EGC02722 (contig1) or EGC01022 (BQ173439)	*E. granulosus*	21.4 / 16.0	5.8 / 5.7	12	82	2	R.QITINDLPVGR.S
									R.DYGVLIEDQGIALR.G
202	Thioredoxin peroxidase	EGC00918 (contig1)	*E. granulosus*	- / 16.2	- / 5.7	18	129	3	R.SMTINDLPVGR.S + Oxidation (M)
									R.EYEVLLEDAGVALR.G
									K.APNFAGTAVVDGQFK.E
203	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1), EGC02722 (contig1), EGC05011 (CN653605) or EGC01022 (BQ173439)	*E. granulosus*	21.4 / 17.6	5.8 / 6.1	6	47	1	R.DYGVLIEDQGIALR.G
204	peptidyl-prolyl cys-trans isomerase (Cyclophilin)	EGC00016 (contig1)	*E. granulosus*	17.3 / 16.9	6.4 / 6.1	26	91	3	K.FEDENFNHK.H
									K.CFFDISIGGKPAGR.I
205	peptidyl-prolyl cys-trans isomerase (Cyclophilin)	EGC00016 (contig1)	*E. granulosus*	17.3 / 17.3	6.4 / 6.4	42	198	5	K.FEDENFNHK.H
									R.IVFALFDDVPK.T
									K.CFFDISIGGKPAGR.I
									K.HVVFGEVESGEDVVK.D
									R.IIPGFMCQGGDFTAGNGTGGK.S + Oxidation (M)
206	peptidyl-prolyl cys-trans isomerase (Cyclophilin)	EGC00016 (contig1)	*E. granulosus*	17.3 / 17.2	6.4 / 6.7	33	131	4	K.FEDENFNHK.H
									R.IVFALFDDVPK.T
									K.CFFDISIGGKPAGR.I
									R.IIPGFMCQGGDFTAGNGTGGK.S + Oxidation (M)
207	peptidyl-prolyl cys-trans isomerase (Cyclophilin)	EGC00016 (contig1)	*E. granulosus*	17.3 / 17.1	6.4 / 7.0	33	211	4	K.FEDENFNHK.H
									R.IVFALFDDVPK.T

208	Ca-binding protein (similar to antigen SM20 de *S. mansoni*)	EL755545 or EL762844	*T. solium*	– / 13.7	– / 4.5	26 or 12	75	2	**K.CFFDISIGGKPAGR.I** **R.IIPGFMCQGGDFTAGNGTGGK.S + Oxidation (M)** **R.RGEIDVDDLR.W**
209	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1), EGC02722 (contig1) or EGC01022 (BQ173439)	*E. granulosus*	21.4 / 13.5	5.8 / 5.2	10	41	2	K.ILQNEQDEEDLR.Q R.IPMLADTNHK.I + Oxidation (M)
210	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1), EGC02722 (contig1) or EGC01022 (BQ173439)	*E. granulosus*	21.4 / 14.2	5.8 / 5.3	5	29	1	**R.QITINDLPVGR.S** **R.QITINDLPVGR.S**
211	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1) or EGC02722 (contig1)	*E. granulosus*	21.4 / 13.2	5.8 / 5.3	23	98	4	R.IPMLADTNHK.I + Oxidation (M)
212	Ferritin	EGC00020 (contig1)	*E. granulosus*	18.6 / 12.7	4.9 / 5.2	14	70	2	**R.QITINDLPVGR.S** R.DYGVLIEDQGIALR.G **K.HGEVCPANWQPGSK.T** **K.ASEEEREHAIK.L** **K.IEHEVNESLLALR.G**
213	FK506-binding protein	EGC00607 (contig1)	*E. granulosus*	11.6 / 9.9	5.8 / 6.3	8	34	1	**R.DRGQPFQFK.L**
214	Cytosolic Cu/Zn-superoxide dismutase (SOD)	BU493162	*E. multilocularis*	15.5 / 11.6	6.2 / 6.5	29	172	4	**K.NHGAPDAAER.H** **K.IHAEFEGLKPGK.H** **K.VISLSGEHSVIGR.S** R.HVGDLGNVVAGADGK.A
215	Cytosolic Cu/Zn-superoxide dismutase (SOD)	BU493162	*E. multilocularis*	15.5 / 11.4	6.2 / 6.7	20	125	3	**K.NHGAPDAAER.H** **K.IHAEFEGLKPGK.H** **K.VISLSGEHSVIGR.S**
	Fatty acid binding protein (FABP)	EGC00017 (contig1) or EGC01240 (contig1)	*E. granulosus*	15.0 /11.4	7.7 /6.7	6	54	1	**K.FKEVTPDSR.E**

a) Theoretical and observed molecular weight (M_w) and isoelectric point (p*I*) for each identification. (-) indicates that full sequence is unavailable

b) MASCOT score is – 10 x log(*P*), where *P* is the probability that the observed match is a random event. Peptide scores > 24 for *E. granulosus* database and > 42 for other databases searched, indicate identity or extensive homology ($p < 0.05$)

c) Individual peptide sequences, including any modification. Only peptides with individual ion scores above the significant threshold were considered for protein identification and their sequences are shown in bold.

Supplementary Table 2: Proteins identified in *E. granulosus* metacestode components PSC (A), HCF (B) and GL (C)

A) PSC

	Protein	LophDB cluster ID or accession number	Transcription data[a]	Organism	KOG[b]	Sequence coverage (%)	MASCOT Score[c]	Number of matched peptides	Peptide sequence[d]	Charge state
1	Actin	EGC00006 (contig1)	2 ESTs (CWGR, 2)	*E. granulosus*	Z	45	683	32	R.GILTLK.Y	2
									R.DLTDYLMK.I	2
									R.GYSFTTTAER.E	2
									R.HQGVMVGMGQK.D	2
									R.AVFPSIVGRPR.H	2
									R.AVFPSIVGRPR.H	3
									K.IWHHTFYNELR.V	2
									K.IWHHTFYNELR.V	3
									R.VAPEEHPVLLTEAPLNPK.A	2
									R.VAPEEHPVLLTEAPLNPK.A	3
									R.TTGIVLDSGDGVTHSVPIYEGYALPHAILR.L	3
									R.TTGIVLDSGDGVTHSVPIYEGYALPHAILR.L	4
2	Actin[e]	EGC00006 (contig2)	14 ESTs (CWGR, 6; PSGR, 6; PSPGR, 2)	*E. granulosus*	Z	37	705	32	K.IVAPPER.K	2
									R.DLTDYLMK.I	2
									R.DLTDYLMK.I + Oxidation (M)	2
									R.GYSFTTTAER.E	2
									K.EITALAPSTMK.I	2
									K.EITALAPSTMK.I + Oxidation (M)	2
									K.SYELPDGQVITIGNER.F	2
									R.GILTLK.Y	2
									R.HQGVMVGMGQK.D	2
									R.AVFPSIVGRPR.H	2
									R.AVFPSIVGRPR.H	3
									K.IWHHTFYNELR.V	2
									K.IWHHTFYNELR.V	3
									R.VAPEEHPVLLTEAPLNPK.A	2
									R.VAPEEHPVLLTEAPLNPK.A	3
									R.TTGIVLDSGDGVTHTVPIYEGYALPHAILR.L	4
3	ß-tubulin[f]	EGC04051 (contig1)	3 ESTs (CWGR, 2; PSGR, 1)	*E. granulosus*	Z	13	82	3	R.FPGQLNADLR.K	1
									K.LAVNMVPFPR.L + Oxidation (M)	1
									R.VSEQFTAMFR.R + Oxidation (M)	1
4	ß-tubulin[f]	EGC03115 (CN649797)	1 EST (PSGR, 1)	*E. granulosus*	Z	7	63	1	R.INVYYNEASGGK.Y	1
5	Myosin heavy chain	Q5C296	-	*S. japonicum*	Z	4	189	4	K.NLSDEIHDLTEQLGEGGR.S	3
									K.EELQAALEEAESALEQEEAK.V	3
									R.LEMEKEELQAALEEAESALEQEEAK.V	3
6	Myosin heavy chain	EX150516	-	*T. solium*	Z	11	75	1	R.LQELVDQLQGK.I	2
7	Myosin regulatory light chain	CV751995	-	*S. japonicum*	Z	6	48	1	R.DGFIDIEDLK.D	2
8	Myosin essential light chain	EGC00125 (contig1)	24 ESTs (PS, 2; PSGR, 11; PSPGR, 11)	*E. granulosus*	Z	16	150	2	K.VPDLLR.C	2
									R.EGQGVVSAAELGHVLSGYGER.L	3
9	Paramyosin[e]	P35417	-	*E. granulosus*	Z*	14	446	20	R.ELEDLLDLER.D	2
									R.LTDLEALR.S	2
									K.FNADMAALK.T	2
									K.LEEAEAFATR.E	2
									R.MILELQDLLDK.T	2
									K.HNTMISEISSEVENLQK.Q	3
									K.YVASQNALNHLK.S	3

									K.SVLEGQLDDLKR.A	2
									R.ITELEDIAEHER.T	3
									K.SQLMLEIDNVLGQLDGALK.A	2
									R.KHNTMISEISSEVENLQK.Q	3
									R.MILELQDLLDKTQIK.M	3
10	Tropomyosin A	AAB65799	-	*E. granulosus*	Z*	12	107	8	K.LQNEVDRLEDELLSEK.E	3
									K.NIQTEVDTVQESLQEAISK.L	3
11	Tropomyosin	EL752226, EL752450 or EL760880	-	*T. solium*	Z	14	129	2	K.ILDLEDELR.I	2
									R.LVHTLQGETDRLEDELVAEK.E	3
12	Tropomyosin	EL753698, EL755360 or EL763451	-	*T. solium*	Z	10	108	4	K.ILDLEDELR.I	2
									K.LDKENAIDLADQLEEQLK.E	3
13	Tropomyosin[f)]	EL755360 or EL758694	-	*T. solium*	Z	16	132	4	R.EEAYEENIR.D	1
									K.EFTFMAEDADR.K + Oxidation (M)	1
									K.SLEISEQEAAQR.E	1
									R.AQEAEAEVAALQR.R	1
14	Dynein light chain	EGC00967 (contig1)	14 ESTs (CWGR, 1; PSGR, 6; PSPGR, 7)	*E. granulosus*	Z	45	295	8	K.DIAAYLK.K	2
									K.DIAAYLKK.E	2
									K.DAMNQFTIEK.D	2
									R.NFGSYVTHETK.N	2
									K.LYSPTWHCIVGR.N	2
									K.LYSPTWHCIVGR.N	3
									K.EFDKLYSPTWHCIVGR.N	3
15	Dynein light chain	EGC00490 (contig1)	4 ESTs (PSGR, 2; PSPGR, 2)	*E. granulosus*	Z	16	272	19	R.FGSEIAYVK.D	2
									K.EQTLASYVR.K	2
16	Dynein light chain	EGC00628 (contig1)	17 ESTs (CWGR, 3; PSGR, 5; PSPGR, 9)	*E. granulosus*	Z	44	245	11	K.DIAAHIK.K	2
									K.AVDCAQDAMEK.F	2
									R.NFGSYVTHETK.N	2
									K.FNIEKDIAAHIK.K	3
									R.LFNPTWHCIVGR.N	2
									R.LFNPTWHCIVGR.N	3
17	Dynein light chain	EGC00250 (contig1)	2 ESTs (PS, 1; CWGR, 1)	*E. granulosus*	Z	21	123	3	R.AVDCAQDAMNK.Y	2
									R.NFGSYVTHETK.H	2
18	Dynein light chain	EGC03046 (contig1)	5 ESTs (CWGR, 2; PSGR, 1; PSPGR, 2)	*E. granulosus*	Z	11	61	1	R.TAIDAATEAISR.T	2
19	Mycophilin/SM22/calponin	EGC00027 (contig1)	9 ESTs (CWGR, 3; PSGR, 2; PSPGR, 3; PSPSL, 1)	*E. granulosus*	Z*	23	148	7	K.GYGVPVADLFQTVDLFEK.K	2
									R.DKDQENEALEWIEALTGLK.L	3
									K.INENATMPFK.I	2
20	Actin-filament fragmenting protein	EL741324, EL745737, EL748769 or EL763435	-	*E. granulosus*	Z	9	93	2	R.IEVTEVPISR.H	2
									K.SILEINSEVLK.V	2
21	EgAFFP[e)]	AAK00052	-	*E. granulosus*	Z*	9	77	3	R.ATSPFLMVWR.V	2
									R.EVEGYESQLFK.S	2
									K.SSAAAFLQDLESK.R	2
22	Calcium-binding protein/Centrin	EGC01120 (contig1)	6 ESTs (PSGR, 2; PSPGR, 4)	*E. granulosus*	ZD	25	42	1	K.VSAGELMAVFGDEVTHEELK.A	3
23	protein homolog[f)]	EGC03178 (contig1)	4 ESTs (PSGR, 4)	*E. granulosus*	DZ	6	63	1	R.DDGVTPYFVFIR.D	1
24	HSP90[e)]	EGC00627 (contig1)	15 ESTs (CWGR, 6; PSGR, 5; PSPGR, 1; PSPSL, 3)	*E. granulosus*	O	5	168	3	K.ESLANSAFIEVLK.K	2
									K.ADLVNNLGTIAR.S	2
									K.LGIHEDSTNR.Q	3
25	HSP70[e)]	EGC00004 (contig1)	8 ESTs (CWGR, 1; PSGR, 6; PSSL, 1)	*E. granulosus*	O*	21	130	4	K.VINSGGKPK.I	2
									K.DAGAISGLNVLR.N	2
									K.VEIIANDQGNR.T	2

									K.NQVAMNPSNTVFDAK.R	2
26	HSP20 related protein[e)]	EGC00939 (contig1)	8 ESTs (CWGR, 2; PSGR, 6)	*E. granulosus*	O	33	591	30	R.SIPLPPSVDR.N	2
									R.GDASMSESVGR.S	2
									K.NFKPEEITIK.T	2
									K.NFKPEEITIK.T	3
									K.AFVTPEVVDASK.T	2
									K.GLAIQPTEAQER.Q	2
									K.EGLEIVTADDGSK.K	2
									R.QSLHDDVTNMHR.N	3
									R.EFHPELEYTQPGELDFLK.D	3
									R.EFHPELEYTQPGELDFLKDAYEVGK.D	4
27	78 kDa glucose regulated protein (grp78)[f)]	AAC37259	-	*E. granulosus*	O*	3	54	2	K.FDLTGIPPAPR.G	1
									R.ITPSYVAFSGDGER.L	1
28	14-3-3 epsilon[e)]	EGC00123 (BF643012)	1 EST (PS, 1)	*E. granulosus*	O	9	337	16	K.IFFLK.M	2
									K.EILDLLDK.T	2
									K.DATLIMQLLR.D	2
									K.DATLIMQLLR.D + Oxidation (M)	2
29	14-3-3 epsilon[e)]	EGC02229 (contig1)	4 ESTs (CWGR, 1; PSGR, 2; PSPSL, 1)	*E. granulosus*	O	6	175	4	K.QMANEILSLLEK.T	2
30	14-3-3 zeta[e)]	EGC02757 (contig1)	6 ESTs (PSGR, 3; PSPGR, 3)	*E. granulosus*	O	27	607	16	K.DHASLVSIAK.L	2
									K.ICNDVLALLSK.N	2
									K.ATSLAEAELSVTHPIR.L	3
									K.AAFDAAITEVDSIKDETYK.D	3
									K.DSTLIMQLLR.D	2
31	14-3-3 zeta[f)]	EGC02546 (contig1)	4 ESTs (PSPGR, 3; PSPSL, 1)	*E. granulosus*	O	8	57	2	R.FDKELNNEER.N	1
									K.AYEEATEIANEK.L	1
32	Thioredoxin peroxidase (EgTPx)[e)]	EGC00084 (contig1) or EGC02722 (contig1)	10 ESTs (CWGR, 5; PSGR, 4; PSPGR, 1) or 26 ESTs (CWGR, 15; PSGR, 4; PSPGR, 7)	*E. granulosus*	O*	19	961	21	R.GLFIIDDK.G	2
									R.IPMLADTNHK.I	2
									R.IPMLADTNHK.I	3
									R.LLDAFQFTDK.H	2
									R.QITINDLPVGR.S	2
33	Thioredoxin peroxidase[f)]	EGC00918 (contig1)	10 ESTs (CWGR, 1; CWSL, 3; PSPGR, 1; PSPSL, 5)	*E. granulosus*	O	18	129	3	R.SMTINDLPVGR.S + Oxidation (M)	1
									R.EYEVLLEDAGVALR.G	1
									K.APNFAGTAVVDGQFK.E	1
34	Thioredoxin (EgTRX)	EGC00470 (contig1)	11 ESTs (PSGR, 7; PSPGR, 4)	*E. granulosus*	O	15	82	2	R.VVGANEAGIR.E	2
									R.VTAMPTLIVFK.N	2
35	Glutathione-S-transferase	EGC00080 (contig1)	2 ESTs (PSPGR, 2)	*E. granulosus*	O	10	503	12	K.LPNFEAYLGEK.E	2
									K.LTQSGAILEYIADR.H	2
36	Glutathione-S-transferase[f)]	EGC03317 (CN650164)	1 EST (PSGR, 1)	*E. granulosus*	O	8	27	1	K.AQMPFNQLPVLEVTTPR.G + Oxidation (M)	1
37	Glutathione-S-transferase[f)]	EGC04109 (contig1)	4 ESTs (CWGR, 4)	*E. granulosus*	O	12	53	3	K.REEIITFR.V	1
									K.LILEHQNFK.Y	1
									R.CLGQQYNLLGR.N	1
38	peptidyl-prolyl cys-trans isomerase (Cyclophilin)[e)]	EGC00016 (contig1)	24 ESTs (PS, 1; CWGR, 2; PSGR, 9; PSPGR, 11; SST, 1)	*E. granulosus*	O*	43	474	35	R.ALCTGEK.G	2
									R.IVFALFDDVPK.T	2
									K.HSKPMMLSMANAGK.N	3
									K.CFFDISIGGKPAGR.I	3
									K.HVVFGEVESGEDVVK.D	2
									K.HVVFGEVESGEDVVKDMEAVGSSSGK.T	3
39	FK506-binding protein[f)]	EGC00607 (contig1)	6 ESTs (PSGR, 4; PSPGR, 2)	*E. granulosus*	O	8	34	1	R.DRGQPFQFK.L	1

40	Ubiquitin-conjugating enzyme E2L	EGC00780 (contig1)	6 ESTS (CWGR, 1; CWSL, 2; PSSL, 1; PSPSL, 2)	*E. granulosus*	O	14	93	4	**K.IVDVIQALQALVANPEIEHPLR.A**	3
41	Calreticulin[f)]	ABQ96270	-	*E. granulosus*	O	14	162	4	**R.YSAPFTKPLSSK.G**	1
									K.GDFDDREDGGIR.T	1
									K.HEQNIDCGGGYVK.L	1
									K.TSETGSLVADFDMIPSK.T + Oxidation (M)	1
42	Polyubiquitin	EGC00094 (contig1), EGC00094 (contig2), EGC01137 (contig1), EGC00481 (contig1), EGC00481 (contig2), EGC02671 (contig1), EGC02662 (CN648925), EGC02744 (CN649084), EGC02689 (CN648987) or EGC02773 (CN649145)	2 ESTs (PSGR, 2); 3 ESTs (PS, 3); 3 ESTs (PSGR, 1; PSPGR, 2); 1 EST (PSGR, 1); 4 ESTs (PSGR, 2; CWGR, 1; PSPGR, 1); 2 ESTs (PSPGR, 2); 1 EST (PSPGR, 1); 1 EST (PSPGR, 1); 1 EST (PSPGR, 1); 1 EST (PSPGR, 1)	*E. granulosus*	OR	15	68	5	**R.MQIFVK.T**	2
									K.ESTLHLVLR.L	2
43	Phosphoenolpyruvate carboxykinase[e)]	EGC04068 (contig1)/ 458 (our clustering)	5 ESTs (CWGR, 5)	*E. granulosus*	C	70	1023	33	**R.VIEWALR.R**	2
									K.SEATAAAEFK.G	2
									K.HGVMIGAQVK.S	2
									R.IFHVNWFR.L	2
									R.IFHVNWFR.L	3
									R.FAWPGFGNNIR.V	2
									K.FLDDQVGVDLPK.A	2
									K.QYLLEDIEETTK.F	2
									K.YLEHWLSMEKPGR.H	3
									K.YLEHWLSMEKPGR.H	2
									R.VSGEDIAVDSPIGLLPK.K	2
									R.RVSGEDIAVDSPIGLLPK.K	3
									K.KGSINMEGLDCDWDAIFGLEK.Q	3
44	Phosphoenolpyruvate carboxykinase[e)]	EGC03250 (contig1)	5 ESTs (CWGR, 4; PSGR, 1)	*E. granulosus*	C	33	444	24	**K.TFICTPDR.F**	2
									K.DIFICDGSK.A	2
									K.GILGQWISPK.D	2
									R.FQTVPHVAEGVK.G	2
									R.FQTVPHVAEGVK.G	3
									K.AEADKITDELLK.S	2
									K.AEADKITDELLK.S	3
									K.FSDLPQVAQPFVGHWANVCTPK.D	3
45	Phosphoenolpyruvate carboxykinase[e)]	EGC04111 (cluster1)/ 191 (our clustering)	2 ESTs (CWGR, 2)	*E. granulosus*	C	38	348	7	**K.TIISHIPDDR.L**	2
									K.TIISHIPDDR.L	3
									K.FVCAAFPSACGK.T	2
									K.TNMAMLTPSLPGWK.G	2
									R.VMCVGDDIAWMR.F	2
									R.AINPEAGFFGVAPGTNHK.T	3
									R.FCCPASQCPIIHPK.V	3
46	Citrate synthase[e)]	EGC04146 (contig1)	2 ESTs (CWGR, 2)	*E. granulosus*	C	10	54	2	**R.DFALTHMPDDPLIK.I**	3
47	Citrate synthase[f)]	EGC00287 (contig1)	3 ESTs (CWGR, 3)	*E. granulosus*	C	6	56	1	**K.VGEVTVDMMYGGMR.G + 3 Oxidation (M)**	1
48	NADP-dependent isocitrate dehydrogenase[f)]	DR748835	-	*E. multilocularis*	C	8	79	2	K.YFDLGLENR.D	1
									R.EPIICSNIPR.L	1
49	Lactate dehydrogenase	EL748237	-	*T. solium*	C	25	123	3	**R.VIGSGTILDSAR.F**	2
									R.VSVGGVNLSTVYPK.F	2
									K.AVHKDVIDSAYEIIR.L	3

50	Lactate dehydrogenase	EL746063, EL749317, EL761523 or EL762110	-	*T. solium*	C	8	60	1	**K.TLLETTSGIK.W**	2
51	ATP synthase beta subunit[f]	EGC05083 (CN653763)	1 EST (CWGR, 1)	*E. granulosus*	C	8	76	1	**R.LILEVAQHLGESTVR.T**	1
52	ATP synthase beta subunit[f]	BW636734, BW640268, BW641311 or BW643972	-	*D. ryukyuensis*	C	7	139	2	**K.VVDLLAPYAR.G**	1
									R.AHGGYSVFAGVGER.T	1
53	Malate dehydrogenase precursor (mitochondrial)	Q704F5	-	*E. granulosus*	C	12	127	3	**K.VVIIPAGVPR.K**	2
									R.IGNAGTEVVNAK.A	2
									K.IAILGASGGIGQPLALLMK.Q	2
54	Malate dehydrogenase, cytoplasmic[e]	EGC00028 (contig1)	3 ESTs (CWGR, 3)	*E. granulosus*	C*	33	890	18	**K.ENFTALTR.L**	2
									K.DQGIILHLLDIPEAK.T	2
									K.DQGIILHLLDIPEAK.T	3
									K.TVLDGVVMELQDCAFTVLAGIVPTHCLK.E + Oxidation (M)	3
									K.TVLDGVVMELQDCAFTVLAGIVPTHCLK.E + Oxidation (M)	4
									K.VLVVGNPANTNCLIMSK.Y	2
55	Inorganic pyrophosphatase[f]	EGC00895 (BQ173221)	1 EST (CWGR, 1)	*E. granulosus*	C	10	62	2	**R.YVNNVFPHK.G**	1
									K.CFGESNSTEYR.M	1
56	Fructose-bisphosphate aldolase[e]	EGC00369 (contig1)	44 ESTs (CWGR, 13; CWSL, 13; PSGR, 1; PSSL, 7; PSPSL, 10)	*E. granulosus*	G	29	331	15	K.ANGAASLGK.F	2
									R.ENASAIVAPGK.G	2
									R.FVPYLCAEK.M	2
									K.DGKPFVELLR.E	2
									K.VGQAEFLQLAK.A	2
									R.VTEQVLSFVYK.A	2
									K.TKDGKPFVELLR.E	3
									R.ELLFTTDPEFAK.H	2
									R.FAAINLENTEENRR.A	3
									K.ISSHNPSYLAMLENANVLAR.Y	3
57	Glyceraldehyde-3-phosphate dehydrogenase[e]	EGC00305 (contig1)	8 ESTs (CWGR, 4; PSGR, 2; PSPGR, 2)	*E. granulosus*	G	59	461	33	**K.LTGMAFR.V**	2
									R.AGIALNDTFVK.L	2
									R.VVDLISYMFK.R	2
									R.VPTPNVSVVDLTCK.L	2
									R.YKGDVK.V	2
									K.YDSAHGR.Y	2
									K.LVIDSHR.I	2
									K.ASAHFQGGAK.K	2
									K.KVIISAPSADAPMFVVGVNHEK.Y	3
58	Putative glucose phosphate isomerase[e]	Q56JA3	-	*E. granulosus*	G	13	292	10	**K.LIELAK.A**	2
									K.LLVDYSK.N	2
									R.AVLHVALR.N	2
									R.AFTDGVLVGK.I	2
									K.EWLLEQLKDPK.A	2
									K.EWLLEQLKDPK.A	3
									K.HVRPETTLFIIASK.T	3
									K.VHFVSNIDGTHIAETLK.H	3
59	UDP-glucose pyrophosphorylase[f]	EL748674	-	*T. solium*	G	12	96	1	**K.TLDSGINIIQLEEAAGAAIR.N**	1
60	Phosphoglycerate mutase[e]	EGC03341 (contig1)	4 ESTs (PSPGR, 4)	*E. granulosus*	G	13	249	7	**R.ALPFWFDSIVPAIK.Q**	2
									K.TLNHVLEVNDLHWIPVYK.S	3
									K.TLNHVLEVNDLHWIPVYK.S	4
61	Phosphoglycerate kinase	EL740229 or EL741199	-	*T. solium*	G	13	98	2	R.AHASMVGCK.L	2
									K.ALESPTRPFLAILGGAK.V	3
62	Enolase[e]	EGC03002 (CN649593)	1 EST (PSGR, 1)	*E. granulosus*	G	48	283	14	**R.IDEFMIK.L**	2

									K.LDGSPNKGK.L	2
									R.DADKNAYMGK.G	2
									K.NVNEVIAPALIK.E	2
									K.LAMQEFMILPTGAK.S	2
									R.AAVPSGASTGVHEAVELR.D	3
									K.DVVLPVPSFNVLNGGSHAGNK.L	3
63	Enolase[e)]	EGC04828 (CN653186)	1 EST (PSGR, 1)	*E. granulosus*	G	21	435	11	**K.AVYAGEHFR.N**	2
									R.AAGWGVMVSHR.S	2
									K.VNQIGSVTESIK.A	2
64	Aldo-keto reductase family member[e)]	EGC00464 (contig1)/ 1577 (our clustering)	2 ESTs (CWGR, 1; PSGR, 1)	*E. granulosus*	R	13	97	2	**K.AMEELVTAGLVK.S**	2
									K.TPAQVLLR.H	2
65	Aldo-keto reductase family member	EGC00799 (BQ173071)	1 EST (CWGR, 1)	*E. granulosus*	R	6	108	2	**K.AMEELVSAGLVK.S**	2
66	Dj-1 protein	EGC00903 (BQ173235)	1 EST (CWGR, 1)	*E. granulosus*	RV	10	75	2	**R.LAELLTDR.A**	2
									R.GPGTATAFGLR.L	2
67	Calmodulin	EGC00516 (contig1) or EGC00348 (BI244069)	19 ESTs (CWGR, 1; PSGR, 8; PSPGR, 10) or 1 EST (CWGR, 1)	*E. granulosus*	T	10	40	1	**R.VFDKDGNGLISAAELR.H**	3
68	Sarcoplasmic calcium-binding protein[e)]	EGC04217 (contig1)	4 ESTs (CWGR, 4)	*E. granulosus*	T	4	36	1	**K.LFGALINYK.R**	2
69	Ca-binding protein (similar to antigen SM20 de *S. mansoni*)[f)]	EL755545 or EL762844	-	*T. solium*	T	26 or 12	75	2	**R.RGEIDVDDLR.W**	1
									K.ILQNEQDEEDLR.Q	1
70	EF-hand calcium-binding protein (EgCaBP II)	AAK39122	-	*E. granulosus*	T*	31	64	1	**M.PSAVEAFLQSVDKDGSGTIDTK.E**	3
71	Hydatid disease diagnostic antigen P-29[e)]	EGC00013 (contig1)	3 ESTs (PSPGR, 2; PSPSL, 1)	*E. granulosus*	T*	46	494	14	K.AAPQLSK.M	2
									K.TSYPTR.T	2
									K.TKLEEVR.L	2
									K.AKWEAEVR.K	2
									R.LDLDSDKTK.L	2
									R.AGELVNKNEK.T	2
									K.MLTEASDVHQR.M	2
									R.VHQESLTIFEK.T	2
									K.NFLNTTLSEAQK.A	2
									R.TSDLIHEIDQMK.A	2
									R.TSDLIHEIDQMK.A	3
									K.LGTALEQVASQSEK.A	2
									K.ITTTDKLGTALEQVASQSEK.A	3
72	Elongation factor 1 alpha	BAF63675/ EGC00741 (contig1)/ EGC00201 (contig1)	3 ESTs (CWGR, 1; PSPGR, 2)/ 3 ESTs (PS, 1; CWGR, 2)	*E. granulosus*	J	11	284	7	**R.LPLQDVFK.I**	2
									K.ISGIGTVPVGR.V	2
									K.YLVTIIDAPGHR.D	2
									K.YLVTIIDAPGHR.D	3
									K.GPTLLASIDLVEPPTRPVDKPLR.L	3
									K.GPTLLASIDLVEPPTRPVDKPLR.L	4
73	Annexin[f)]	EGC00272 (contig1)	5 ESTs (PS, 1; CWGR, 3; PSPGR, 1)	*E. granulosus*	U	7	118	2	**K.AHVNASALYR.A**	1
									R.SLEQDVIHDVSGDFER.V	1
74	Annexin[f)]	EGC00278 (contig1)	3 ESTs (PS, 1; CWGR, 2)	*E. granulosus*	U	8	82	1	**R.LGTDEEAFITILCTR.S**	1
75	Rab5[f)]	2542 (our clustering)/ EGC04099	2 ESTs (CWGR, 2)	*E. granulosus*	U	9	57	2	K.YAQENGLLFK.E	1
									R.YHSLAPMYYR.G + Oxidation (M)	1
76	Ferritin[f)]	EGC00020 (contig1)	4 ESTs (CWGR, 1; PSGR, 2; PSPGR, 1)	*E. granulosus*	P*	14	70	2	**K.ASEEEREHAIK.L**	1
									K.IEHEVNESLLALR.G	1
77	Cytosolic Cu/Zn-superoxide dismutase (SOD)[f)]	BU493162	-	*E. multilocularis*	P	29	172	4	**K.NHGAPDAAER.H**	1
									K.IHAEFEGLKPGK.H	1

									K.VISLSGEHSVIGR.S	1
									R.HVGDLGNVVAGADGK.A	1
78	Mn-superoxide dismutase (SOD)[f]	EGC00326 (contig1)/ 757 (our clustering)	2 ESTs (CWGR, 1; PSPSL, 1)	*E. granulosus*	P	16	83	3	**K.NQRPDYVK.A**	1
									K.GDASTIISLQPAFK.F	1
									K.HHAAYVNNLNIVEEK.M	1
79	Aminoacylase (ACY1)	EGC01496 (contig1) or EGC04785 (CN653081)	3 ESTs (CWSL, 2; PSPSL, 1) or 1 EST (PSGR, 1)	*E. granulosus*	E	6 or 4	42	1	**K.AISFSPLR.N**	2
80	Aminoacylase (ACY1)	EGC02921 (CN649454)	1 EST (PSGR, 1)	*E. granulosus*	E	5	59	1	**K.SVGIQQLEAVR.R**	2
81	Aspartate aminotransferase[f]	EL742110, EL753611, EL759324 or EL759714	-	*T. solium*	E	10	81	2	**R.DSGIAVQYYR.Y**	1
									R.NATVQCLSGTGSLR.I	1
82	Aspartate aminotransferase[f]	EL741119, EL744715 or EL748976	-	*T. solium*	E	8	43	1	**K.NVAYVAQAIHAVTK.-**	1
83	Fatty acid binding protein (FABP)[e]	EGC00017 (contig1)	15 ESTs (CWGR, 1; PSGR, 7; PSPGR, 7)	*E. granulosus*	I	25	93	3	**R.LGVDFVTR.K**	2
									K.VDEVVCVR.T	2
									K.MGNLVKPNLIVTDLGGGK.Y	3
84	UDP-glucose 4-epimerase[e]	EGC00902 (contig1)	2 ESTs (CWGR, 1; CWSL, 1)	*E. granulosus*	M	13	224	8	**R.SDPEWSVINLR.Y**	2
									K.YIGELILEDLTR.S	2
									R.IIDLLDKDALTSLFHEFK.F	3
									R.IIDLLDKDALTSLFHEFK.F	4
85	Methylthioadenosine phosphorylase[f]	EGC02514 (contig1)/ 1398 (our clustering)	2 ESTs (PSGR, 1; PSPSL, 1)	*E. granulosus*	F	21	138	3	**K.HSIMPGNVNFR.A + Oxidation (M)**	1
									K.FMPNTYIPGEARPEHDVPR.L + Oxidation (M)	1
									K.VGIIGGSGLDDPEIFENQHEVR.V	1
86	PP2A inhibitor I2PP2A/SET protein[f]	EGC00630 (contig1)	5 ESTs (PSGR, 4; PSPGR, 1)	*E. granulosus*	L	5	54	1	**R.AVEVQEFDDITSGYK.I**	1
87	putative MVP protein[e]	Q56J97	-	*E. granulosus*	no KOG	13	195	5	**R.LVTGPLTFIR.K**	2
									K.EYCILNPVKR.D	3
									R.IPGEQWLFEGPGVYKPR.K	3
88	LIM and SH3 domain protein	EGC00966 (contig1)	2 ESTs (PSGR, 1; PSPGR, 1)	*E. granulosus*	no KOG	9	36	1	**K.HYQEMVLAK.S**	2
89	Kunitz-type proteinase inhibitor	EGC01164 (BQ173695)	1 EST (PSPGR, 1)	*E. granulosus*	no KOG	11	40	1	**K.AGMCLGYFPK.W**	2
90	Putative calcium-binding EF-hand protein or Antigenic protein EpC1	Q56J99/Q869D3	-	*E. granulosus*	no KOG*	12	72	1	**K.LFDELDKDK.S**	2
91	Tegumental protein (EgTeg)	EGC00319 (contig1)	38 ESTs (CWGR, 14; PSGR, 9; PSPGR, 15)	*E. granulosus*	no KOG*	27	905	31	**K.LESLVTQLGR.T**	2
									K.NFIIDQVDDTLR.E	2
									R.EYNADSSRPIKLESLVTQLGR.T	3
									R.EYNADSSRPIKLESLVTQLGR.T	4
92	Antigen Eg19[f]	EGC00615 (contig1)	26 ESTs (PSGR, 8; PSPGR, 18)	*E. granulosus*	no KOG*	17	115	2	**R.DDESVGGDEGRPLPHEVSHEGK.Q**	1
									K.GDEVDGEDRDDESVGGDEGRPLPHEVSHEGK.Q	1
93	no significant hit (hypothetical protein)	EGC02967 (contig1)	4 ESTs (PSGR, 4)	*E. granulosus*	no KOG	11	31	3	**K.QALIAMINELR.E**	2
94	no significant hit (hypothetical protein)	EGC03348 (contig1)	2 ESTs (PSPGR, 2)	*E. granulosus*	no KOG	6	51	2	**R.MITGLLEK.H + Oxidation (M)**	2
95	Vimentin	AAI18270	-	*B. taurus*	-	2	249	7	**K.ILLAELEQLK.G**	2

a) EST data from *E. granulosus* protoscolex cDNA (PS), protoscolex full length enriched cDNA (PSGR), protoscolex trans-spliced cDNA (PSSL), pepsin-treated protoscolex full length enriched cDNA (PSPGR), pepsin-treated protoscolex trans-spliced cDNA (PSPSL), protoscoleces signal sequence trap library cDNA (SST), germinal layer full length enriched cDNA (CWGR) and germinal layer trans-spliced cDNA (CWSL).

b) KOG functional classes: (Z) Cytoskeleton; (O) Posttranslational modification, protein turnover, chaperones; (C) Energy production and conversion; (G) Carbohydrate transport and metabolism; (R) General function prediction only; (T) Signal transduction mechanisms; (J) Translation, ribosomal structure and biogenesis; (U) Intracellular trafficking, secretion, and vesicular transport; (P) Inorganic ion transport and metabolism; (E) Amino acid transport and metabolism; (I) Lipid transport and metabolism; (M) Cell wall/membrane/envelope biogenesis; (S) Function unknown; (F) Nucleotide transport and metabolism; (V) Defense mechanisms; (D) Cell cycle control, cell division, chromosome partitioning; (L) Replication, recombination and repair; no KOG, protein not related to any KOG category; (*) antigens or immunomodulatory proteins (proteins arbitrarily classified based on their prior immunological characterization according to published literature).

c) MASCOT score is – 10 x log(*P*), where *P* is the probability that the observed match is a random event. Peptide scores >24 for *E. granulosus*, >30 for *B. taurus* and >42 for other database searched, indicate identity or extensive homology ($p < 0.05$).

d) Individual peptide sequences, including any modification. Only peptides with individual ion scores above the significant threshold were considered for protein identification and their sequences are shown in bold. Non-unique peptide are shown underlined.

e) Protein identified by both 2-DE/MALDI-TOF MS/MS and LC-MS/MS analyses.

f) Protein only identified by 2-DE/MALDI-TOF MS/MS analysis.

B) HCF

	Protein	LophDB cluster ID or accession number	SignalP[a]	SecretomeP[b]	Transcription data[c]	Organism	KOG[d]	Sequence coverage (%)	MASCOT Score[e]	Number of matched peptides	Peptide sequence[f]	Charge state
1	Actin	EGC00006 (contig2)	N	0.501	14 ESTs (CWGR, 6; PSGR, 6; PSPGR, 2)	*E. granulosus*	Z	12	143	3	K.EITALAPSTMK.I	2
											K.SYELPDGQVITIGNER.F	2
											R.VAPEEHPVLLTEAPLNPK.A	3
2	Paramyosin	P35417	N	0.549	-	*E. granulosus*	Z*	6	228	5	R.LTDLEALR.S	2
											K.LEGLDSQLTR.L	2
											R.EAAESNLQVSER.K	2
											R.ITELEDIAEHER.T	3
											R.VQELQTALEDERR.A	3
3	Myosin essential light chain	EGC00125 (contig1)	N	0.241	24 ESTs (PS, 2; PSGR, 11; PSPGR, 11)	*E. granulosus*	Z	6	50	1	R.LSDEEIDEIIK.L	2
4	Dynein light chain	EGC00521 (contig1)	N	0.267	7 ESTs (PSGR, 1; PSPGR, 6)	*E. granulosus*	Z	19	156	3	R.YGDLTILMYR.L	2
											R.YGDLTIL<u>M</u>YR.L + Oxidation (M)	2
											R.LKDMLDEDIGR.H	3
5	Dynein light chain	EGC03031 (contig1)	TRUNC	TRUNC	3 ESTs (PSGR, 2; PSPGR, 1)	*E. granulosus*	Z	26	85	2	R.SWPQNLAR.R	2
											K.EIIYDLLNEAENR.S	2
6	HSP20 related protein	EGC00939 (contig1) or EGC04177 (contig1)	N	0.518	8 ESTs (CWGR, 2; PSGR, 6) or 2 ESTs (CWGR, 2)	*E. granulosus*	O	3	56	1	K.AFVTPEVVDASK.T	2
7	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1), EGC02722 (contig1) or EGC01022 (BQ173439)	N	0.582	10 ESTs (CWGR, 5; PSGR, 4; PSPGR, 1), 26 ESTs (CWGR, 15; PSGR, 4; PSPGR, 7) or 1 [illegible]	*E. granulosus*	O*	12	111	3	R.QITINDLPVGR.S	2
											R.DYGVLIEDQGIALR.G	2
8	Polyubiquitin	EGC00481 (contig1), EGC00094 (contig1), EGC02671 (contig1) or EGC02744 (CN649084)	N	0.527	5 ESTs (1 CWGR, 3 PSGR, 1 PSPGR); 5 ESTs (3 PS, 2 PSGR); 2 ESTs (2 PSPGR); 1 EST (PSPGR)	*E. granulosus*	OR	19	81	3	R.MQIFVK.T	2
											K.ESTLHLVLR.L	2
											K.TITLEVEPSDTIENVKAK.I	3
9	Citrate synthase	EGC00287 (contig1)	N	0.530	3 ESTs (CWGR, 3)	*E. granulosus*	C	12	83	2	R.DVLEEAITK.H	2
											K.GLVTETSVLDPDEGIR.F	2
10	Citrate synthase	EGC04146 (contig1)	TRUNC	TRUNC	2 ESTs (CWGR, 2)	*E. granulosus*	C	26	128	6	R.ALGLPIERPK.S	2
											K.LVPPYLESLGK.V	2
											R.DFALTHMPDDPLIK.I	2
11	Fructose-bisphosphate aldolase	EGC00369 (contig1)	N	0.420	44 ESTs (CWGR, 13; CWSL, 13; PSGR, 1; PSSL, 7; PSPSL, 10)	*E. granulosus*	G	34	521	12	K.YSVEDNAR.A	2
											K.DGKPFVELLR.E	2
											K.VGQAEFLQLAK.A	2
											R.VTEQVLSFVYK.A	2
											K.GLLAADESTSTIGK.R	2
											K.TKDGKPFVELLR.E	3
											R.ELLFTTDPEFAK.H	2
											K.GLLAADESTSTIGKR.F	3
											R.FAAINLENTEENRR.A	3
											K.ISSHNPSYLAMLENANVLAR.Y	3
											R.TVPVAVPGIVFLSGGQSELDATR.N	2
											R.TVPVAVPGIVFLSGGQSELDATR.N	3
12	Putative glucose phosphate isomerase	Q56JA3	N	0.548	-	*E. granulosus*	G	3	73	1	K.AFGINEENMFEFWDWVGGR.Y	2
13	Histone H2B	EGC00502 (contig1) or EGC01157 (BQ173683)	N	0.398 or 0.513	9 ESTs (PSGR, 7; PSPGR, 2) or 1 EST (PSPGR, 1)	*E. granulosus*	B	6	43	1	R.LLLPGELAK.H	2
14	Histone H2A	EGC03425 (contig1) or EGC03590 (CN650779)	N	0.641 or 0.626	6 ESTs (CWGR, 2; PSPGR, 4) or 1 EST (PSPGR, 1)	*E. granulosus*	B	6	37	1	R.AGLQFPVGR.V	2

15	Tetraspanin family member	EGC00129 (contig1) or EGC03207 (CN649957)	N	0.654	PSGR, 6; PSPGR, 6) or 1 EST (PSGR, 1)	*E. granulosus*	R	17	47	2	K.ALYELQMK.L	2
											K.GLGSGSLSGSDPTLK.A	2
16	Antigen 5 (Ag5)	EGC03443 (contig1)	Y	0.625	2 ESTs (CWGR, 1; PSPGR, 1)	*E. granulosus*	E*	5	64	1	**G.LELTLDPDELVK.A**	2
17	Antigen 5 (Ag5)	2704 (our clustering) or DR748735	Y	0.625	-	*E. granulosus* or *E. multilocularis*	E*	23 or 5	62	1	**R.VDSPFDVALLR.L**	2
18	Antigen B subunit 1 (AgB8/1)	CAA81235	Y	0.922	-	*E. granulosus*	no KOG*	33	200	8	**K.ELEEVFQLLR.K**	2
											K.YFFERDPLGQK.V	2
											K.YFFERDPLGQK.V	3
											K.VVDLLKELEEVFQLLR.K	3
											K.VVDLLKELEEVFQLLR.K	2
19	Tegumental protein (EgTeg)	EGC00319 (contig1)	TRUNC	TRUNC	38 ESTs (CWGR, 14; PSGR, 9; PSPGR, 15)	*E. granulosus*	no KOG*	18	94	2	**K.LESLVTQLGR.T**	2
											K.NFIIDQVDDTLR.E	2
20	LDL-receptor	EL757670	TRUNC	TRUNC	-	*T. solium*	no KOG	5	91	1	**R.GQTGFSSLQIR.R**	2
21	(Diazepam-binding inhibitor) (DBI) (Endozepine)	P07107	-	-	-	*B. taurus*	-	51	158	5	**K.AYIDKVEELK.K**	2
											K.TKPADEEMLFIYSHYK.Q	3
											K.QATVGDINTERPGMLDFK.G	3
											K.QATVGDINTERPGMLDFK.G + Oxidation (M)	3
22	Adenylyl cyclase-associated protein 1	NP_001030182	-	-	-	*B. taurus*	-	3	123	2	**R.SALFAGINQGESITHALK.H**	3
23	Albumin	NP_851335	-	-	-	*B. taurus*	-	12	371	15	K.YLYEIAR.R	2
											K.KQTALVELLK.H	2
											K.LVNELTEFAK.T	2
											K.HLVDEPQNLIK.Q	2
											R.HPEYAVSVLLR.L	2
											K.LGEYGFQNALIVR.Y	2
											R.KVPQVSTPTLVEVSR.S	2
24	Beta-galactoside-binding lectin (Galectin-1)	P11116	-	-	-	*B. taurus*	-	11	82	2	**R.LNLEAINYLSAGGDFK.I**	2
25	Beta-2-microglobulin precursor	CAA48828	-	-	-	*B. taurus*	-	11	63	2	K.IQVYSR.H	2
											K.HVTLEQPR.I	2
26	Calgranulin A (S100 calcium binding protein A8)	NP_001107197	-	-	-	*B. taurus*	-	13	59	1	**K.VGLEAHEEIHKE.-**	3
27	Complement component 1, s subcomponent	NP_001070018	-	-	-	*B. taurus*	-	2	69	1	**R.VEDPESTLFGSITR.Y**	2
28	Cystatin B	P25417	-	-	-	*B. taurus*	-	45	117	3	K.GRHDELTYF.-	2
											K.VQVDEDDFVHIR.V	3
											R.VFESLPHENKPVALTSYQTNK.G	4
29	Fc gamma receptor I (CD64)	NP_776963	-	-	-	*B. taurus*	-	9	127	3	**K.ELFPAPVLR.T**	2
											R.VFTEGDPLALR.C	2
											R.TTSSEYQTFIAK.K	2
30	Hemoglobin alpha chain	CAB56828	-	-	-	*B. taurus*	-	17	144	3	R.MFLSFPTTK.T + Oxidation (M)	2
											K.VGGHAAEYGAEALER.M	2
											K.VGGHAAEYGAEALER.M	3
31	Hemoglobin beta chain	NP_776342	-	-	-	*B. taurus*	-	40	254	6	K.VDEVGGEALGR.L	2
											R.LLVVYPWTQR.F	2
											K.VKVDEVGGEALGR.L	2
											K.EFTPVLQADFQK.V	2
											K.VVAGVANALAHR.Y	2
											K.LLGNVLVVVLAR.N	2
32	IgG1 heavy chain constant region	AAB37381	-	-	-	*B. taurus*	-	8	128	4	**K.VHNEGLPAPIVR.T**	2
											K.VHNEGLPAPIVR.T	3
											R.EPQVYVLAPPQEELSKS	2

33	IgG2a heavy chain constant region	AAB37380	-	-	-	*B. taurus*	-	9	44	2	R.VVSALPIQHQDWTGGK.E	3
											R.EPQVYVLDPPKEELSK.S	3
34	IgM heavy chain constant region	AAB62251	-	-	-	*B. taurus*	-	19	440	11	K.QISLSWFR.D	2
											R.FWTFPEVLR.D	2
											K.ALETHTYFER.H	2
											K.ALETHTYFER.H	3
											K.SSLICQATDFSPK.Q	2
											K.AEVLSPVVSVFVPPR.N	2
											K.GFAPADVFVQWLQR.G	2
											K.DVAMKPPSVYLLPPTR.E	3
											K.DVAMKPPSVYLLPPTR.E + Oxidation (M)	3
35	Immunoglobulin J chain	NP_786967	-	-	-	*B. taurus*	-	9	77	1	**R.IIPSAEDPSQDIVER.N**	2
36	Ig lambda chain C region	B30554	-	-	-	*O. aries*	-	18	58	1	**K.SAPSVTLFPPSKEELDTNK.A**	3
37	Lymphocyte cytosolic protein 1 (L-plastin)	AAI03002	-	-	-	*B. taurus*	-	3	74	2	K.ISFDEFIK.V	2
											R.GSVSDEEMLELR.E	2
38	Osteopontin	AAA30462	-	-	-	*B. taurus*	-	9	90	3	R.SNVQSPDATEEDFTSHIESEEMHDAPK.K	3
											R.SNVQSPDATEEDFTSHIESEEMHDAPK.K	4
											R.SNVQSPDATEEDFTSHIESEEMHDAPK.K + Oxidation (M)	4
39	SH3 domain binding glutamic acid-rich protein like 3	AAI02049	-	-	-	*B. taurus*	-	20	290	6	**R.IQYQLVDISQDNALR.D**	2
											R.IQYQLVDISQDNALRDEMR.A	3
											R.IQYQLVDISQDNALRDEMR.A + Oxidation (M)	3
40	Thioredoxin	NP_776393	-	-	-	*B. taurus*	-	24	151	5	**K.YAFQEALNSAGEK.L**	2
											K.MIKPFFHSLSEK.Y	3
41	Thymosin beta-4	XP_001789311	-	-	-	*B. taurus*	-	44	139	3	**K.TETQEKNPLPSK.E**	3
											K.TETQEKNPLPSK.E	2
											K.NPLPSKETIEQEK.Q	3
42	Thymosin beta-10	AAG03074	-	-	-	*B. taurus*	-	30	67	1	**K.NTLPTKETIEQEK.Q**	3

a) Signal peptide prediction by SignalP. Y and N indicate the presence or absence of a signal sequence, respectively. TRUNC indicates that sequence is truncated at the N-terminus and therefore the prediction of secretion could not be performed. Predictions of secretion for Ag5 were performed with reference sequence AAL14214 due to unavailability of complete sequences for EGC03443 and 2704 clusters or DR748735 EST.

b) SecretomeP scores. Values > 0.5 not having predicted signal peptide indicate the possibility of non-classical secretion in mammalian cells. TRUNC indicates that sequence is truncated at the N-terminus and therefore the prediction of secretion could not be performed.

c) EST data from *E. granulosus* protoscolex cDNA (PS), protoscolex full length enriched cDNA (PSGR), protoscolex trans-spliced cDNA (PSSL), pepsin-treated protoscolex full length enriched cDNA (PSPGR), pepsin-treated protoscolex trans-spliced cDNA (PSPSL), protoscoleces signal sequence trap library cDNA (SST), germinal layer full length enriched cDNA (CWGR) and germinal layer trans-spliced cDNA (CWSL).

d) KOG functional classes: (Z) Cytoskeleton; (O) Posttranslational modification, protein turnover, chaperones; (C) Energy production and conversion; (G) Carbohydrate transport and metabolism; (R) General function prediction only; (E) Amino acid transport and metabolism; (B) Chromatin structure and dynamics; no KOG, protein not related to any KOG category; (*) antigens or immunomodulatory proteins (proteins arbitrarily classified based on their prior immunological characterization according to published literature).

e) MASCOT score is – 10 x log(P), where P is the probability that the observed match is a random event. Peptide scores >24 for *E. granulosus*, >30 for *B. taurus* and >42 for other database searched, indicate identity or extensive homology ($p < 0.05$).

f) Individual peptide sequences, including any modification. Only peptides with individual ion scores above the significant threshold were considered for protein identification and their sequences are shown in bold.

C) GL

	Protein	LophDB cluster ID or accession number	Transcription data[a]	Organism	KOG[b]	Sequence coverage (%)	MASCOT Score[c]	Number of matched peptides	Peptide sequence[d]	Charge state
1	Actin	EGC00006 (contig1)	2 ESTs (CWGR, 2)	*E. granulosus*	Z	47	402	10	**K.AGFAGDDAPR.A**	2
									R.DLTDYLMK.I	2
									R.GYSFTTTAER.E	2
									R.HQGVMVGMGQK.D	2
									R.AVFPSIVGRPR.H	2
									K.DSYVGDEAQSKR.G	2
									R.VAPEEHPVLLTEAPLNPK.A	3
									R.VAPEEHPVLLTEAPLNPK.A	2
									R.TTGIVLDSGDGVTHSVPIYEGYALPHAILR.L	3
									R.TTGIVLDSGDGVTHSVPIYEGYALPHAILR.L	4
2	Actin	EGC00006 (contig2)	14 ESTs (CWGR, 6; PSGR, 6; PSPGR, 2)	*E. granulosus*	Z	36	504	11	**K.AGFAGDDAPR.A**	2
									R.DLTDYLMK.I	2
									R.GYSFTTTAER.E	2
									R.HQGVMVGMGQK.D	2
									R.AVFPSIVGRPR.H	2
									K.DSYVGDEAQSKR.G	2
									R.VAPEEHPVLLTEAPLNPK.A	3
									R.VAPEEHPVLLTEAPLNPK.A	2
									R.TTGIVLDSGDGVTHTVPIYEGYALPHAILR.L	4
									R.TTGIVLDSGDGVTHTVPIYEGYALPHAILR.L	3
									K.EITALAPSTMK.I	2
									K.SYELPDGQVITIGNER.F	2
3	Paramyosin	P35417	-	*E. granulosus*	Z*	6	232	5	**K.SQLMLEIDNVLGQLDGALK.A**	2
									R.TIEELTTTISEMEVR.F	2
									R.MILELQDLLDKTQIK.M	3
									R.MILELQDLLDKTQIK.M	2
4	Thioredoxin peroxidase (EgTPx)	EGC00084 (contig1), EGC02722 (contig1) or EGC01022 (BQ173439)	10 ESTs (CWGR, 5; PSGR, 4; PSPGR, 1), 26 ESTs (CWGR, 15; PSGR, 4; PSPGR, 7) or 1 EST (PSGR, 1)	*E. granulosus*	O*	18	112	3	**R.QITINDLPVGR.S**	2
									R.GLFIIDDKGVLR.Q	2
									R.DYGVLIEDQGIALR.G	2
5	14-3-3 protein	EGC02757 (contig1), EGC02229 (contig1) or EGC00123 (BF643012)	6 ESTs (PSGR, 3; PSPGR, 3), 4 ESTs (CWGR, 1; PSGR, 2; PSPSL, 1) or 1 EST (PS)	*E. granulosus*	O	3	31	1	**R.NLLSVAYK.N**	2
6	Glutathione-S-transferase	EGC00080 (contig1)	2 ESTs (PSPGR, 2)	*E. granulosus*	O	7	32	1	**K.FSLGLDFPNLPYYIDGDFK.L**	2
7	Citrate synthase	EGC00287 (contig1)	3 ESTs (CWGR, 3)	*E. granulosus*	C	49	251	8	R.EVVASFR.K	2
									R.ASLPSHVLK.I	2
									R.DVLEEAITK.H	2
									K.GLVTETSVLDPDEGIR.F	2
									K.STSSGSLRDVLEEAITK.H	2
									K.STSSGSLRDVLEEAITK.H	3
									K.SFPTTMHPMSQLVSATAALNSESK.F	3
									K.APGGKEPLPEGILFLLLTGQIPSEEQVK.S	2
8	Citrate synthase	EGC04146 (contig1)	2 ESTs (CWGR, 2)	*E. granulosus*	C	54	265	8	**K.SLSTDGLR.T**	2
									R.ALGLPIERPK.S	2
									K.LVPPYLESLGK.V	2
									K.EMSFYTVLFGVSR.A	2

									K.EMSFYTVLFGVSR.A + Oxidation (M)	2
									R.DFALTHMPDDPLIK.I	3
									R.DFALTHMPDDPLIK.I	2
									R.VVPGYGHAVLR.K	2
9	Phosphoenolpyruvate carboxykinase	EGC04068 (contig1)/458 (our clustering)	5 ESTs (CWGR, 5)	*E. granulosus*	C	33	184	5	K.SEATAAAEFK.G	2
									K.FLDDQVGVDLPK.A	2
									R.RPEGIPLVYEAR.T	2
									K.QYLLEDIEETTK.F	2
									R.VSGEDIAVDSPIGLLPK.K	2
10	Phosphoenolpyruvate carboxykinase	EGC03250 (contig1)	5 ESTs (CWGR, 4; PSGR, 1)	*E. granulosus*	C	18	97	3	K.GILGQWISPK.D	2
									R.FQTVPHVAEGVK.G	3
									R.TLYVIPFSMGPIGSPLAK.Y	2
11	Phosphoenolpyruvate carboxykinase	EGC04111 (cluster1)/ 191 (our clustering)	2 ESTs (CWGR, 2)	*E. granulosus*	C	6	31	1	K.TNMAMLTPSLPGWK.G	2
12	Lactate dehydrogenase	EGC00284 (contig2)	20 ESTs (CWGR, 20)	*E. granulosus*	C	29	174	4	R.VLGSGTMLDTAR.F	2
									K.LIPTLVEQSPK.C	2
									K.AVVDSAYEIIR.M	2
									K.QSLLASVETLQK.I	2
13	Pyruvate dehydrogenase E1 component	EGC05022 (CN653636)	1 EST (CWGR, 1)	*E. granulosus*	C	6	56	1	R.GESVASVIGELMGK.S	2
14	Malate dehydrogenase precursor (mitochondrial)	Q704F5	-	*E. granulosus*	C	30	405	8	R.SNTFIAEAK.G	2
									K.VVIIPAGVPR.K	2
									R.LFGVTTLDITR.S	2
									R.IQNAGTEVVNAK.A	2
									K.LNEYEIELVKK.A	3
									K.LNEYEIELVKK.A	2
									K.IAILGASGGIGQPLALLMK.Q	2
									K.QSLFVSEIALYDIANAAGVAADLSHIETR.A	3
15	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	BP187957, EE283416, DN290221, DN294018, DN304358, EG350980, EC386157, EC386168, EC386565, EG404969, EG406839, EG414978, EL619196, BW635094, BW636689, BW638802, EE667132, EE669215, EE672236, EE673373 or BU720808	-	*D. japonica*, *S. mediterranea*, *O. viverrini*, *D. ryukyuensis* or *S. japonicum*	C	6	47	1	R.LIDDMVAQALK.S	2
16	Ornithine aminotransferase	EGC00294 (BI244007)	1 EST (CWGR, 1)	*E. granulosus*	E	40	283	4	K.VLPMNTGVEGGETALK.L	2
									K.LVEALIDQAQQLTLVSR.A	2
									R.AFYTNLLGEYEEYITK.L	2
17	Ornithine aminotransferase	EL752974, EL753252, EL754566, EL756558, EL757797, EL761379 or EL763277	-	*T. solium*	E	23	179	4	R.ESMATLPK.S	2
									R.VPSDGYLR.K	2
									K.YNVLWISDEVQTGLGR.T	2
									K.LKNPNVAAFMVEPIQGEAGVR.V	3
18	Serine hydrolase similar to OVCA2	EGC04361 (CN652347)	1 EST (PSPGR, 1)	*E. granulosus*	E	7	26	1	K.EGFRTFIGQFLPR.V	2
19	Fructose-bisphosphate aldolase	EGC00369 (contig1)	44 ESTs (CWGR, 13; CWSL, 13; PSGR, 1; PSSL, 7; PSPSL, 10)	*E. granulosus*	G	25	115	6	R.ENASAIVAPGK.G	2
									K.VGQAEFLQLAK.A	2

									R.ELLFTTDPEFAK.H	2
									R.FAAINLENTEENRR.A	3
									K.ISSHNPSYLAMLENANVLAR.Y	3
									R.TVPVAVPGIVFLSGGQSELDATR.N	2
20	Calcium-binding protein	EGC03147 (contig1)	2 ESTs (PSGR, 2)	*E. granulosus*	T	11	44	1	**R.SEEGLLEYVVSVVK.S**	2
21	EF-hand calcium-binding protein (EgCaBPII)	AAK39122	-	*E. granulosus*	T*	31	83	1	**M.PSAVEAFLQSVDKDGSGTIDTK.E**	3
22	UDP-glucose 4-epimerase	EGC00902 (contig1)	2 ESTs (CWGR, 1; CWSL, 1)	*E. granulosus*	M	4	32	1	**K.YIGELILEDLTR.S**	2
23	Ferritin	EGC00020 (contig1)	4 ESTs (CWGR, 1; PSGR, 2; PSPGR, 1)	*E. granulosus*	P*	7	42	1	**K.IEHEVNESLLALR.G**	2
24	Antigen B subunit 1 (AgB8/1)	CAA81235	-	*E. granulosus*	no KOG*	20	53	1	**K.VVDLLKELEEVFQLLR.K**	2
25	Tegumental protein (EgTeg)	EGC00319 (contig1)	38 ESTs (CWGR, 14; PSGR, 9; PSPGR, 15	*E. granulosus*	no KOG*	18	268	6	**K.LESLVTQLGR.T**	2
									K.NFIIDQVDDTLR.E	2
26	Acyl-CoA-binding protein (ACBP) (Diazepam-binding inhibitor) (DBI) (Endozepine)	P07107	-	*B. taurus*	-	39	50	2	**K.TKPADEEMLFIYSHYK.Q**	3
									K.QATVGDINTERPGMLDFK.G	3
27	Albumin	NP_851335	-	*B. taurus*	-	35	1210	35	**K.VLTSSAR.Q**	2
									K.QTALVELLK.H	2
									K.KQTALVELLK.H	2
									K.LVNELTEFAK.T	2
									R.FKDLGEEHFK.G	3
									R.FKDLGEEHFK.G	2
									K.HLVDEPQNLIK.Q	2
									K.TVMENFVAFVDK.C	2
									K.SLHTLFGDELCK.V	2
									R.RHPEYAVSVLLR.L	2
									R.RHPEYAVSVLLR.L	3
									K.YICDNQDTISSK.L	2
									K.LGEYGFQNALIVR.Y	2
									K.VPQVSTPTLVEVSR.S	2
									K.DDPHACYSTVFDK.L	2
									K.DAFLGSFLYEYSR.R	2
									R.KVPQVSTPTLVEVSR.S	3
									R.MPCTEDYLSLILNR.L	2
									R.RPCFSALTPDETYVPK.A	2
									R.RPCFSALTPDETYVPK.A	3
									R.HPYFYAPELLYYANK.Y	2
									R.RHPYFYAPELLYYANK.Y	2
									R.RHPYFYAPELLYYANK.Y	3
									K.GLVLIAFSQYLQQCPFDEHVK.L	3
									K.GLVLIAFSQYLQQCPFDEHVK.L	2
28	Alpha-1 acid glycoprotein (Orosomucoid)	AAI02741	-	*B. taurus*	-	7	61	1	**K.VESDREHFVDLLLSK.H**	3
29	Alpha-2-HS-glycoprotein (Fetuin)	NP_776409	-	*B. taurus*	-	2	71	1	**K.HTLNQIDSVK.V**	2
30	Alpha-2-macroglobulin	NP_001103265	-	*B. taurus*	-	1	59	1	**R.ASFSVLGDILGSAMR.D**	2
31	Annexin A5 (Annexin V)	AAI02236	-	*B. taurus*	-	9	183	3	**K.GLGTDEESILTLLTSR.S**	2
									R.SIPAYLAETLYYAMK.G	2
32	Apolipoprotein A-IV	NP_001032557	-	*B. taurus*	-	13	207	4	**K.AMVQQLDTLR.Q**	2
									R.LTPYADQLQTK.I	2
									K.SELTQQLNTLFQDK.L	2
									K.LGPLAGDVEDHLSFLEK.D	3
33	Cathepsin K	NP_001029607	-	*B. taurus*	-	8	134	2	**R.APDSVDYR.K**	2

									R.VGPISVAIDASLTSFQFYR.K	2
34	Complement component 3	AAI12453	-	*B. taurus*	-	3	240	6	**K.LYNVEATSYALLALLAR.K**	2
									R.SQFPESWLWTVIEDLK.Q	2
									R.EPGQDLVVLPLTITSDFIPSFR.L	2
35	Complement component 5	XP_589483	-	*B. taurus*	-	2	107	3	K.LQGTLPVEAR.E	2
									K.DMNYVNPVIK.W	2
									K.DSFDHLVGGIPVTLSAK.T	2
36	Complement component 6	AAI14015	-	*B. taurus*	-	1	45	2	**R.VPIFYSSK.R**	2
									K.DLQLSDVFLK.A	2
37	Complement component 9	NP_001030441	-	*B. taurus*	-	14	336	9	**K.LVPIYDLIPVK.M**	2
									R.VWDGNTLTYYR.R	2
									R.AIEDYINEFSVR.K	2
									R.TENHEESIQILR.T	2
									R.RPWNVASLTYDTK.A	2
									K.GMGQTETNPEADLFDDVITFIR.G	2
									K.GMGQTETNPEADLFDDVITFIR.G + Oxidation (M)	2
									K.KGMGQTETNPEADLFDDVITFIR.G + Oxidation (M)	3
38	Creatine kinase	AAI02334	-	*B. taurus*	-	4	135	3	**R.LGFSEVELVQMVVDGVK.L**	2
39	Endopin 2B	AAI02747	-	*B. taurus*	-	4	46	1	**K.LFNDLDVLTNLILLNYIFFK.A**	2
40	Enolase 1 (alpha)	AAD33073	-	*B. taurus*	-	8	150	3	R.IGAEVYHNLK.N	2
									R.GNPTVEVDLFTAK.G	2
									R.YITPDELANLYK.S	2
41	Fatty acid-binding protein, liver (L-FABP)	CAA60506	-	*B. taurus*	-	14	82	2	**K.FIITAGSK.V**	2
									K.AVGMPDDIIQK.G	2
42	Fructose-1,6-bisphosphatase 1	NP_001029619	-	*B. taurus*	-	5	50	1	**K.SPIILGSPEDVTEFLEIYKK.H**	3
43	Glucose phosphate isomerase (Neuroleukin)	AAI03417	-	*B. taurus*	-	2	94	1	**K.ILLANFLAQTEALMR.G**	2
44	Glutathione S-transferase isoform 1	AAB72239	-	*B. taurus*	-	16	106	3	K.FLQPGSQR.K	2
									R.YLPAFENVLK.S	2
									K.NDGSLMFQQVPMVEIDGMK.L	2
45	Glutathione S-transferase mu 4	AAI26697	-	*B. taurus*	-	7	46	1	**K.LGLDFPNLPYLIDGAHR.L**	3
46	Hemoglobin alpha chain	CAB56828	-	*B. taurus*	-	50	310	7	R.VDPVNFK.L	2
									R.MFLSFPTTK.T	2
									K.LRVDPVNFK.L	2
									K.VGGHAAEYGAEALER.M	2
									K.TYFPHFDLSHGSAQVK.G	3
									K.AVEHLDDLPGALSELSDLHAHK.L	4
									K.AVEHLDDLPGALSELSDLHAHK.L	3
47	Hemoglobin beta chain	NP_776342	-	*B. taurus*	-	66	517	9	**K.AAVTAFWGK.V**	2
									K.VLDSFSNGMK.H	2
									K.VVAGVANALAHR.Y	2
									K.LLGNVLVVVLAR.N	2
									R.LLVVYPWTQR.F	2
									K.VKVDEVGGEALGR.L	2
									K.EFTPVLQADFQK.V	2
									R.FFESFGDLSTADAVMNNPK.V	2
48	IgG1 heavy chain constant region	AAB37381	-	*B. taurus*	-	13	87	3	**K.VHNEGLPAPIVR.T**	2
									R.EPQVYVLAPPQEELSK.S	2
									K.FSWFVDDVEVNTATTKPR.E	3
49	IgM heavy chain constant region	AAB62251	-	*B. taurus*	-	3	111	3	**K.GFAPADVFVQWLQR.G**	2

50	Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	AAX08719	-	*B. taurus*	-	2	61	1	**R.LWAYLTIQQLLEQMVSALDAEK.Q**	2
51	Lactate dehydrogenase-A	BAA14169	-	*B. taurus*	-	12	111	3	K.FIIPNIVK.Y	2
									K.LLVVSNPVDILTYVAWK.I	2
									K.DQLIQNLLKEEHVPQNK.I	3
52	Lumican	AAA30608	-	*B. taurus*	-	7	40	2	R.FSALQYLR.L	2
									K.LPSGLPVSLLTLYLDNNK.I	2
53	Matrix metallopeptidase 9 (MMP-9)	NP_777169	-	*B. taurus*	-	2	101	1	**K.LGLGPEVAQVTGALPRPEGK.V**	3
54	Matrix metallopeptidase 12 (MMP-12) (macrophage elastase)	XP_605366	-	*B. taurus*	-	4	88	2	**K.GPNLFLVAVHEIGHSLGLDHSSDTR.A**	4
55	Phosphoglycerate mutase	Q3SZ62	-	*B. taurus*	-	7	102	1	**R.TLWTVLDAIDQMWLPVVR.T**	2
56	Prostaglandin F synthase	BAA13690	-	*B. taurus*	-	18	65	5	K.FAIEAGFR.H	2
									K.LNDGHFIPVLGFGTFAPR.E	2
									K.LNDGHFIPVLGFGTFAPR.E	3
									R.IKENIQVFDFELTPEDMK.A	2
									R.IKENIQVFDFELTPEDMK.A	3
57	Regucalcin (Senescence marker protein-30)	NP_776382	-	*B. taurus*	-	14	137	3	**R.VTVDAPVSSVALR.Q**	2
									K.GLLQQPEAGGIFK.I	2
									R.YFAGTMAEETAPAVLER.R	2
58	SH3 domain binding glutamic acid-rich protein like 3	AAI02049	-	*B. taurus*	-	10	43	1	**R.VYSTSVTGSR.E**	2
59	Thioredoxin	NP_776393	-	*B. taurus*	-	12	59	1	**K.YAFQEALNSAGEK.L**	2
60	Vitronectin (Serum spreading factor)	NP_001030222	-	*B. taurus*	-	3	64	1	**R.DVWGIEGPIDAAFTR.I**	2

a) EST data from *E. granulosus* protoscolex cDNA (PS), protoscolex full length enriched cDNA (PSGR), protoscolex trans-spliced cDNA (PSSL), pepsin-treated protoscolex full length enriched cDNA (PSPGR), pepsin-treated protoscolex trans-spliced cDNA (PSPSL), protoscoleces signal sequence trap library cDNA (SST), germinal layer full length enriched cDNA (CWGR) and germinal layer trans-spliced cDNA (CWSL).

b) KOG functional classes: (Z) Cytoskeleton; (O) Posttranslational modification, protein turnover, chaperones; (C) Energy production and conversion; (G) Carbohydrate transport and metabolism; (T) Signal transduction mechanisms; (E) Amino acid transport and metabolism; (M) Cell wall/membrane/envelope biogenesis; (P) Inorganic ion transport and metabolism; no KOG, protein not related to any KOG category; (*) antigens or immunomodulatory proteins (proteins arbitrarily classified based on their prior immunological characterization according to published literature).

c) MASCOT score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Peptide scores >24 for *E. granulosus*, >30 for *B. taurus* and >42 for other database searched, indicate identity or extensive homology ($p < 0.05$).

d) Individual peptide sequences, including any modification. Only peptides with individual ion scores above the significant threshold were considered for protein identification and their sequences are shown in bold. Non-unique peptide are shown underlined.

Supporting Information Figure 1. Ag5 isoforms. (A) Alignment of Ag5 amino acid sequences, with peptides that were identified by mass spectrometry highlighted in gray. The arrowhead indicates the signal peptide cleavage site. The solid and dotted lines under the sequence indicate the Ag5 22- and 38 kDa subunits, respectively. Sequences: *E. granulosus* Ag5 (AAL14214), *E. multilocularis* EST (DR748735), *E. granulosus* LophDB cluster EGC03443 (contig1), and *E. granulosus* sequence from our EST clustering (EGC02704). (B) MS/MS spectrum of the isoform-specific tryptic peptide *VDSPFDVALLR*. The precursor ion was *m/z* 616.365300(2+) and the ion species from the y and b series that could be assigned are shown in bold.

A

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AAL14214  MARSRPLWIV FVCLFATAAL GLELTLDPDE LVKAQRESHG GFYFYDSNGA TLMFNRSLFV YRENIYDGWS RWSECSPHTC LEHRYRRCVD DSYTQPVNYL
EGC03443  MARSRPLWIV FVLFATA-AL GLELTLDPDE LVKAQRESHG GFYFYDSNGA TLMFNRSLFV HRENIYDGWS RWSECSPHTC LEHRYRRCVD DSYTQPVNYL
DR748735  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EGC02704  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

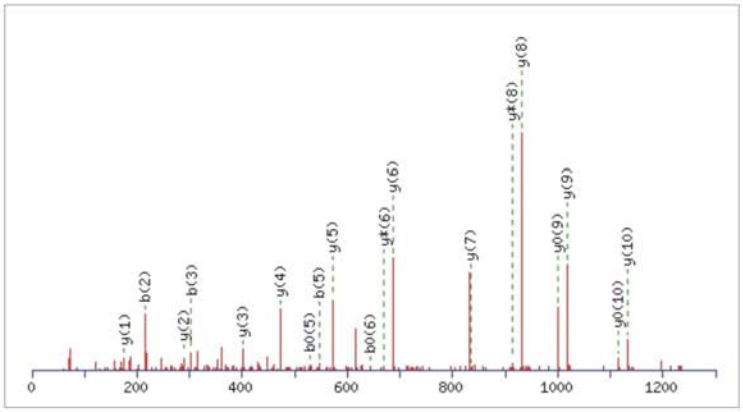
AAL14214  TSSARICPFK YIAEERPCED KSNCIINSKP SEELTRMQEK CGIRGSFDKN TAKPSRRRWK DMDDDEADDA EAEERGEYES ERLPYSLKIL GGKSAKSKSW
EGC03443  TSSARICPFK YIAEERPCED KSNCIINSKP SGELTRMQEK CGIRG----- ---------- ---------- ---------- ---------- ----------
DR748735  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EGC02704  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

AAL14214  PWHVGIYKAA NYNASEGLTR LKSENIICGG TLITPRWVLT AAHCLKPIFG SSNALPFGIP APLNTDEMKP IFLLVRAGDT VLEGTRTRNE QESDHVVAWS
EGC03443  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
DR748735  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------WS
EGC02704  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

AAL14214  SFIRIGLPNV LISPFDVALL RLETPVNIES DGAGVACVPK NADATPAEDA VCFSVGWGEK SRPISKPRRR RPTFFNPFFW PFGRLWERRP QRPTSLNEIR
EGC03443  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
DR748735  SFIRDWVPQR VDSPFDVALL RLETPVNIES DGAGVACVPK NADATPAEDA VCFSVGWGEK SRPLSKPQRR RPTFFNPFFW SFGRLWERRP QRPTSLNEIR
EGC02704  ------VAQR VDSPFDVALL RLETP----- ---------- --------DA VCFSVGWGEK SRPISKPRRR RPTFFNPFFW PFGRLWERRP QRPTSLNEIR

AAL14214  VSIDPPEKRF HHDDENEAQI CAGSSNKGVC AGDTGGGLFC RNEEDGRWYV YGVMGSGPTQ YCKSRRWLYN SVGSVIQWIN RYAV 484
EGC03443  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---- 144
DR748735  VSIDPPEKCF HHDDENEAQI CAGSSNKGVY AGDTGGGLFC RNEEDGRWYV YGVMGSGPTQ YYKSRRWLYN SVGSVIQWIN RYAV 186
EGC02704  VSIDPPEMCF HHDDENEAQI CAGSFNKGVC AGDTGGGLFC RNEEDGRWYV YGVMGSGPTQ YCKSRRWLYN SVGSVIQWIN RYAV 155
```

B



#	b	b^0	Seq	y	y*	y^0	#
1	100.0757		V				11
2	**215.1026**	197.0921	D	**1132.5997**	1115.5732	**1114.5891**	10
3	**302.1347**	284.1241	S	**1017.5728**	1000.5462	**999.5622**	9
4	399.1874	381.1769	P	**930.5407**	**913.5142**	912.5302	8
5	**546.2558**	**528.2453**	F	**833.4880**	816.4614	815.4774	7
6	661.2828	**643.2722**	D	**686.4196**	**669.3930**	668.4090	6
7	760.3512	742.3406	V	**571.3926**	554.3661		5
8	831.3883	813.3777	A	**472.3242**	455.2976		4
9	944.4724	926.4618	L	**401.2871**	384.2605		3
10	1057.5564	1039.5459	L	**288.2030**	271.1765		2
11			R	**175.1190**	158.0924		1

CAPÍTULO II

Análises estruturais de oligômeros e agregados de alto peso molecular do antígeno B de *Echinococcus granulosus*

Manuscrito a ser submetido para publicação na revista *Biochemistry*

Structural analyses of *Echinococcus granulosus* antigen B oligomers and high-molecular-weight aggregates

Monteiro, K. M., Cardoso, M. B., Follmer, C., da Silveira, N. P., Kitajima, E. W., Zaha, A., Ferreira, H. B.

Structural analyses of *Echinococcus granulosus* antigen B oligomers and high-molecular-weight aggregates[†]

[†] This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Karina M. Monteiro,[‡] Mateus B. Cardoso,[§] Cristian Follmer,[⊥] Nádya P. da Silveira,[#] Elliot W. Kitajima,[¶] Arnaldo Zaha,[‡] Henrique B. Ferreira *,[‡]

[‡] Laboratório de Biologia Molecular de Cestódeos, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[§] Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, SP, Brazil.

[⊥] Departamento de Físico-Química, Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

[#] Laboratório de Instrumentação e Dinâmica Molecular, Instituto de Química, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[¶] Departamento de Entomologia, Fitopatologia e Zoologia Agrícola, Escola Superior de Agricultura Luiz de Queiroz (ESALQ), Universidade de São Paulo, Piracicaba, SP, Brazil.

* Corresponding author. Email: henrique@cbiot.ufrgs.br. Telephone: 55-51-3316-7768. Fax: 55-51-3316-7309.

[1]Abbreviations: AFM, atomic force microscopy; AgB, antigen B; CD, circular dichroism; CHD, cystic hydatid disease; DLS, dynamic light scattering; HCF, hydatid cyst fluid; LS, light scattering; MS, mass spectrometry; TEM, transmission electronic microscopy.

Abstract

Antigen B (AgB) is the major protein secreted by the pathogenic larval stage of the tapeworm *Echinococcus granulosus,* playing key roles in host-parasite interactions. Here, the structure of the different AgB aggregation states was characterized using recombinant subunits (AgB8/1, AgB8/2 and AgB8/3) as models. We also identified the subunit composition of AgB expressed and secreted by the parasite in the hydatid cyst fluid (HCF) during infection. Using dynamic light scattering, it was demonstrated that AgB recombinant subunits aggregate in different molecular assemblies under physiological pH and temperature, forming oligomers and high-molecular-weight aggregates as those observed for AgB purified from HCF. The AgB recombinant subunits showed different aggregative tendencies, with AgB8/3 forming a higher proportion of high-molecular-weight aggregates. AgB8/3 aggregates are also the most similar, both in morphology and size, to those of parasite-produced AgB as revealed by microscopy analyses. These results suggest a major role for AgB8/3 subunit in AgB assembling. Pressure-induced dissociation experiments revealed that disulfide bonds formation confers greater stability to AgB8/2 and AgB8/3 homo-oligomers. Mass spectrometry (MS) analysis of AgB samples purified from individual hydatid cysts resulted in the identification of AgB8/1, AgB8/3 and AgB8/4 subunits, indicating that they have a major contribution for *E. granulosus* AgB structural, biological, and immunological properties. Our MS results also suggest a possible interaction between AgB and antigen 5, another major protein secreted by the *E. granulosus* larval stage.

Cystic hydatid disease (CHD)[1] is a worldwide zoonosis caused by infection with the larval stage (metacestode or hydatid cyst) of the tapeworm *Echinococcus granulosus*. This emergent/re-emergent helminthic disease represents an increasing public and animal health concern, causing important socio-economic impact in endemic areas (*1,2*). The *E. granulosus* metacestode resides within mammal tissues (mainly liver and lungs), what requires the use of several strategies to avoid the host immune response, which include the exposition and secretion of numerous immunomodulatory molecules (*3*).

Antigen B (AgB) is the major protein secreted by *E. granulosus* in the hydatid cyst fluid (HCF), and it has being the most studied *E. granulosus* protein due to its role in parasite biology and its potential for application in CHD control tools (*4,5*). AgB has been involved in several host-parasite interaction mechanisms that promote parasite establishment and survival in the intermediate host, such as protease inhibition (*6*), lipid binding (*7*) and immunomodulation (*8,9*). Furthermore, AgB is highly immunogenic in human infections, being a antigen of high diagnostic value for CHD immunodiagnosis (*10,11*). However, despite being extensively studied, several aspects of AgB molecular structure remain obscure (*12*).

AgB is homologous to hydrophobic ligand binding proteins (HLBPs), a family of cestode helix-rich proteins which bind hydrophobic compounds (*7*). It is an oligomeric lipoprotein composed by related 8 kDa subunits (*13,14*), which are encoded by a multigene family that includes at least five members (*EgAgB1-5*) (*15,16*). In SDS-PAGE, under reducing conditions, AgB dissociates into components spaced by 8 kDa (8-, 16-, 24- and 32-kDa), corresponding to multimers of 8-kDa subunits (*14,17*). In solution, AgB present different aggregation states, forming 120-160-kDa oligomers and high-molecular-weight aggregates (*13,18*).

AgB, as other proteins, can undergo multiple aggregation pathways leading to assemblies characterized by different molecular structures and morphologies (*19,20*), which could be involved in different biochemical interactions and play different physiological roles (*21,22*). AgB aggregation states and aggregation pathway are largely unknown, as well as the resulting subunit composition of AgB oligomers and high-molecular-weight aggregates. Additional studies are therefore required to characterize AgB molecular assemblies and to elucidate which subunits compose the *E. granulosus* AgB and whether they are organized as homo- or hetero-oligomers/aggregates, for example. AgB subunits could present differential expression within individuals (*15*) and/or throughout the parasite's development (*23*). Furthermore, different 8-kDa subunits have distinct physical-chemical and immunological properties (*10,18*), and may have different biological roles. Therefore, AgB subunit composition is expected to have repercussions on adaptive parasite-host relationships and also on the outcome of commonly used AgB-based hydatid disease immunodiagnostic methods (*12,15*).

Recently, we have demonstrated the self-assembly of AgB recombinant subunits in homo-oligomers with similar properties to those of parasite produced AgB, validating them as valuable tools to study AgB structure (*18*). In the present work, AgB recombinant subunits were used to investigate AgB aggregation and to structurally characterize the molecular assemblies formed by different 8-kDa subunits under physiological conditions. We have also analyzed the subunit composition of AgB samples purified from individual hydatid cysts, in order to identify the 8-kDa subunits that actually contribute to the formation of AgB oligomers and high-molecular-weight aggregates and thus to their functional, structural and immunological properties.

Experimental procedures

Protein expression and purification. AgB recombinant subunits AgB8/1, AgB8/2 and AgB8/3 were expressed in *Escherichia coli* as glutathione S-transferase fusion proteins, purified by affinity chromatography and recovered using thrombin cleavage as described previously (*18*). *E. granulosus* AgB samples were purified from parasite-enriched HCF fractions from individual cysts that were prepared as described previously (*13*). AgB purification was carried out as described by Monteiro *et al.* (*18*).

Formation of AgB recombinant homo-aggregates. AgB recombinant subunits are purified in the form of stable homo-oligomers of 120-160 kDa (*18*), forming higher-order aggregates when incubated at 37 °C for 15 min, at concentration of 125 or 375 μM in PBS. Herein, "AgB oligomers" refer to AgB oligomeric form of 120-160 kDa, and any assemblies of higher-order will be refered as "AgB aggregates or high-molecular-weight aggregates".

Dynamic light scattering (DLS). DLS experiments with AgB recombinant subunits were performed on a Brookhaven Instruments standard setup (BI-200 M goniometer, BI-9000 AT digital correlator) with a He-Ne laser (λ = 632.8 nm) as light source. Proteins samples were diluted in PBS to a final concentration of 125 μM. After centrifugation (14,000 rpm, 10 min, 4 °C), the supernatant was filtered through 0.22 μm pore diameter membranes (Millipore) into dust free cylindrical cuvettes in a laminar flow box. DLS measurements of parasite-produced AgB were performed on a DynaPro instrument (Protein Solutions Inc.), since it allows use of smaller sample volumes and the amount of *E. granulosus* AgB

samples was limited. AgB was analyzed in PBS at a 50 μg/ml concentration and DynaPro V.5 software was used for data analysis.

Light scattering measurements were conducted at a scattering angle of 90° and a water bath was used to control the temperature. The time-dependent fluctuations in the scattered intensity due to the random thermal motion of the particles were analyzed using a digital correlator and processed as an autocorrelation function (*24*). Normalized electric field autocorrelation functions $g_1(t)$, calculated from $g_2(t)$, were analyzed using the program GENDIST which employs the algorithm REPES (*25*) to performed the inverse Laplace transformation as demonstrated in Eq. (1),

$$g_2(t) - 1 = \beta \left[\int A(\tau)\exp(-t/\tau)d\tau\right]^2 \quad (1)$$

wherein t is the delay time of the correlation function and β is an instrument parameter. The resulting $A(\tau)$ is a distribution of relaxation times that generally consists of several peaks representing individual dynamic processes. Herein, the distributions of the relaxation times are shown in the equal area representation (*26*) as $\tau A(\tau)$ vs. log τ.

The mean relaxation time τ or the relaxation frequency Γ (τ^{-1}) characteristic of a dynamic process can be quantitatively associated to an apparent diffusion coefficient (D), determined according to the relation:

$$D = \Gamma/q^2 \quad (2)$$

Finally, the hydrodynamic radius (R_h) is derived from the diffusion coefficient (D) using the well-known Stokes-Einstein relation:

$$R_h = k_B T/6\pi\eta D \quad (3)$$

k_B is the Boltzmann constant, T is the absolute temperature, and η the viscosity of the solvent in the same temperature.

The percentage of each aggregate population in solution was estimated by the respective contribution to the total intensity of scattered light, dividing the scattered light intensity of each population by the total.

Transmission electron microscopy (TEM) of AgB aggregates. Formvar/carbon-coated grids were placed onto 15 μl drops of each protein sample (375 μM in PBS). After 15 min, the excess of sample was removed with filter paper and the grids were washed three times with distilled water. The bound particles were stained with 30 μl of uranyl acetate (1% aqueous solution) for 5 min and examined in a Zeiss EM 900 electron microscope operating at 50 kV.

Atomic force microscopy (AFM) of AgB aggregates. Three microliters of each protein sample at 375 μM concentration were deposited onto silicon substrates previously cleaned with ethanol and acetone. After 60 s, the excess of sample was removed by wicking with filter paper and the substrates were gently rinsed twice with 50 μl of MilliQ water to remove salts and loosely bound proteins. The samples were dried in the atmosphere at room temperature and then imaged with a DI Nanoscope IIIa microscope in the noncontact mode. The images were taken in the air, ambient conditions, at a scan rate of 0.8 Hz. AFM images were analyzed with WSxM v4.0 software (Nanotec) (*27*).

Pressure-induced dissociation of AgB recombinant oligomers. Recombinant AgB 120-160-kDa oligomers (0.2 mg/ml in 25 mM Tris, pH 7.5) were subjected to high hydrostatic pressure treatment at 25 °C, in the absence or presence of 2-mercaptoethanol. The high pressure cell equipped with optical windows has been described (*28*) and was purchased

from ISS (Champaign, IL). Average size distribution of proteins in solution was evaluated by light scattering (LS), through exciting the samples at 320 nm and collecting the scattered light at 90°, from 315 to 325 nm, in an ISS K2 spectrofluorometer. In a compression/decompression cycle, pressure was increased from 1 to 3000 bar in steps of 270 bar, and then decreased in similar steps. At each step, samples were allowed to equilibrate for 15-20 min before LS data collection. The secondary structure content of proteins before and immediately after the compression was monitored by circular dichroism (CD), as described in (*29*).

Tryptophan emission spectra were obtained by setting the excitation at 280 nm and collecting the emission in the 300-400 nm range. The mean energy of the fluorescence emission at pressure p evaluated by the center of spectral mass $\langle \nu_p \rangle$ is given by

$$\langle \nu_p \rangle = \Sigma \nu_i F_i / \Sigma F_i \quad (4)$$

where F_i is the fluorescence emitted at wavelength ν_i.

Mass spectrometry (MS) analysis of AgB. AgB samples (50 μg) from individual hydatid cysts were in-solution digested with tripsin and desalted as described by Monteiro *et al* (*30*). The peptides were analyzed by on-line liquid chromatography/mass spectrometry (LC-MS/MS) using a Waters nanoACQUITY UPLC system coupled to a Waters Micromass Q-Tof MicroTM mass spectrometer (Waters MS Technologies, UK). The peptides were eluted from the reverse-phase column towards the mass spectrometer at a flow rate of 200 nl/min, with a 10-50% water/acetonitrile 0.1% formic acid linear gradient over 30 min. Analyses were performed using the data-dependent acquisition (DDA) mode. Automatic switching was done from MS to MS/MS at precursor ion counts greater than 8, and the MS/MS collision energy was dependent on the precursor ion m/z and charge state.

MS data processing and protein identification. MS/MS raw data were processed using the ProteinLynx Global Server 2.0 software (Waters, UK), and the peak lists were exported in the micromass pkl format. Searches were performed in local *E. granulosus* and platyhelminthes EST databases described in (*30*), and in public databases. Database searches were performed using MASCOT software (Matrix Science, UK) using the following search parameters: a maximum of one missed cleavage, fixed carbamidomethyl alkylation of cysteines, variable oxidation of methionines and 0.1 mass unit tolerance on parent and fragment ions. The significance threshold was set at $p < 0.05$ and only peptides with individual ion scores above this significance threshold (typically >24 for *E. granulosus* and >42 for platyhelmithes databases) were considered for protein identification.

Results

Formation of AgB aggregates in aqueous solution. The aggregation states of AgB recombinant subunits in solution were monitored by DLS as function of temperature. The relaxation time distributions for each AgB recombinant subunit are shown in the Figure 1. At room temperature (23 °C), all recombinant subunits are organized in homo-oligomers with calculated R_h of ~ 4 nm, which correspond to the previously characterized oligomeric forms of 120-160 kDa (*18*). When the temperature was raised to 37 °C (physiological temperature), we detected the formation of soluble high-molecular-weight protein aggregates with R_h of ~ 100-200 nm and larger than 2 μm, represented by the slow relaxation modes (~ 3.5 μs and 6 μs) mainly observed in the AgB8/2 and AgB8/3 DLS measurements (Figure 1B and 1C). The aggregation was less pronounced for AgB8/1 subunit and more pronounced for AgB8/3, with the latter showing a higher proportion of high-molecular-weight aggregates than AgB8/2 or AgB8/1 (Table 1). The formation of AgB high-order aggregates was temperature-dependent, but, once formed, these aggregates remained stable upon temperature decreasing, as detected by DLS (data not shown). Different temperature-dependent aggregation properties were observed between AgB recombinant subunits, with an AgB8/3>AgB8/2>AgB8/1 aggregative tendency. The aggregation states presented by AgB8/2 and AgB8/3 are more similar to those observed for AgB purified from ***E. granulosus*** HCF, which presents, at room temperature, molecular species with calculated R_h of ~20 nm, ~100-200 nm and >2 μm (data not shown).

Ultrastructural characterization of AgB high-molecular-weight aggregates. AgB recombinant aggregates formed by incubation of subunits at 37 °C and the parasite-produced aggregates present in HCF had their ultrastructure characterized by TEM (Figure

2) and AFM (Figure 3). While AgB8/1 aggregation is less pronounced, with TEM images revealing only few small aggregates (Figure 2A), the analysis of AgB8/2 (Figure 2B), AgB8/3 (Figure 2C) and purified AgB (Figure 2D) preparations, showed large aggregates with a chain-like structure. The AFM analysis provided three-dimensional images of AgB aggregates, which present a near-globular shape, with recombinant aggregates presenting distinct size and morphology/arrangement (Figure 3A-C). Some AgB8/1 and AgB8/2 aggregates appeared as clusters of smaller globular oligomers/aggregates (insets in Figure 3A and B), while AgB8/3 appeared as large globular aggregates (Figure 3C), with size and morphology more similar to those of AgB purified from *E. granulosus* HCF (Figure 3D).

Pressure-induced dissociation of AgB recombinant 120-160-kDa oligomers. The stability of the different AgB recombinant homo-oligomers was probed by subjecting them to treatment with high hydrostatic pressures (Figure 4). The oligomeric state of protein during the pressure treatment was monitored by changes in LS. The LS signal of AgB8/1 homo-oligomer decreased by ~ 60% after compression (Figure 4A), which indicates its partial dissociation into smaller molecular species. The AgB8/2 and AgB8/3 homo-oligomers are in turn more poorly dissociated by the pressure treatment (LS decreased only ~ 30%) (Figure 4B and C, respectively), even at the highest pressure attained in our experimental setup (3000 bar). Furthermore, the CD spectra recorded before and immediately after the compression of protein samples demonstrated that AgB8/2 and AgB8/3 secondary structure was little or not perturbed by the pressure treatment (Figure 4E and F, respectively). Thus, marked differences were observed in the stability of the AgB recombinant homo-oligomers, with those of AgB8/2 and AgB8/3 presenting the higher stabilities. However, despite differences in the degree of dissociation of the AgB

recombinant oligomers, the process appears to be irreversible for all of them, as shown by the maintenance of the LS values after pressure removal (Figure 4A-C).

AgB8/2 and AgB8/3 subunits contain single cysteine residues, absent from the AgB8/1 subunit, that are involved in subunit dimerization (*18*). These residues may have an effect on AgB8/2 and AgB8/3 oligomerization, as well as on the maintenance of their oligomeric structure. Therefore, we evaluated the effect of the reducing agent 2-mercaptoethanol on the pressure-induced dissociation of AgB recombinant oligomers (Figure 5). As expected for a Cys-less subunit, 2-mercaptoethanol had no effect on dissociation of the AgB8/1 oligomer (Figure 5A), while the AgB8/2 and AgB8/3 oligomers exhibited a greater dissociation, although still incomplete (~ 50%), in the presence of the reducing agent (Figure 5B and C, respectively).

The extent of protein dissociation/unfolding during the pressure treatment was evaluated by changes in the center of spectral mass of Trp fluorescence emission. Trp residues are not present in AgB8/1 and AgB8/3 subunits and, therefore, these experiments were performed only with AgB8/2 recombinant oligomers. Figure 6 shows that the pressure treatment promoted a progressive but slight red shift of the Trp fluorescence emission, suggesting partial structural loss. Even at higher pressures (3.0 kbar), the maximum emission of Trp reached only 340.5 nm, suggesting incomplete exposure of Trp residue to the aqueous environment, since the maximum emission of Trp shift to 350 nm when it is fully exposed to the polar solvent. After decompression, the initial center of mass was completely restored, indicating reversibility of the pressure-induced conformational changes.

The presence of the 2-mercaptoethanol, at atmospheric pressure, already causes conformational changes in AgB8/2 oligomer, as can be seen in Figure 6 by the ~3 nm

difference in the spectral center of mass of Trp emission in the presence or absence of the reducing agent. At reducing conditions, a higher, but still partial, protein denaturation was observed, with the maximum emission of Trp reaching ~343 nm.

Subunit composition of parasite-produced AgB. Mass spectrometry (LC-MS/MS) was used to analyze the subunit composition of AgB purified from *E. granulosus* HCF. AgB samples analyzed were the same samples used in DLS and microscopy studies, which were immunopurified from independent HCF from two different single cysts. The same subunit composition was observed for the two analyzed samples, with identification of peptides corresponding to AgB8/1, AgB8/3 and AgB8/4 subunits (Figure 7A). The identified peptide *LVALGNDLTAICQK* can be related both to AgB8/2 and AgB8/4 sequence, but, since no other peptides corresponding to AgB8/2 were detected, this peptide was assigned to the AgB8/4 subunit. AgB8/5 subunit was also not detected in either AgB sample. In both AgB samples analyzed, in addition to AgB related peptides, we also detected peptides corresponding to antigen 5 (Ag5) (Figure 7B), another secreted protein abundant in HCF. Peptides related to both Ag5 subunits were identified, along with the tryptic peptide *VDSPFDVALLR*, that corresponds to a more recently described *E. granulosus* HCF Ag5 isoform (*30*).

Discussion

AgB is the major protein secreted by ***E. granulosus*** pathogenic larval stage, being involved in several host-parasite interactions to promote parasite survival (*4,5*). Although significant advances have been made in molecular characterization of AgB, knowledge about its structure is still very limited (*12*). The elucidation of AgB subunit composition and overall structure has both functional and practical implications, regarding its role in host-parasite interplay and its use in CHD diagnostic, therapeutic and control approaches (*12,15,23*). A better understanding of AgB structure and assembly mechanism may greatly assist in the future development of drugs able to inhibit and/or interfere with interactions between subunits, leading to disruption of the AgB oligomers/aggregates and impairing AgB-mediated ***E. granulosus*** biological functions.

In this work, we structurally characterized the different aggregation states observed in AgB purified from ***E. granulosus*** HCF, including the high-molecular-weight aggregates. Recombinant AgB subunits (AgB8/1, AgB8/2 and AgB8/3) were used as models to study AgB aggregation and to gain some insights into structural properties of different AgB subunits. DLS analysis of AgB recombinant subunits revealed that different aggregative protein states can be formed in solution under physiological conditions. From a monodisperse population of 120-160-kDa recombinant oligomers, it was possible to reproduce ***in vitro***, at physiological pH and temperature, the formation of large soluble aggregates similar to those observed in the parasite-produced AgB. These results indicate that the high-order AgB aggregates are not artifacts of the protein purification process. They can actually be formed ***in vivo*** and their formation seems to be triggered by host physiological conditions. Although these high-molecular-weight aggregates constitute a

minor population of the AgB forms found in HCF, they are soluble and formed under physiological conditions, suggesting a possible biological relevance.

Cytotoxicity has been shown to be an inherent feature of proteins able to form fibrillar aggregates *in vitro*, independently of their sequence, origin, or relation with amyloid diseases (*31*,*32*). Recent studies have also shown that, in some cases, pre-fibrillar globular aggregates represent the molecular species responsible for this toxicity (*31*-*33*). A preliminary study performed *in vitro* by Siracusano *et al.* (*4*) showed that AgB increases apoptotic rates in peripheral blood mononuclear cells from patients with active CHD. Further *in vitro* studies can determine whether AgB is able to form fibrillar aggregates and could be implicated in an additional *E. granulosus* mechanism of host immune evasion based on its toxicity to host cells.

AgB and its recombinant counterparts are structured proteins that are highly thermostable, maintaining most of their secondary structure upon thermal denaturation (*13*,*18*). Thus, AgB subunit aggregation is likely to involve a structured native conformation, unlike the major aggregation pathways that starts from fully or partially unfolded conformational states, as those of intrinsically unstructured proteins or proteins that have undergone unfolding/misfolding by chemical or physical treatments (*34*). Recent observations have demonstrated that proteins in native-like conformational states are able to self-assemble, showing that protein unfolding is not essential for aggregation, which may be initiated from locally unfolded states that become accessible via thermal fluctuations occurring under physiological conditions (*35*,*36*). Possible local conformational changes in AgB structure that could promote protein aggregation were not yet analyzed and therefore additional studies are required to ascertain whether such changes are involved in AgB aggregation. On the other hand, in a previous work using

molecular modeling of AgB subunits, it was suggested they would self-assemble into oligomers due to their amphiphilic character, thus generating a structure thermodynamically more stable, where non-polar regions would be segregated from the solvent in a hydrophobic core and polar regions would be exposed solvating the molecule (*18*). In this scenario, the temperature dependency observed in the formation of AgB high-molecular-weight aggregates is in agreement with the temperature dependency of the hydrophobic effect, which shows an increase from low to medium temperatures (*37,38*). Therefore, we believe that the association of AgB subunits into oligomers and high-order aggregates would be driven mostly by hydrophobic interactions.

This is also in line with the differential aggregative tendency observed between AgB recombinant subunits (AgB8/3>AgB8/2>AgB8/1), which may be related with different degree of charge separation/distribution detected in each AgB subunit structural models (*18*). The high-molecular-weight aggregates formed by AgB subunits presented different dimensions and morphology, as shown by the microscopy analyses. Besides presenting a higher aggregative tendency, AgB8/3 recombinant aggregates are more similar to those formed by the parasite-produced protein, both in morphology and size. These results suggest that AgB8/3 subunit may play a major role in the assembling of AgB oligomers and high-order aggregates, acting as a nucleation center to which other subunits could be attached. It is noteworthy that the AgB8/3 subunit was identified in both AgB samples analyzed by MS. However, further analyses are required to establish whether the specific fraction of high-molecular-weight aggregates observed in the purified AgB contains, or is formed only by, the AgB8/3 subunit. Differences in the morphology and size observed in the ultrastructure of purified and recombinant AgB aggregates could be

related to a different and probably heterogenous subunit composition of *E. granulosus* AgB and to its possible post-translational modifications and interactions with lipids.

The oligomeric form of 120-160 kDa is the primary and most populated protein state of AgB, being found in all recombinant subunit preparations. Therefore, we chose this oligomeric state to probe the stability of the molecular assemblies formed by each AgB recombinant subunit. Pressure-induced dissociation experiments revealed that disulfide bonds confer greater stability to the molecular assemblies formed by AgB8/2 and AgB8/3 subunits. Therefore, subunits containing cysteine residues identified in the parasite-produced AgB by this and other studies (*17*) could contribute to increase stability of the AgB oligomeric structure.

The subunit composition of AgB samples purified from HCF of individual hydatid cysts were analyzed by MS. In both AgB samples analyzed, we detected the same subunit composition, identifying AgB8/1, AgB8/3 and AgB8/4 subunits. These results indicated that these subunits are in fact expressed and secreted by the parasite during the infection and therefore would have a major contribution for the *E. granulosus* AgB structural, functional and immunological properties. The AgB8/2 and AgB8/5 subunits were not detected in AgB samples analyzed by MS, which could be expressed at very low levels. In *E. multilocularis,* AgB8/5 transcript was detected only in immature adult worms, suggesting that this subunit is not expressed in parasite's larval stage, different from AgB8/2 subunit that was detected at RNA and protein level in parasite vesicles and protoscoleces (*23*). González *et al*. (*17*) detected AgB8/1, AgB8/2 and AgB8/4 subunits in the 8-, 16-, and 24-kDa bands of *E. granulosus* AgB that are resolved on SDS-PAGE, but did not identify the AgB8/3 subunit. These discrepancies observed in relation to the AgB8/2 and AgB8/3 subunit expression could be explained by differences between

Echinococcus species and *E. granulosus* cysts and/or strains. Indeed, differences in AgB8/2 subunit expression level were previously described between different *E. granulosus* strains (*39*).

Ag5-derived peptides were detected during the analysis of the subunit composition of AgB purified from HCF, suggesting the association between the two major proteins secreted by the *E. granulosus* metacestode. The expression and secretion of these proteins in large amounts by the parasite suggests their involvement in key processes in *E. granulosus* biology (*4,5,40,41*). Since the knowledge about both AgB and Ag5 structure and function is still limited, further studies will be required to determine whether this interaction has biological implications on the *E. granulosus* metacestode survival. Additional studies are also needed to determine whether the association between AgB and Ag5 result in a reciprocal or non-reciprocal influence on their properties and functions, such as antigenicity and immunomodulation.

Although further progress is required to elucidate important aspects of the AgB structure, this work provided important information on the AgB aggregation and subunit composition, as well as on structural properties of different AgB subunits, contributing to a significant increase in the current knowledge about the protein structure. Therefore, we hope that our results may help to better understand the role of this protein into host-parasite interplay during *E. granulosus* infection and improve its application in CHD immunodiagnostic, therapeutic and vaccination approaches.

Acknowledgments

We thank the Uniprote-MS (CBIOT, UFRGS) for the support for MS analyses, specially to Dr. Paulo M. Pinto. K.M.M. is a recipient of PhD fellowship from CNPq.

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Tables

Table 1: Percentage of oligomers and high-molecular-weight aggregates formed in solution by the different AgB recombinant subunits, as monitored by DLS.

AgB recombinant subunit	R_h 4 nm (%)	R_h 100-200 nm (%)	R_h > 2μm (%)
AgB8/1	98	-	2
AgB8/2	62	25	13
AgB8/3	14	50	36

Figures

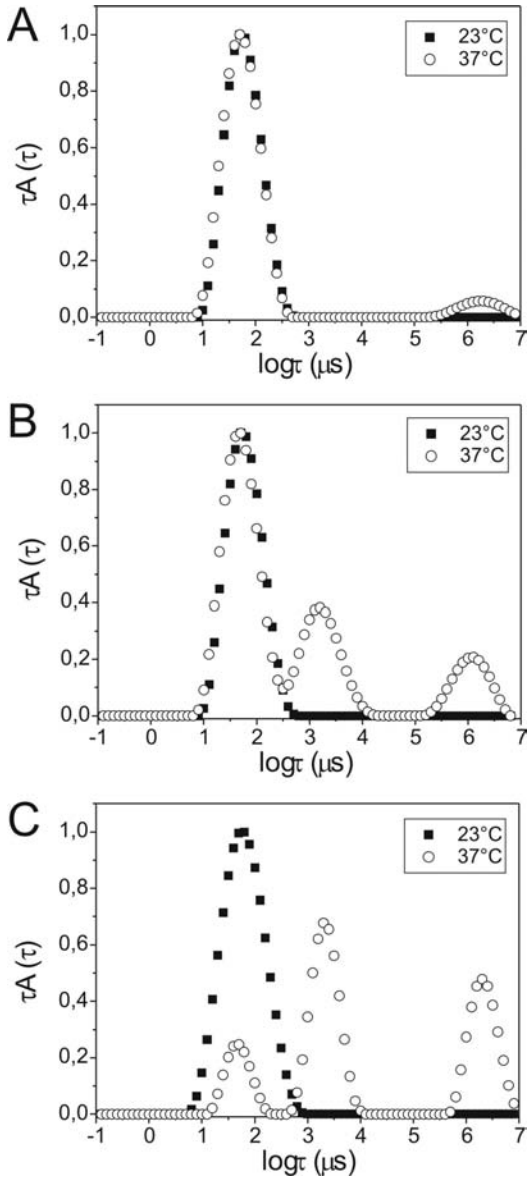

Figure 1: Detection of AgB oligomeric states in solution by DLS. The relaxation time distributions from AgB8/1 (A), AgB8/2 (B), and AgB8/3 (C), as a function of temperature. DLS was recorded at the scattering angle of 90° from 125 μM of protein samples in PBS at 23 °C (■) and 37 °C (○).

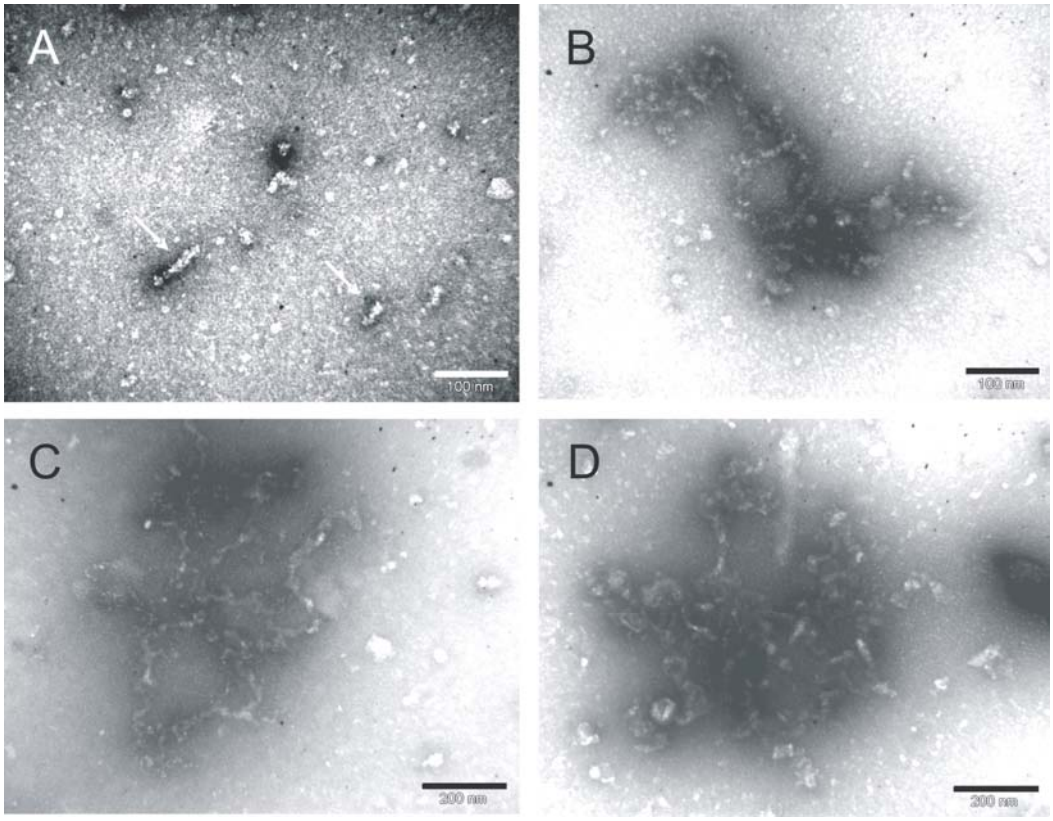

Figure 2: Ultrastructure of AgB high-molecular weight aggregates characterized by TEM. TEM images from AgB8/1 (A), AgB8/2 (B), AgB8/3 (C), and AgB (D) high-molecular high aggregates. AgB recombinant subunits are incubated under aggregating condition before sample preparation for TEM analysis. Scale bar, 100 nm (A and B) or 200 nm (C and D).

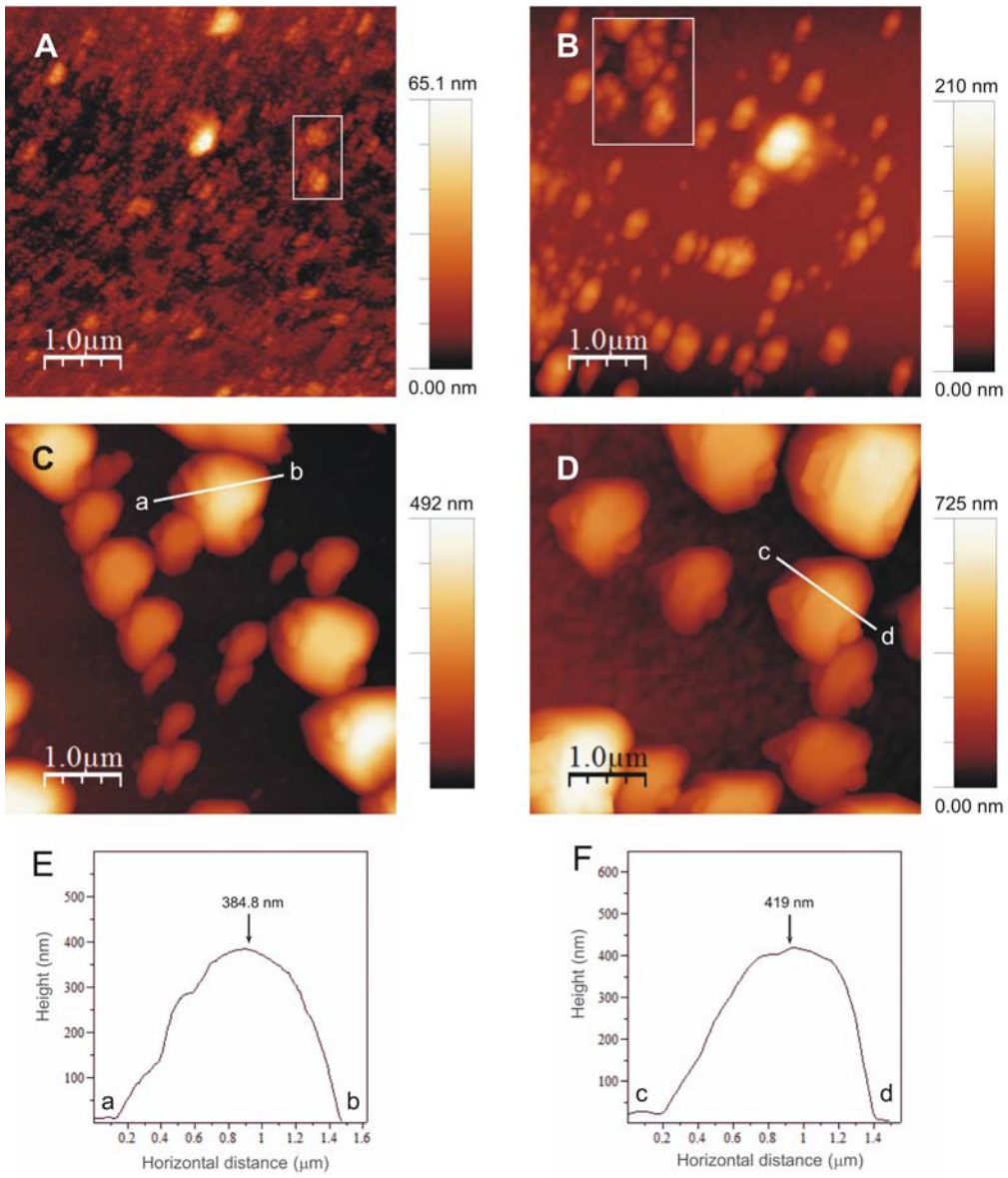

Figure 3: Morphology of AgB aggregates analyzed by AFM. AFM height images from AgB8/1 (A), AgB8/2 (B), AgB8/3 (C), and AgB (D) aggregates. Recombinant aggregates were formed by AgB subunit incubation at 37 °C for 15 min before samples preparation for AFM analysis. The insets in A and B show, respectively, some AgB8/1 and AgB8/2 aggregates formed by clusters of smaller protein structures. E and F show the AFM surface profile along A-B and C-D axes in C and D, respectively.

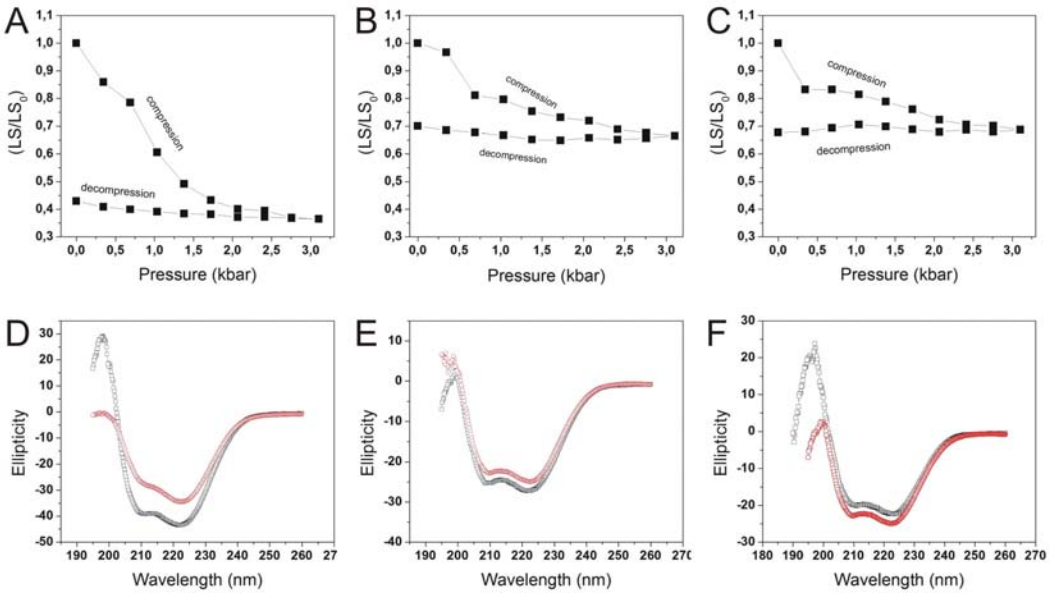

Figure 4: Pressure-induced dissociation of AgB8/1 (A and D), AgB8/2 (B and E), and AgB8/3 (C and F) 120-160-kDa oligomers and its effects in protein secondary structure. Protein samples (0.2 mg/ml in 25 mM tris buffer pH 7.5) were submitted to pressure treatment (and decompression) at 25° C and the light scattering was monitored at the steady state [LS was recorded and divided by the initial value (LS/LS_0)]. The circular dichroism spectra (D-F) were recorded before (black) and immediately after (red) pressure treatment.

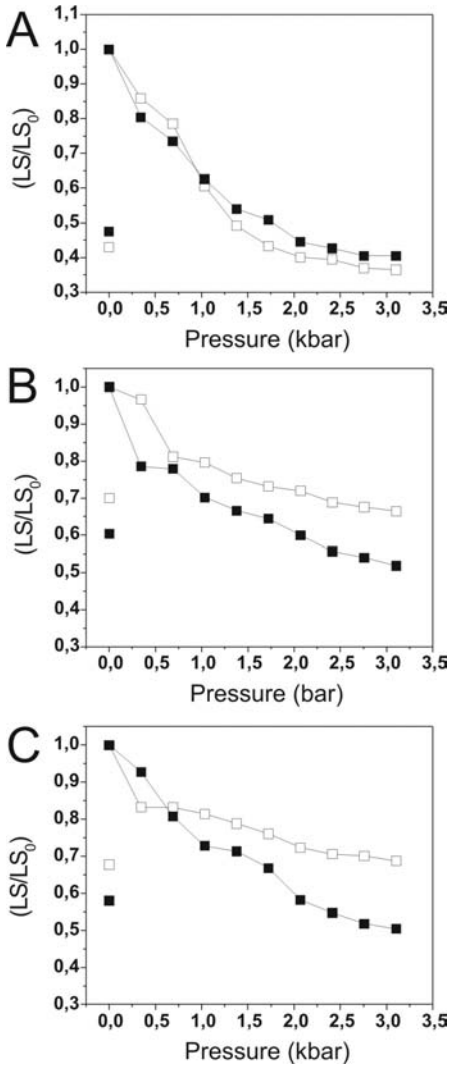

Figure 5: Effect of reducing agent 2-mercaptoethanol on the pressure-induced dissociation pattern of AgB8/1 (A), AgB8/2 (B), and AgB8/3 (C) oligomers. Protein samples (0.2 mg/mL in 25 mM Tris buffer pH 7.5), in absence (open symbols) or presence (solid symbols) of 2-mercaptoethanol, were submitted to pressure treatment at 25° C and the light scattering was monitored at the steady state [LS was recorded and divided by the initial value (LS/LS_0)]. Symbols on the left indicate the values of (LS/LS_0) after decompression.

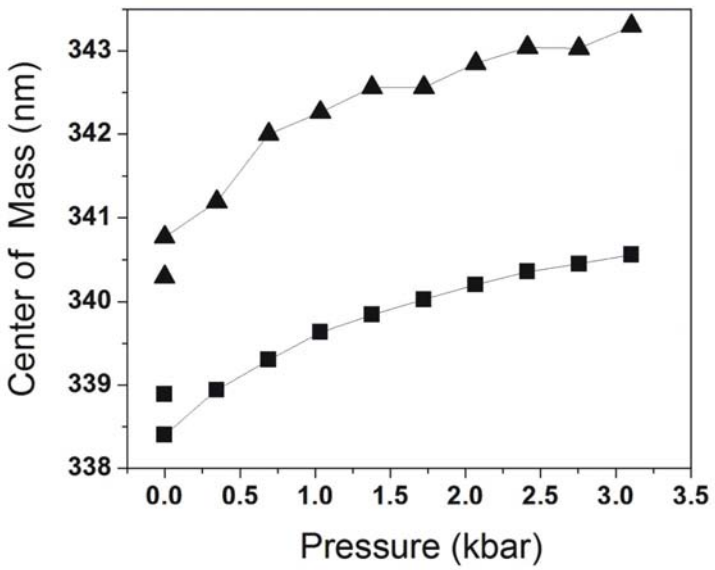

Figure 6: Effect of pressure treatment on AgB8/2 Trp fluorescence spectrum emission described by the center of spectral mass. The center of spectral mass of AgB8/2 Trp emission was followed as a function of pressure at 25 °C, in absence (■) or presence (▲) of 2-mercaptoethanol. Symbols on the left indicate the values of center of mass after decompression.

Figure 7: Peptides from AgB and Ag5 subunits identified by MS. Alignment of the amino acid sequences of the AgB subunits (A) and Ag5 isoforms (B), with peptides that were identified by mass spectrometry highlighted in gray. The identified peptide which is shared by the AgB8/2 and AgB8/4 sequences is shown underlined (dashed line) in the AgB8/2 sequence. The arrowhead indicates the signal peptide cleavage site. Sequence accession numbers: CAA81235 (AgB8/1), AAC47169 (AgB8/2), EGC03328 (LophDB cluster AgB8/3), Q6UZD9 (AgB8/4), BAE94835 (AgB8/5), AAL14214 (*E. granulosus* Ag5), DR748735 (*E. multilocularis* Ag5 EST), and EGC02704 (*E. granulosus* Ag5 sequence from our EST clustering).

3. Discussão geral, conclusões e perspectivas

Neste trabalho, diferentes abordagens proteômicas foram utilizadas para a identificação de proteínas do parasito e do hospedeiro presentes nos principais componentes do metacestódeo de *E. granulosus* (protoescólices, camada germinativa e líquido hidático) durante a infecção do hospedeiro intermediário bovino, gerando informações importantes tanto sobre a resposta montada pelo hospedeiro contra a presença do cisto hidático, como sobre as estratégias utilizadas pelo parasito para promover a sua sobrevivência dentro do hospedeiro. Uma abordagem imunoproteômica de 2-DE imunoblot foi também utilizada na identificação de proteínas antigênicas, revelando potenciais alvos para abordagens imunodiagnósticas e terapêuticas para a hidatidose cística.

Durante a infecção do hospedeiro intermediário, os protoescólices são normalmente sequestrados da resposta imune dentro da estrutura cística do metacestódeo (Riganò *et al*., 2001). Porém, se liberados no caso de uma ruptura acidental do cisto, os protoescólices necessitam de diferentes mecanismos de interação com o hospedeiro para estabelecer a infecção secundária (Virginio *et al*., 2007). Da mesma forma, diferentes estratégias de sobrevivência são necessárias para o desenvolvimento de vermes adultos de *E. granulosus* a partir de protoescólices ingeridos pelo hospedeiro definitivo canídeo (Zhang *et al*., 2003; González *et al*., 2009). Neste contexto, dentre as proteínas identificadas em extratos de protoescólices, destacam-se proteínas de detoxificação (TPx, TRx, SOD e GST), imunomoduladoras (EgTeg e paramiosina) e inibidores de proteases (inibidor do tipo Kunitz, *Eg*KU-3), as quais podem estar envolvidas em diferentes estratégias utilizadas para sobrevivência dentro do corpo do hospedeiro intermediário e definitivo, permitindo o desenvolvimento de cistos secundários e vermes adultos, respectivamente. Algumas dessas

proteínas reconhecidamente atuam na interface parasito-hospedeiro intermediário/definitivo, como por exemplo EgTPx (Li *et al*., 2004; Margutti *et al*., 2008), EgTeg (Ortona *et al*., 2005) e EgKU-3 (González *et al*., 2009), interferindo com as defesas do hospedeiro nos estágios inicias da infecção por ***E. granulosus***.

As proteínas ciclofilina e FKBP (*FK506-binding protein*), identificadas no extrato de protoescólices de ***E. granulosus***, são alvos interessantes para o desenho de novas drogas antiparasitárias, uma vez que estão envolvidas em mecanismos de suscetibilidade a diferentes compostos (Bell *et al*., 2006). Essas proteínas possuem atividade de peptidil-prolil cis-trans isomerase (PPIase ou rotamase), estando envolvidas na tradução, dobramento, montagem e transporte de proteínas, transcrição e ***splicing*** de RNA, e regulação de complexos multi-protéicos atuando com chaperonas. Drogas como a ciclosporina A e análogos da rapamicina exercem seus efeitos antiparasitários através da ligação à essas proteínas, inibindo, assim, suas atividades biológicas. Essas drogas possuem reconhecido efeito protoscolicida em ***E. granulosus*** e têm sido propostas como novos agentes terapêuticos para o tratamento da hidatidose cística em humanos (Colebrook *et al*., 2002; Cumino *et al*., 2010).

Na busca de proteínas antigênicas de ***E. granulosus***, com potencial para utilização em abordagens imunodiagnósticas e vacinação, foram realizados 2-DE imunoblots com soros de pacientes com hidatidose cística. Entre as proteínas antigênicas detectadas no extrato de protoescólices encontram-se proteínas de choque térmico, como a HSP70, HSP20 e grp78. HSPs representam antígenos dominantes em infecções por diferentes parasitos, induzindo fortes respostas imunes celular e humoral (Maresca & Kobayashi, 1994). Em ***E. granulosus***, as proteínas da família HSP70 foram descritas como antigênicas em infecções humanas (Mühlschlegel *et al*., 1995; Colebrook & Lightowlers, 1997). A

proteína HSP20 foi identificada como antigênica em uma abordagem imunoproteômica utilizando 2-DE *western blot* de proteínas de protoescólices de ***E. multilocularis*** contra soros de cães infectados (Kouguchi ***et al.***, 2010). A triagem de bibliotecas de cDNA e análises de RT-PCR e *western blot* revelaram que a HSP20 é expressa em todos os estágios de vida de ***E. multilocularis***, sendo proposta para o desenvolvimento de uma vacina contra o parasito (Merckelbach ***et al.***, 2003; Kouguchi ***et al.***, 2010).

A camada laminar é altamente permeável a macromoléculas, permitindo o trânsito de proteínas entre parasito e hospedeiro. A presença de proteínas do hospedeiro, como albumina e imunoglobulina, na camada germinativa e no líquido hidático de ***E. granulosus***, têm sido documentada (Coltorti & Varela-Díaz, 1974; Shapiro ***et al.***, 1992; Chemale ***et al.***, 2003). A grande quantidade de proteínas séricas do hospedeiro presentes na camada germinativa e líquido hidático de ***E. granulosus*** impediu a análise desses componentes parasitários por 2-DE. Esforços anteriores realizados por Chemale ***et al.*** (2003) na tentativa de padronizar as condições de 2-DE para análise de proteínas de líquido hidático, demonstraram que a contaminação massiva dessa amostra com proteínas séricas abundantes, como albumina e imunoglobulinas, impede a resolução de outras proteínas menos representadas. Nesse contexto, também não foi possível analisar as proteínas antigênicas presentes na camada germinativa e no líquido hidático utilizando metodologias de 2-DE imunoblot como realizado para protoescólices.

Metodologias de afinidade (Cibacron Blue e Proteína G) (Govorukhina ***et al.***, 2003) e ultra-filtração centrífuga (Tirumalai ***et al.***, 2003) foram utilizadas na tentativa de eliminar ou diminuir a quantidade de proteínas séricas abundantes presentes nas amostras de camada germinativa e líquido hidático. A metodologia de ultra-filtração foi eficiente na eliminação das proteínas séricas abundantes de alta massa molecular, como albumina,

imunoglobulina, transferrina e lipoproteínas (dados não mostrados). Porém, a posterior análise por LC-MS/MS da fração de baixa massa molecular resultante do processo de ultra-filtração indicou que a contaminação do líquido hidático de ***E. granulosus*** com outras proteínas séricas do hospedeiro era ainda muito significativa, prejudicando a identificação das proteínas de ES do parasito.

A seleção de amostras com menor quantidade de proteínas séricas abundantes do hospedeiro por SDS-PAGE e a padronização de uma abordagem de imunopurificação utilizando anticorpos anti-protoescólices permitiu a análise das proteínas de camada germinativa e líquido hidático por LC-MS/MS. Porém, a contaminação das amostras do metacestódeo de ***E. granulosus*** com proteínas séricas do hospedeiro ainda representa um problema para a análise proteômica do parasito, sendo necessária a padronização de novas estratégias de afinidade e/ou fracionamento na tentativa da eliminação desses contaminantes.

A camada germinativa e o líquido hidático são os principais componentes de interação com o hospedeiro durante a infecção pelo metacestódeo de ***E. granulosus***. Por isso, estes componentes parasitários foram analisados na busca de proteínas potencialmente envolvidas em mecanismos de interação parasito-hospedeiro, constituindo alvos interessantes para abordagens imunodiagnósticas e terapêuticas.

A paramiosina foi identificada em todos os componentes do metacestódeo de ***E. granulosus***. Esta proteína é um componente estrutural do músculo de invertebrados, tendo sido identificada em um grande número de parasitos helmintos de humanos e animais domésticos (Gobert & McManus, 2005). A paramiosina é uma proteína altamente imunogênica, sendo capaz de induzir proteção contra a infecção por ***Schistosoma*** (Zhou ***et al***., 2000) e ***Taenia solium*** (Vázquez-Talavera ***et al***., 2001). Além de seu potencial vacinal,

a paramiosina possui diferentes propriedades imunomoduladoras. Loukas *et al.* (2001) demonstraram que a paramiosina de esquistossomos funciona como um receptor Fc, ligando imunoglobulinas do hospedeiro num mecanismo de evasão da resposta imune. A evasão das respostas do hospedeiro pode se dar pelo mascaramento de antígenos de superfície ou pela perturbação das funções dependentes de Fc, como a interação com fagócitos (Pleass & Woof, 2001). A paramiosina de helmintos é também capaz de ligar componentes do sistema complemento, como C1, C8 e C9, inibindo a ativação dos passos iniciais e terminais da cascata do sistema complemento (Laclette et al., 1992; Deng *et al*., 2003).

As tetraspaninas constituem uma família de proteínas de superfície celular que possuem quatro domínios transmembrana típicos. Essas proteínas foram especificamente identificadas no tegumento de *Schistosoma mansoni*, sendo caracterizadas como antígenos protetores contra a esquistossomose (Tran *et al*., 2006). Em *E. multilocularis*, tetraspaninas foram identificadas no tegumento de protoescólices e na camada germinativa do metacestódeo (Dang *et al*., 2009a). Sete tetraspaninas de *E. multilocularis* (TSP1-TSP7) apresentaram eficácias protetoras variáveis contra a hidatidose alveolar, sendo propostas para o desenvolvimento de uma vacina contra o parasito (Dang *et al*., 2009b). A tetraspanina T24 de *T. solium* apresentou um alto valor diagnóstico para a cisticercose, exibindo alta sensibilidade e especificidade (Hancock *et al*., 2006). Sendo assim, as tetraspaninas apresentam potencial para o desenvolvimento de ferramentas diagnósticas e terapêuticas para diferentes helmintíases.

A tetraspanina de *E. granulosus* identificada no líquido hidático possui aproximadamente 95% e 75% de identidade, em nível de aminoácidos, com a TSP5 de *E. multilocularis* e com a T24 de *T. solium*, respectivamente. A proteína TSP5 de *E.*

multilocularis reduziu em mais de 70% o número de lesões císticas nos camundongos vacinados quando comparados com os não vacinados (Dang *et al*., 2009b). Quanto a sua localização extracelular, a presença de tetraspaninas em produtos de ES já foi demonstrada para *Ancylostoma caninum* (Mulvenna *et al*., 2009), e sua presença nas secreções/excreções de helmintos pode ser resultado do processo de muda do tegumento.

A proteína EgTeg foi identificada em todos os componentes do metacestódeo de *E. granulosus*, sugerindo um papel importante na manutenção do parasito. Essa proteína é altamente imunogênica e possui diferentes propriedades imunomoduladoras, inibindo a quimiotaxia de células polimorfonucleares e induzindo preferencialmente citocinas Th2 (IL-4) e anticorpos não-fixadores de complemento (IgG4) (Ortona *et al*., 2005). Desta forma, essa proteína contribui significativamente para o estabelecimento e manutenção da infecção crônica pelo metacestódeo de *E. granulosus*. A EgTeg contém um domínio conservado do tipo cadeia leve da dineína (Ortona *et al*., 2005), o mesmo domínio encontrado em outras duas proteína identificadas no líquido hidático, fazendo dessas proteínas alvos interessantes para estudos futuros sobre a sua localização tegumentar e suas propriedades imunológicas e imunomoduladoras.

Parasitos helmintos são sensíveis ao estresse oxidativo gerado pelo seu próprio metabolismo e pela resposta imune do hospedeiro, necessitando, assim, de um eficiente sistema de defesa antioxidante para assegurar a sua sobrevivência no interior do corpo do hospedeiro (Henkle-Dührsen & Kampkötter, 2001). Além da proteína EgTPx, a qual parece desempenhar um papel principal na proteção da forma larval patogênica de *E. granulosus* contra os danos oxidativos, nós encontramos GSTs nos protoescólices e camada germinativa do metacestódeo. Além disso, uma isoforma foi reconhecida como antigênica nos ensaios de 2-DE imunoblot.

GSTs têm sido identificadas em diversos parasitos helmintos, inclusive no tegumento e produtos de ES (Knudsen *et al*., 2005; Pérez-Sánchez *et al*., 2008; Liu *et al*., 2009). Essas enzimas constituem alvos interessantes para intervenções quimioterápicas e imunológicas contra diferentes infecções helmínticas, uma vez que desempenham funções importantes na sobrevivência destes parasitos e são induzidas em resposta à diferentes estímulos, como estresse oxidativo ou tratamento com diferentes drogas (Brophy & Barrett, 1990; Brophy & Pritchard, 1994). As GSTs têm a capacidade de neutralizar produtos citotóxicos formados durante o ataque de espécies reativas de oxigênio à membrana celular, evidenciando o seu potencial protetor contra a resposta imune do hospedeiro (Brophy & Pritchard, 1994). Elas desempenham também papel na resistência a drogas, tendo potencial de detoxificar compostos antihelmínticos. Além disso, essas enzimas apresentam propriedades imunogênicas, demonstrando potencial vacinal contra diferentes infecções helmínticas, principalmente esquistossomose (Capron *et al*., 2001) e fasciolose (Sexton *et al*., 1990). Harispe *et al*. (2009) demonstraram que a GST de ***E. granulosus*** pode participar da detoxificação de produtos tóxicos da peroxidação de lipídeos e tem a capacidade de ligar diversas drogas antihelmínticas. As propriedades funcionais desempenhadas pela GST na biologia de ***E. granulosus***, associadas à sua característica induzível (Fernández *et al*., 2000) e antigênica, destacam-na como um alvo interessante para o desenvolvimento de abordagens quimioteráticas e imunológicas para a hidatidose cística.

Neste trabalho, foi identificado pela primeira vez um receptor de LDL putativo presente no líquido hidático de ***E. granulosus***, o qual pode desempenhar funções importantes para a sobrevivência do metacestódeo dentro do hospedeiro. Estudos têm documentado a presença de receptores de LDL na superfície tegumentar de helmintos

como *Schistosoma mansoni* (Xu & Caulfield, 1992; Tempone *et al.*, 1997) e *Wuchereria bancrofti* (Rao & Sritharan, 1999). Como parasitos helmintos não possuem a capacidade de sintetizar seus próprios esteróis e ácidos graxos (Smith *et al.*, 1970; Barrett, 1981), tem sido sugerido que estes receptores possam atuar na aquisição de lipídeos do hospedeiro para a nutrição do parasito, síntese e manutenção de membranas, e evasão da resposta imune (Furlong, 1991). Neste último caso, a ligação de lipoproteínas do hospedeiro na superfície do parasito pode reduzir a resposta imune do hospedeiro através da ocultação de antígenos parasitários de superfície do reconhecimento pelos mecanismos de defesa celular e humoral do hospedeiro (Furlong *et al*., 1992).

Muitas proteínas citoplasmáticas e de membrana foram identificadas no líquido hidático de *E. granulosus*. Diversos estudos têm demonstrado a presença dessas proteínas nos produtos de ES de diferentes helmintos (Knudsen *et al*., 2005; Guillou *et al*., 2007; Liu *et al.*, 2009), correspondendo provavelmente a produtos de morte celular e/ou muda do tegumento. Essas proteínas podem também ser secretadas através de vias não-clássicas (Bendtsen *et al.*, 2004), as quais parecem estar envolvidas na secreção de aproximadamente 50-60% das proteínas presentes nos produtos de ES *in vitro* de outros helmintos (Cass *et al*., 2007; Hewitson *et al*., 2008; Liu *et al.*, 2009). Independente da sua origem, algumas proteínas são altamente conservadas nos produtos de ES de diferentes helmintos, sugerindo que os mecanismos moleculares envolvidos na evasão da resposta imune do hospedeiro podem ser conservados entre diferentes espécies (Liu *et al.*, 2009). Entre essas proteínas destacam-se a enolase, actina, frutose-bisfosfato aldolase, GAPDH e GST. A análise proteômica do líquido hidático de *E. granulosus* por LC-MS/MS suporta a identificação de algumas dessas proteínas, porém outras foram identificadas apenas nos produtos de ES de protoescólices em cultura (Virginio, 2007). Essa é uma diferença

importante entre os dados obtidos nesse trabalho, que foi realizado com amostras do parasito coletadas durante a infecção do hospedeiro, e os dados de proteínas gerados pelos estudos que analisam os produtos de ES de parasitos cultivados *in vitro*. É esperado que a composição dos produtos de ES seja alterada pela remoção do parasito dos tecidos do hospedeiro e manutenção em uma mistura química, e estudos têm demonstrado diferenças entre as proteínas de ES de parasitos *in vivo* e *in vitro* (Carmena *et al*., 2005; Morphew *et al*., 2007).

O metacestódeo de *E. granulosus* vive dentro dos tecidos do hospedeiro por longos períodos de tempo, sendo uma grande estrutura antigênica e, portanto, apresentando elevado potencial para estimular uma resposta inflamatória (Díaz *et al*., 1999). A resposta imune inata e adaptativa montada pelo hospedeiro contra a infecção por *E. granulosus* foi extensivamente revisada por Zhang *et al*. (2003, 2008) e Zhang & McManus (2006). Recentemente, Gottstein *et al*. (2010) avaliaram o perfil de expressão gênica do tecido hepático que circunda o metacestódeo de *E. multilocularis*, identificando biomarcadores relacionados à imunopatologia da infecção crônica inicial. O estudo da reação periparasitária pode auxiliar na caracterização da resposta montada pelo hospedeiro para controlar o parasito, bem como dos eventos imunopatológicos resultantes dessa resposta (Gottstein & Piarroux, 2008). Portanto, foram analisadas neste trabalho as proteínas do hospedeiro bovino que se encontravam em íntimo contato com o cisto hidático de *E. granulosus*, isto é, presentes no líquido hidático e adsorvidas/ligadas à membrana germinativa, gerando informações importantes sobre a imunopatologia da hidatidose cística, bem como indicando potenciais mecanismos utilizados pelo parasito na evasão dessa resposta imune.

O sistema complemento é um importante mecanismo efetor da imunidade inata e adquirida, tendo o potencial de interagir com e causar danos ao parasito. Embora o cisto hidático contenha moléculas capazes de ativar o sistema complemento, incluindo anticorpos IgG e IgM, identificados tanto na camada germinativa quanto no líquido hidático, ele não ativa o sistema complemento de forma eficiente (Ferreira ***et al.***, 2000). Isso é provavelmente resultado de adaptações físicas e bioquímicas do parasito ao ambiente do hospedeiro, como, por exemplo, a secreção da camada laminar e a exposição de mecanismos de evasão eficientes. Díaz ***et al.*** (1997) caracterizaram um mecanismo de evasão da via alternativa de ativação do complemento baseado no sequestro do fator H do hospedeiro pela parede do cisto hidático de ***E. granulosus***. Além disso, o mio-inositol hexakisfosfato (InsP_6), um componente majoritário da camada laminar, foi descrito como inibidor da C3 convertase da via alternativa de ativação do sistema complemento ***in vitro*** (Irigoín ***et al.***, 2002). Porém, os mecanismos utilizados pelo cisto hidático no controle da ativação da via clássica do complemento não são conhecidos, e inibidores atuando nos passos iniciais de ativação do complemento (C1, por exemplo) não foram identificados até o momento (Díaz ***et al.***, 1999; Ferreira ***et al.***, 2000; Breijo ***et al.***, 2008).

Diferentes componentes do sistema complemento (C3, C5, C6 e C9) foram identificados na camada germinativa e o componente C1s foi encontrado especificamente ligado a uma proteína de ES do parasito presente no líquido hidático. Estes resultados sugerem que o metacestódeo de ***E. granulosus*** possui potenciais mecanismos de evasão do sistema complemento, atuando sobre diferentes passos de ativação da cascata do complemento, incluindo um mecanismo de inibição específico da via clássica envolvendo a ligação de C1s. Em diferentes parasitos, proteínas capazes de ligar e inibir a ativação do sistema complemento já foram caracterizadas, como a paramiosina, já descrita aqui, e a

calreticulina (Laclette *et al*., 1992; Gobert & McManus, 2005; Naresha *et al*., 2009; Schroeder *et al*., 2009). A identificação das proteínas envolvidas nas interações entre ***E. granulosus*** e o complemento pode auxiliar na caracterização das estratégias moleculares utilizadas pelo parasito para subverter o sistema imune e permitir a sobrevivência dentro do hospedeiro.

As calgranulinas ou proteínas S100 são DAMPs (***damage-associated molecular pattern molecules***), moléculas que têm sido descritas como importantes fatores pró-inflamatórios da imunidade inata (Roth *et al*., 2003; Foell *et al*., 2007). Em resposta ao dano celular, infecção ou inflamação, as células fagocíticas secretam calgranulinas, as quais no meio extracelular mediam respostas inflamatórias através do recrutamento leucócitos. Algumas funções extracelulares das calgranulinas estão envolvidas em mecanismos de defesa do hospedeiro durante a infecção, com todos os membros da família S100 mostrando atividade antiparasitária (Marti *et al*., 1996; Gottsch *et al*., 1999; Moroz *et al*., 2003). As calgranulinas A, B e C foram detectadas na superfície ou no extrato de vermes adultos de *Onchocerca volvulus*, sugerindo um papel para essas moléculas na resposta inflamatória do hospedeiro contra a infecção pelo parasito (Edgeworth *et al*., 1992; Marti *et al*., 1996). Em *Brugia malayi*, a calgranulina C demonstrou atividade filaricida e filaristática *in vitro* (Gottsch *et al*., 1999). A análise por LC-MS/MS das proteínas imunopurificadas do líquido hidático de ***E. granulosus*** demonstrou a interação específica entre a calgranulina A e proteínas de ES do parasito. Além de sugerir a participação da calgranulina A na resposta imune do hospedeiro contra o cisto hidático, essa interação poderia representar um mecanismo de defesa do parasito, ligando a calgranulina para inativar ou bloquear o seu sinal pró-inflamatório. Ao contrário dessa hipótese, a paramiosina de ***B malayi*** foi identificada como proteína de ligação à

calgranulina C, e o complexo paramiosina-calgranulina mostrou-se altamente imunogênico, produzindo uma reação inflamatória intensa e induzindo uma queratite severa (Akpek *et al*., 2002).

Embora anticorpos (IgG1, IgG2 e IgM) tenham sido identificados na camada germinativa e no líquido hidático do metacestódeo de *E. granulosus*, não foi possível determinar se essas interações se dão via região Fab, na forma de complexos imunes com antígenos parasitários; ou via região Fc, em associação com moléculas parasitárias que atuam como receptores Fc. Proteínas que ligam Fc foram identificadas em diversos parasitos (Pleass & Woof, 2001), e receptores Fc específicos para IgG1 e IgG3 humanas foram detectados na superfície de protoescólices de *E. granulosus* (Baz *et al.*, 1998). A paramiosina, já discutida aqui e identificada em todos os componentes do metacestódeo de *E. granulosus*, foi caracterizada como um receptor de Fc em *Taenia crassiceps*, *Schistosoma mansoni* e *Schistosoma japonicum* (Kalinna e McManus, 1993; Loukas *et al*., 2001). A presença de anticorpos IgM, para o qual nenhum receptor parasitário foi descrito, pode indicar a ligação dos anticorpos à antígenos específicos via região Fab. Além disso, a fixação do componente C3, o qual foi identificado na camada germinativa e requer uma região Fc livre na imunoglobulina para iniciar a via clássica de ativação do complemento, também sugerem a ligação desses anticorpos à antígenos parasitários específicos (Braschi & Wilson, 2006). Contudo, embora as imunoglobulinas identificadas no metacestódeo de *E. granulosus* provavelmente se encontrem em complexos imunes com antígenos específicos, a possibilidade de interação desses anticorpos com receptores parasitários não pode ser excluída.

Os resultados obtidos na análise proteômica do metacestódeo de *E. granulosus* contribuíram para um melhor entendimento da biologia do parasito e da sua interação com

o hospedeiro intermediário bovino. Esse estudo permitiu também a identificação de potenciais alvos para o desenvolvimento de estratégias de diagnóstico, tratamento e controle da hidatidose cística.

A segunda parte desse trabalho refere-se à caracterização estrutural de oligômeros e agregados de alta massa molecular formados pelo AgB, principal proteína secretada pelo metacestódeo de *E. granulosus*, a qual está envolvida em diferentes mecanismos de interação com o hospedeiro implicados na sobrevivência do parasito (Siracusano *et al*., 2008a). Embora seja a proteína mais estudada de *E. granulosus*, devido ao seu papel na biologia do parasito e seu potencial imunodiagnóstico, muitos aspectos da estrutura molecular do AgB continuam desconhecidos (Monteiro *et al*., 2008).

Utilizando subunidades recombinantes do AgB (AgB8/1, AgB8/2 e AgB8/3), foi demonstrado que a proteína forma diferentes estados agregativos em condições fisiológicas de pH e temperatura, sugerindo, portanto, que estas estruturas moleculares podem desempenhar um papel na biologia de *E. granulosus*.

Estudos recentes têm demonstrado que uma ampla variedade de proteínas não relacionadas com doenças amiloidogênicas, incluindo proteínas de bactérias e fungos, são capazes de agregar *in vitro* formando fibrilas amilóides indistinguíveis daquelas formadas sob condições patológicas (Guijarro *et al*., 1998; Fandrich *et al*., 2001; Bucciantini *et al*., 2002; Stefani & Dobson, 2003). Essas descobertas, envolvendo proteínas sequência e estruturalmente não relacionadas, levaram a hipótese de que a agregação pode ser uma propriedade geral das cadeias polipeptídicas, em vez de uma característica restrita a um pequeno número de sequências (Dobson, 2001). Além disso, a citotoxicidade tem se mostrado uma característica inerente das proteínas capazes de formar fibrilas *in vitro*, independente da sua sequência ou origem (Bucciantini *et al*., 2002). Embora existam

evidências da toxicidade das fibrilas maduras em algumas doenças amiloidogênicas, estudos recentes têm sugerido que, ao menos em alguns casos, os agregados não-fibrilares que precedem a formação das fibrilas amilóides maduras podem ser as espécies moleculares responsáveis pela toxicidade observada (Zhu *et al.*, 2000; Sousa *et al.*, 2001; Bucciantini *et al.*, 2002; Stefani & Dobson, 2003). Estes agregados globulares pré-fibrilares apresentam resíduos hidrofóbicos e outras regiões da cadeia polipeptídica que podem estar mais acessíveis que nas fibrilas maduras, fazendo com que essas espécies interajam de forma inapropriada com uma ampla gama de proteínas celulares, produzindo efeitos tóxicos para a célula (Bucciantini *et al*., 2002).

A formação de agregados fibrilares pelo AgB não foi detectada *in vivo* ou *in vitro* nas condições testadas neste trabalho. Porém, maiores períodos de incubação e tratamentos mais drásticos, como pH extremo ou desnaturantes químicos, precisariam ser testados para avaliar se o AgB é capaz de formar fibrilas *in vitro*. De qualquer forma, um estudo preliminar *in vitro* realizado por Siracusano *et al.* (2008a), demonstrou que o AgB aumenta as taxas apoptóticas em PBMC de pacientes com hidatidose ativa. Assim sendo, o AgB poderia desempenhar um mecanismo adicional de evasão da resposta imune baseado na toxicidade de seus agregados para as células do hospedeiro. Testes *in vitro* para verificar a citotoxicidade dos diferentes estados agregativos do AgB sobre células do hospedeiro seriam necessários para confirmar essa hipótese e caracterizar as bases moleculares desse mecanismo.

A oligomerização e agregação do AgB acontecem em condições fisiológicas e tanto a proteína produzida pelo parasito, quanto suas subunidades recombinantes, formam estruturas termoestáveis à altas temperaturas (Oriol *et al.*, 1971; Monteiro *et al*., 2007), indicando que o mecanismo de oligomerização/agregação do AgB não envolve a

desnaturação da molécula. O processo de agregação protéica comumente observado envolve estados conformacionais parcialmente ou totalmente desnaturados (Chiti & Dobson, 2006). Porém, recentemente, Chiti & Dobson (2009) descreveram uma via diferente de agregação envolvendo conformações nativas estruturadas, na qual proteínas globulares podem agregar a partir de estados localmente desnaturados que se tornam acessíveis através de flutuações térmicas que ocorrem sob condições fisiológicas, sem a necessidade de transições além da barreira energética para a desnaturação. Tais estados conformacionais, os quais são referidos como "estados tipo-nativo" (Bemporad & Chiti, 2009), são responsáveis por uma aumentada propensão à agregação da proteína, podendo levar, ultimamente, a formação de fibrilas (Chiti & Dobson, 2009). Estes estados conformacionais podem ser transientemente formados através de flutuações do estado nativo (Canet *et al*., 2002), ou permanentemente formados devido a mutações ou outros eventos, isto é, estados termodinamicamente mais estáveis que o estado conformacional totalmente dobrado (Banci *et al*., 2005).

A partir das metodologias utilizadas nesse trabalho, não é possível determinar se o AgB sofre flutuações na sua estrutura durante o aumento da temperatura para 37 °C, o que poderia levar à exposição de resíduos mais hidrofóbicos, estimulando uma maior agregação das subunidades do AgB e a consequente formação de estruturas de alta massa molecular. Embora essa hipótese não possa ser formalmente descartada, o efeito hidrofóbico pode desempenhar um papel principal na agregação das subunidades do AgB. As subunidades do AgB mostram caráter anfifílico (Monteiro *et al*., 2007) e o efeito hidrofóbico é máximo em temperaturas médias (Lüdemann *et al*., 1996; Szilvay *et al*., 2006), o que explicaria uma maior associação das subunidades em resposta ao aumento de temperatura.

A análise por MS do AgB purificado do líquido hidático de ***E. granulosus*** revelou a presença das subunidades AgB8/1, AgB8/3 e AgB8/4 nas duas amostras analisadas. González *et al.* (1996) identificaram as subunidades AgB8/1 e AgB8/2 a partir do sequenciamento de peptídeos trípticos derivados das bandas do AgB resolvidas em SDS-PAGE. Alguns peptídeos não puderam ser diretamente relacionados com a seqüência deduzida de aminoácidos de AgB8/1 e AgB8/2, os únicos cDNAs relacionados ao AgB disponíveis na época da realização dos experimentos. Posteriormente, foi possível correlacionar estes peptídeos com a seqüência deduzida de aminoácidos da subunidade AgB8/4, demostrando que esta proteína também está envolvida na formação do AgB produzido pelo parasito (Mamuti *et al.*, 2006a). A análise de um maior número de amostras do AgB permitirá determinar possíveis variações na composição de subunidades do AgB entre diferentes indivíduos ou cepas.

Uma vez que as subunidades do AgB possuem diferentes propriedades estruturais (Monteiro *et al.*, 2007), funcionais (Chemale *et al.*, 2005) e imunológicas (Virginio *et al.*, 2003; Lorenzo *et al.*, 2005a), somente com a elucidação da composição de subunidades do AgB o conhecimento adquirido com o uso das subunidades recombinantes poderá ser corretamente aplicado no estudo da proteína produzida pelo parasito. A participação da subunidade AgB8/4 no AgB expresso e secretado pelo metacestódeo de ***E. granulosus***, aponta a necessidade de clonagem da seqüência codificadora dessa subunidade para posterior caracterização das propriedades estruturais, funcionais e imunológicas da proteína recombinante correspondente.

A subunidade AgB8/2 não foi detectada nas amostras de AgB analisadas por MS, concordando com os resultados obtidos na análise transcricional dos genes que codificam o AgB de *E. granulosus* (Arend *et al.*, submetido para publicação). Quando comparado com

os outros genes que codificam subunidades do AgB, o gene *EgAgB8/2* foi o menos expresso em camada germinativa e protoescólices, não sendo a subunidade AgB8/2 detectada no AgB expresso por esses dois componentes, nem secretado no líquido hidático. Nesse contexto, outra questão envolve a caracterização da subunidade AgB8/2 como um antígeno de alto valor diagnóstico, apresentando alta sensibilidade e especificidade (Rott *et al*., 2000; Virginio *et al*., 2003). Nestes estudos, a subunidade AgB8/4 não foi analisada, e é sabido que AgB8/2 e AgB8/4 apresentam 70% de homologia nas suas sequências de aminoácidos (Arend *et al*., 2004). Portanto, é possível que os epitopos reconhecidos nos experimentos de ELISA estejam presentes em ambas as proteínas. A identificação da subunidade AgB8/4 no AgB produzido pelo parasito reforça essa hipótese, uma vez que a presença dessa subunidades no AgB secretado no líquido hidático indica que ela estaria disponível para estimular uma resposta imune contra os epitopos de reatividade cruzada entre AgB8/2 e AgB8/4.

O estudo dos diferentes estados agregativos do AgB gerou informações importantes sobre a agregação da proteína, sua composição de subunidades e das propriedades estruturais de diferentes subunidades, contribuindo para aumentar o conhecimento da estrutura do AgB e das suas implicações biológicas na interação parasito-hospedeiro durante a infecção pelo metacestódeo de *E. granulosus* e na sua utilização em ferramentas de diagnóstico, tratamento e controle da hidatidose cística.

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ANEXOS

ANEXO I

Artigo publicado durante o doutorado com resultados referentes ao mestrado

Monteiro, K. M.; Scapin, S. M.; Navarro, M. V.; Zanchin, N. I. T.; Cardoso, M. B.; da Silveira, N. P.; Gonçalves, P. F.; Stassen, H. K.; Zaha, A.; Ferreira, H. B. Self-assembly and structural characterization of *Echinococcus granulosus* antigen B recombinant subunit oligomers. ***Biochim. Biophys. Acta***, 1774(2): 278-285, 2007

Available online at www.sciencedirect.com
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Biochimica et Biophysica Acta 1774 (2007) 278–285
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbapap

Self-assembly and structural characterization of *Echinococcus granulosus* antigen B recombinant subunit oligomers

Karina M. Monteiro [a], Sandra M.N. Scapin [b], Marcos V.A.S. Navarro [b], Nilson I.T. Zanchin [b], Mateus B. Cardoso [c], Nádya P. da Silveira [c], Paulo F.B. Gonçalves [d,1], Hubert K. Stassen [d], Arnaldo Zaha [a], Henrique B. Ferreira [a,*]

[a] *Laboratório de Biologia Molecular de Cestódeos, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Cx. Postal 15005, 91501-970, Porto Alegre, RS, Brazil*
[b] *Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, SP, Brazil*
[c] *Laboratório de Instrumentação e Dinâmica Molecular, Instituto de Química, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[d] *Grupo de Química Teórica, Instituto de Química, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*

Received 16 August 2006; received in revised form 7 November 2006; accepted 8 November 2006
Available online 17 November 2006

Abstract

Echinococcus granulosus antigen B is an oligomeric protein of 120–160 kDa composed by 8-kDa (AgB8) subunits. Here, we demonstrated that the AgB8 recombinant subunits AgB8/1, AgB8/2 and AgB8/3 are able to self-associate into high order homo-oligomers, showing similar properties to that of parasite-produced AgB, making them valuable tools to study AgB structure. Dynamic light scattering, size exclusion chromatography and cross-linking assays revealed ~120- to 160-kDa recombinant oligomers, with a tendency to form populations with different aggregation states. Recombinant oligomers showed helical circular dichroism spectra and thermostability similar to those of purified AgB. Cross-linking and limited proteolysis experiments indicated different degrees of stability and compactness between the recombinant oligomers, with the AgB8/3 one showing a more stable and compact structure. We have also built AgB8 subunit structural models in order to predict the surfaces possibly involved in electrostatic and hydrophobic interactions during oligomerization.

Keywords: *Echinococcus granulosus*; Antigen B; Recombinant subunits; Oligomerization; Thermostability; Protease sensitivity

1. Introduction

Echinococcus granulosus (Cestoda, Taeniidae) is the causative agent of cystic hydatid disease (CHD), a worldwide prevalent cestodiasis in humans and domestic ungulates [1]. The antigen B (AgB) is one of the major antigenic components of the metacestode hydatid fluid (HF) and was first characterized as a lipoprotein of 120–160 kDa [2,3]. This antigen is highly immunogenic in human infections and its use has been proposed for the improvement of CHD immunodiagnosis [4,5]. The biological roles of AgB are not clear, but several lines of evidence suggest its involvement in key parasite–host interactions. For instance, it has been described as a protease inhibitor that impairs neutrophil recruitment in vitro [6], and as a factor able to skew Th1/Th2 cytokine ratios towards a nonprotective Th2 cell response [7] and induce immune cells apoptosis in patients with active disease [8]. Moreover, a possible role for AgB in lipid uptake and/or detoxification mechanisms has also been suggested [9].

Structurally, AgB is an oligomeric protein that, under reducing conditions, dissociates into components of 8, 16, 24 and 32 kDa, with their relative abundance decreasing proportionally to the increase of the molecular mass [10]. The components with higher molecular masses correspond to multimers (dimers, trimers, etc.) of monomeric 8-kDa related subunits (AgB8) [11], which are encoded by a multigene family [12]. The

Abbreviations: AgB, antigen B; AgB8, 8-kDa AgB subunit; CD, circular dichroism; CHD, cystic hydatid disease; DLS, dynamic light scattering; HF, hydatid fluid

* Corresponding author. Tel.: +55 51 3316 7768; fax: +55 51 3316 7309.
E-mail address: henrique@cbiot.ufrgs.br (H.B. Ferreira).
[1] Present address: Unilasalle, Canoas, RS, Brazil.

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doi:10.1016/j.bbapap.2006.11.006

existence of at least five *AgB8* gene loci has been proposed for *E. granulosus* and it has been suggested that they would constitute a family of contingency genes [13].

Studies on AgB subunit composition and interaction are required to establish whether AgB is a homo- or a hetero-oligomer, and which or how many different AgB8 subunits are present in an AgB oligomer. Previous studies with AgB components have failed to answer these questions [10,11]. In this context, recombinant AgB8 subunits can be important models for AgB structural analyses.

Our group has produced three AgB8 recombinant subunits (AgB8/1, AgB8/2, AgB8/3) by cloning and expressing their corresponding genes in *Escherichia coli* [12,14,15]. In this work, we present experimental evidences of the self-association of these recombinant subunits into higher molecular mass homo-oligomers, as determined by dynamic light scattering, size exclusion chromatography and cross-linking assays. Spectroscopic characterization showed that recombinant oligomers and purified AgB present similar properties, validating the recombinant oligomers as models for the investigation of AgB structural aspects, such as subunit composition and oligomerization. We have also built AgB8 subunit structural models that allowed the identification of molecular surfaces possibly involved in electrostatic and hydrophobic interactions during oligomerization.

2. Materials and methods

2.1. Protein expression and purification

AgB8/1 [14], AgB8/2 [15] and AgB8/3 [12] subunits were expressed in *E. coli* as fusion proteins with glutathione-*S*-transferase, purified by affinity chromatography on immobilized glutathione and recovered by thrombin cleavage, as previously described [4]. The deduced amino acid sequences of these AgB recombinant subunits are presented in Fig. 1. Expression in *E. coli* yielded 4, 6 and 10 mg of protein per liter of culture for AgB8/1, AgB8/2 and AgB8/3, respectively. On reducing SDS-PAGE, these proteins appear as single bands of ~8 kDa (data not shown). In non-reducing conditions, AgB8/2 and AgB8/3, which contain single cysteine residues, absent in AgB8/1, also appeared as dimers ([12,15], data not shown). AgB was purified from a parasite enriched HF fraction, obtained from a single fertile cyst of cattle lung as described by Oriol et al. [2]. Rabbit polyclonal antibodies raised against recombinant AgB8/2 were coupled to a cyanogen bromide-activated Sepharose 4B (GE Healthcare, Uppsala, Sweden) and AgB purification by immunoaffinity chromatography was carried out as described by Chemale et al. [9]. SDS-PAGE analysis of purified AgB generated the previously described [10] ladder-like pattern of 8-, 16-, 24- and 32-kDa components (data not shown).

2.2. Dynamic light scattering measurements (DLS)

Light scattering was performed on a DynaPro instrument (Protein Solutions Inc.) that monitors the scattered light intensity at 90° from the incident light beam. A Nd:YAG laser (λ=883 nm) was used as light source. The intensity fluctuation of the scattered light was recorded by single-photon-counting electronics. Under the assumption of Brownian motion and a hard sphere model for the particle, the apparent diffusion coefficient (D) was converted to the hydrodynamic radius (R_h) by using the Stokes–Einstein relation: $R_h=\kappa_B T/6\pi\eta_o D$, where κ_B is the Boltzmann constant, T is the absolute temperature, and η_o is the viscosity of the solvent.

The molecular mass (MM) of proteins was estimated assuming a spherical shape for the proteins in solution, using the algorithm $MM=(1.549R_h)^{2.426}$. The linear calibration curves (log R_h versus log MM) were obtained from 18 different standard protein solutions. Data analysis was performed with the Dynapro V.5 software.

Proteins samples at 0.8 mg/ml in PBS were centrifuged (14,000 rpm for 10 min at 4 °C) before DLS analysis at 20 °C. At least 50 measurements of 10 s were acquired for each protein sample.

2.3. Size exclusion chromatography

One milligram of each AgB8 recombinant protein in PBS was individually loaded onto a Superdex 200 HR 10/30 column (GE Healthcare, Uppsala, Sweden) previously equilibrated in PBS. Chromatography was performed on an Äkta FPLC system (GE Healthcare, Uppsala, Sweden) at a flow rate of 0.3 ml/min at room temperature and the absorbance was monitored at 280 nm. The column was calibrated using protein molecular mass standards under the same conditions.

2.4. Cross-linking assay

Cross-linking experiments were carried out as previously described [16]. Briefly, 80 µl of a 2 mg/ml solution of each recombinant protein in PBS was incubated with 0.1% glutaraldehyde (v/v) at room temperature. Ten-microliter aliquots were withdrawn at different times and reactions were stopped by adding SDS sample buffer and boiling for 5 min. Cross-linked products were analyzed under reducing conditions on 15% SDS-PAGE and detected by Coomassie blue staining.

2.5. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were acquired on a Jasco J810 spectropolarimeter at 20 °C in 1 mm path-length cells, using a 50 nm/min scanning rate and a spectral bandwidth of 0.5 nm. Protein CD spectra were recorded at 2.5 µM, 40 µM, 35 µM and 45 µM for AgB, AgB8/1, AgB8/2 and AgB8/3, respectively, over the range of 195–260 nm in buffer containing 10 mM of Na_2HPO_4 and 5.5 mM of NaCl, pH 7.2. Ellipticity was reported as the mean residual ellipticity [θ] (deg cm^2 dmol^{-1}). For the recombinant proteins, the number of residues used corresponded to that of individual monomers. For the purified AgB, the number of residues was estimated as for a 120-kDa protein. The CDNN deconvolution program [17] was employed to estimate the ratio of secondary structure elements in CD spectra. Proteins were also subjected to thermal unfolding from 20 °C to 95 °C, with a scan rate of 1 °C/min. Thermal stability was measured at 222 nm using 0.5 °C intervals and CD spectra were collected at 5 °C intervals over the ranges of 195–260 nm and 200–260 nm for recombinant proteins and purified AgB, respectively. Refolding assays were started at 95 °C and the temperature was lowered to 20 °C, with concomitant monitoring of ellipticity at 222 nm.

2.6. Limited proteolysis assay

Recombinant AgB8 subunits were subjected to proteolysis using trypsin, chymotrypsin and proteinase K at ratios of 1:100, 1:100 and 1:1000 (w/w),

```
AgB8/1 : ---DIGLTSTSRSVMKMFGEVKYFFERDPLGQKVVLLLKELEEVFQILRKKLRMALRSHLRGLIAEGE---- : 65
AgB8/3 : DDDDIEVTKTKKGVMKAISEIKHFFQSDPLGKKLVEVMKDVASVCEMVRKKARMALKEYVRKLVKPDE---- : 68
AgB8/2 : --KLEPKAHMGQVVKKRWGELRDFFRNDPLGQRLVALGNDLTAICQKLQLKIREVLKKYVKNLVEPKDDDSK : 70
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Fig. 1. Alignment of the deduced amino acid sequences of AgB8 recombinant subunits. The aligned sequences correspond to that of mature peptides. The alignment was obtained with ClustalW (http://www.ebi.ac.uk/clustalw/) and GeneDoc (http://www.psc.edu/biomed/genedoc/) softwares. Residues shaded in black are identical or conserved in all three sequences and those shaded in gray, identical or conserved in two of the three sequences. Accession numbers: AAD38373 (AgB8/1), AAC47169 (AgB8/2), and AAK64236 (AgB8/3).

respectively. Digestion was performed with 1 mg/ml of recombinant AgB8 oligomers in PBS at 37 °C. Aliquots were removed at different time intervals and the reactions were stopped with the addition of loading buffer containing 1 mM PMSF, followed by boiling for 5 min. Proteolysis products were resolved on 16.5% tricine–SDS-PAGE gels.

2.7. Fluorescence spectroscopy

Fluorescence emission spectra were collected on an Aminco Bowman Series II Luminescence Spectrometer (SLM Aminco, Spectronic Instruments) using a protein concentration of 10 μM in PBS. For tryptophan intrinsic fluorescence, the samples were excited at 280 nm and the emission spectra were recorded from 300 to 400 nm. An excitation wavelength of 274 nm was used in tyrosine fluorescence measurements and the emission spectra were recorded from 285 to 400 nm. Proteins denatured with 6 M guanidine hydrochloride were used as experimental controls.

2.8. Modeling of AgB subunits

The primary sequences were assembled using the Swiss PDB-Viewer software (http://www.expasy.org/spdbv). The structures obtained were independently heated to 1000 K for 5 ps in vacuum and subsequently cooled slowly to 310 K. Each structure was solvated in boxes containing SPC water [18], Na^+ and Cl^- ions in physiological concentration. The systems were equilibrated for 2.0 ns and subsequently simulated using the GROMACS software [19] for 3.0 ns. The simulation temperature was 310 K at a pressure of 1 bar for weak coupling of a Berendsen thermostat [20] with coupling constant of τ_p=1.0 ps. The LINCS [21] and SETTLE [22] methods allowed the use of an integration step of 2 fs. A cut-off of 1.4 nm was used with periodic conditions. The electrostatic interactions were calculated using the PME method [23]. The average configuration obtained in the last nanosecond of simulation was used for surface electrostatic potential calculation with the APBS software [24], which resolves the Poisson–Boltzmann equation for the system.

3. Results and discussion

3.1. Self-assembly of AgB8 recombinant subunits

The oligomeric state of individual AgB8 recombinant subunits in solution was monitored by DLS, size exclusion chromatography and glutaraldehyde cross-linking. DLS yields the hydrodynamic radius of a species in solution and thus reflects its state of oligomerization [25]. DLS measurements showed radii of 3.8 nm, 4.8 nm, and 5.3 nm for AgB8/1, AgB8/2 and AgB8/3, respectively, which are larger than the radius expected for a monomeric 8-kDa protein. This clearly indicated that these proteins spontaneously associate into high order multimers. The apparent molecular masses, estimated from hydrodynamic radii of particles, were 96 kDa, 193 kDa and 266 kDa for AgB8/1, AgB8/2 and AgB8/3, respectively.

Subsequently, we examined the oligomeric state of recombinant AgB8 subunits by size exclusion chromatography (Fig. 2). The elution profiles revealed major components of 125 kDa, 164 kDa and 113 kDa for AgB8/1, AgB8/2 and AgB8/3, respectively. Secondary peaks containing oligomers of higher molecular masses were observed for the three proteins, being less defined for the AgB8/1 size exclusion chromatography profile. The molecular masses estimated from the chromatography analysis were consistent with those estimated from DLS and the deviations were probably due to the non-spherical shape of AgB8 oligomers. Based on the molecular masses calculated from size exclusion chromatography, the homo-oligomers would consist of approximately 16 AgB8/1, 20 AgB8/2 and 14 AgB8/3 monomers.

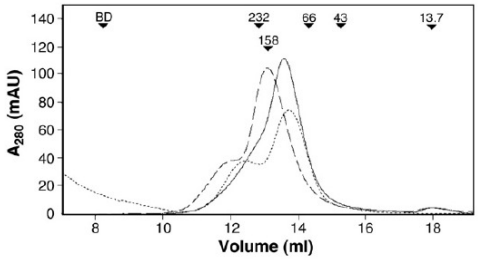

Fig. 2. Gel filtration analysis of recombinant AgB8 oligomers. Elution profiles of AgB8/1 (solid line), AgB8/2 (dashed line) and AgB8/3 (dotted line) oligomers chromatographed on a Superdex 200 column. Blue dextran (BD), catalase (232 kDa), aldolase (158 kDa), albumin (66 kDa), ovalbumin (43 kDa), and ribonuclease (13.7 kDa) were used as molecular mass standards and their positions are indicated by arrowheads (top).

The two peaks observed in size exclusion chromatography suggest the existence of two major populations, with different states of oligomerization. In agreement with this scenario, Oriol et al. [2] reported that the Sephadex G-200 separation profile of a parasite enriched HF fraction showed three major peaks, corresponding to ≥320 kDa, ~240 kDa and ~160 kDa. AgB was found in all of them, although it was obtained in a more purified form from the third one (~160 kDa). When this ~160-kDa fraction was reloaded on the column, it again produced the three peaks of the original profile, which was suggestive of the existence of AgB in several distinct forms of aggregation that would be in reversible equilibrium. This situation is mimicked, at least in a certain extent, by the recombinant AgB8 subunits.

The self-association of AgB subunits was further confirmed by glutaraldehyde cross-linking experiments. Fig. 3 shows a time course where a progressive transition of the monomeric form of AgB subunits into high molecular mass oligomers took place with increasing times of cross-linking. Major cross-linked products are concentrated in the region corresponding to approximately 50–60 kDa for all recombinant homo-oligomers. Less abundant products of higher molecular masses were also detected. The kinetics of multimer cross-linking was different for the three proteins, with oligomers starting to appear after 30, 5, and 1 min of cross-linking for AgB8/1 (Fig. 3A), AgB8/2 (Fig. 3B), and AgB8/3 (Fig. 3C), respectively. Shorter cross-linking times denote closer interactions, indicating a relation of AgB8/3 > AgB8/2 > AgB8/1 regarding the association affinity between subunits within the corresponding homo-oligomers.

DLS, size exclusion chromatography and cross-linking analyses indicated that AgB8 recombinant subunits in solution are able to self-assemble into high molecular mass oligomers, in a non-covalent manner, with molecular masses close to that of purified AgB (120–160 kDa). The recombinant proteins apparently have an intrinsic tendency to form populations with different states of aggregation, as the purified AgB. However, unlike purified AgB, the recombinant oligomers completely dissociate in monomers on SDS-PAGE. Post-translational modifica-

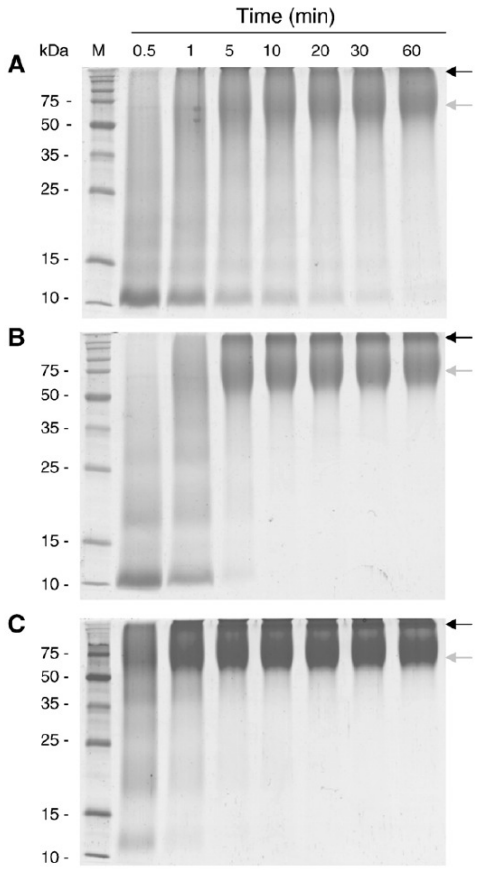

Fig. 3. Cross-linking of AgB8/1 (A), AgB8/2 (B) and AgB8/3 (C) recombinant oligomers. Proteins were cross-linked with 0.1% of glutaraldehyde for 0.5, 1, 5, 10, 20, 30 and 60 min, as indicated (top), and separated on 15% SDS-PAGE followed by Coomassie blue staining. The gray arrows indicate the cross-linked products of approximately 50–60 kDa. Cross-linked products with higher molecular masses are indicated by black arrows. Molecular mass standards (lane M) are indicated on the left.

tions, previously suggested for AgB subunits, such as lipidation [2], and unavailable for their recombinant counterparts could be responsible for the stability of the AgB multimers with low molecular masses (16, 24 or 32 kDa) under SDS-PAGE denaturing conditions. Differential non-covalent interactions with hydrophobic ligands, demonstrated for both purified AgB and recombinant AgB subunits [9] may also play a role in different levels of oligomer stabilization and deserve future investigation.

3.2. Secondary structure and thermostability of recombinant and purified oligomers

In order to obtain information about oligomer structure and stability, the proteins were analyzed by CD. The CD spectra were indicative of a high α-helical content, with double minima at 208 and 222 nm (Fig. 4), both for the recombinant oligomers and purified AgB. Spectra deconvolution with CDNN indicated a secondary structure ratio of ~35–40% α-helix, ~33% random coil and ~17% β-turn, which are consistent with values previously described [3,26]. Under thermal denaturation, the CD signal at 222 nm showed a relatively small loss of signal, indicating that proteins maintained most of their secondary structures (Fig. 5). Refolding assays demonstrated that even the small loss of secondary structure content under thermal denaturation was reversible (data not shown). The oligomer stability is most probably due to the highly organized quaternary structure. These data support previous studies which described AgB as an antigenically thermostable lipoprotein [2] and demonstrate that the recombinant and purified oligomers are very similar in secondary structure content and thermostability. Such degree of similarity with purified AgB, make the recombinant AgB8 homo-oligomers useful models to study structural and functional aspects of AgB. The availability of these recombinant AgB8 homo-oligomers can be helpful to overcome problems associated to structural and functional characterization of parasite-produced oligomers, with unknown and probably heterogeneous subunit composition [13,27] and post-translational modifications.

3.3. Insights into oligomer conformation

In order to obtain information on oligomer conformation, we performed limited proteolysis and intrinsic fluorescence experiments. Limited proteolysis has been widely used to probe conformational features of proteins, such as compactness, and to identify protein domains [28,29]. We digested recombinant oligomers with both specific (trypsin and chymotrypsin) and non-specific (proteinase K) proteolytic enzymes. AgB susceptibility to proteolytic digestion was not assessed due to its unknown post-translation modification content, which would probably interfere with the digestion outcome. Fig. 6 shows the proteolytic products of the recombinant oligomers with the

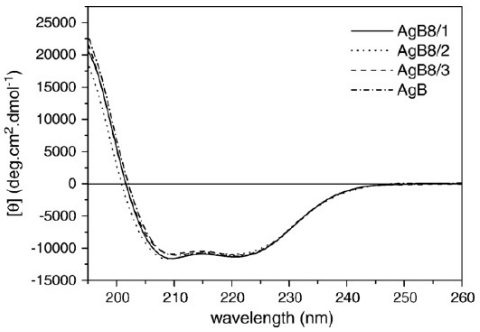

Fig. 4. Far UV CD spectra of recombinant AgB8 oligomers and purified AgB acquired at 20 °C in buffer containing 10 mM Na_2HPO_4 and 5.5 mM NaCl, pH 7.2. CD spectra are typical of α-helical proteins with double minima at 208 and 222 nm.

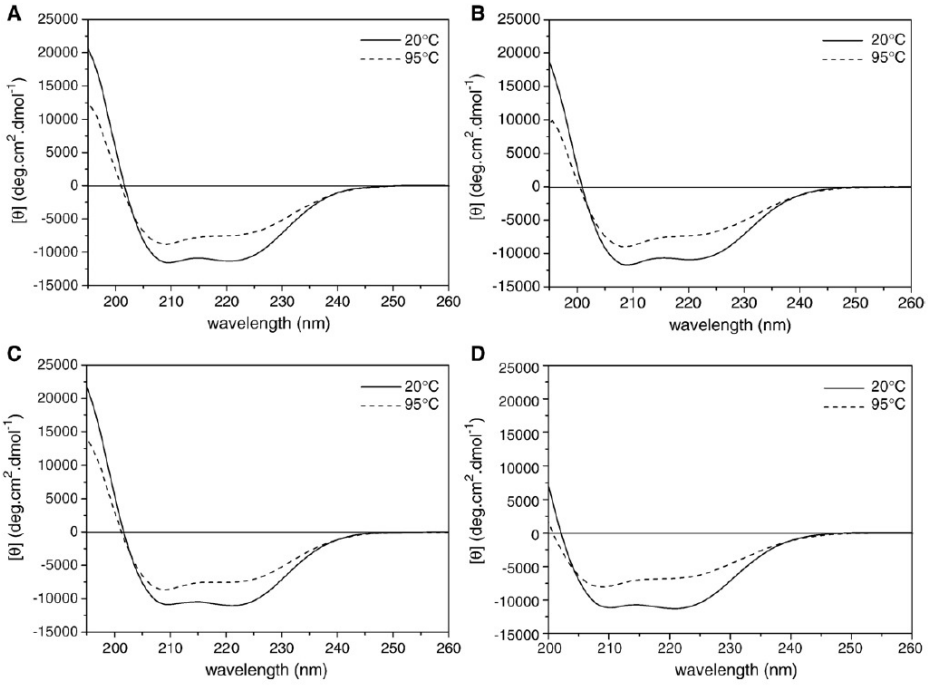

Fig. 5. Thermal denaturation of recombinant AgB8 oligomers and purified AgB monitored by circular dichroism. CD spectra of (A) 40 μM AgB8/1, (B) 35 μM AgB8/2, (C) 45 μM AgB8/3, and (D) 2.5 μM AgB are shown at 20 °C (solid line) and 95 °C (dashed line).

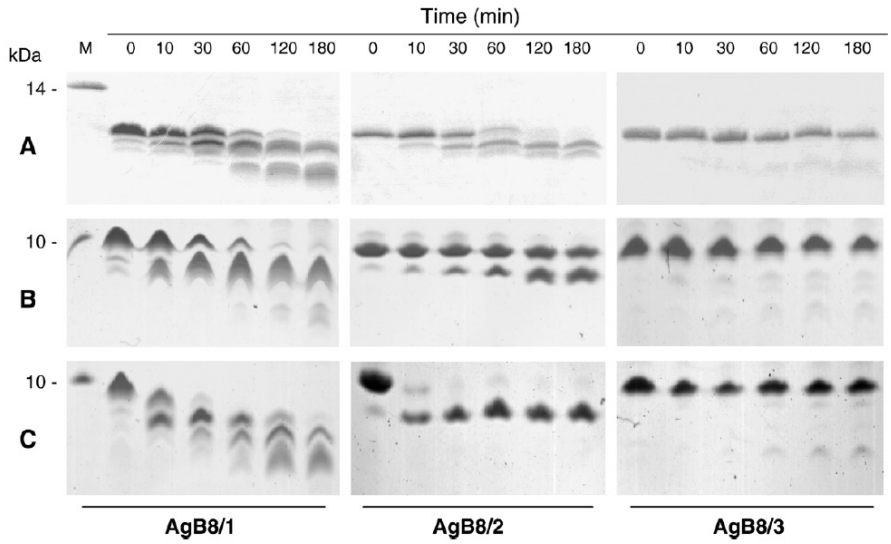

Fig. 6. Coomassie stained 16.5% tricine–SDS-PAGE gels of recombinant AgB8 proteolysis products following digestion with (A) trypsin 1:100 (w/w), (B) chymotrypsin 1:100 (w/w) and (C) proteinase K 1:1000 (w/w). Aliquots were removed at different time intervals and the reactions were stopped with the addition of 1 mM PMSF and loading buffer, followed by boiling. The incubation times are indicated on the top of each lane and molecular mass standards (lane M) are indicated on the left.

panel of proteases as a function of incubation time. AgB8/1 and AgB8/2 oligomers were partially or fully digested by protease treatment, while AgB8/3 remained mostly intact throughout the course of the experiment. The AgB8/3 oligomer higher resistance to proteolysis denotes a conformation more stable than that of the other two recombinant oligomers. Such a different stability is consistent with the observation that recombinant AgB8/3 yields in *E. coli* are higher than those of AgB8/1 and AgB8/2 (see Materials and methods section), since a direct relationship is expected between recombinant protein stability and its expression level [30]. Since all proteins are equally stable at higher temperatures and show similar secondary structure content and number of cleavage sites, the proteolysis assays point to a higher compactness of the AgB8/3 oligomeric structure. AgB8/1, on the other hand, was the more labile oligomer, showing digestion products as soon as a protease was added (time 0 in Fig. 6) and full digestion at final times, reflecting a more loose association between subunits. The observed AgB8/1, AgB8/2 and AgB8/3 differential susceptibility to protease treatment is in agreement with our cross-linking results (see Fig. 3), reinforcing the idea that AgB8/3 oligomers would have a more compact structure.

Initial host responses to the establishment of hydatid cysts involve infiltration of innate immune system cells [31] and one of their effector mechanisms is the production and secretion of proteolytic enzymes. With that in mind and considering that AgB is a major component of the HF, the differential susceptibility of AgB8 subunits to protease digestion and their representativity and relative contribution to the structure of AgB oligomers would be of major concern to understand possible functional relationships between AgB and host proteases within the hydatid cyst. In the same context, intracellular proteolysis is a key step in antigen presentation events and an increase in protein stability is known to reduce the antigenicity of proteins [32,33]. Therefore, the AgB8/3 three-dimensional structure could potentially limit its processing and presentation to helper T cells, which may explain, at least in part, why this subunit elicits a lower specific humoral response in hydatid disease patients than that elicited by AgB8/1 or AgB8/2 ([4]; our unpublished data).

To determine the tryptophan and tyrosine microenvironments in oligomer structures we performed intrinsic fluorescence experiments. AgB8/2 fluorescence presented a maximum emission near 330 nm, indicating that the single AgB8/2 Trp residue (Trp16 in mature protein) is in a hydrophobic environment (Fig. 7A). The same was observed for purified AgB oligomers, indicating that they include Trp-containing AgB8 subunits and that the AgB8 Trp residues would be also buried into the oligomer structure. Trp residues are not present in AgB8/1 and AgB8/3 proteins, but they contain Tyr residues, which can also be used to measure the intrinsic fluorescence of a protein. The AgB8/1 and AgB8/3 fluorescence emission spectra showed a maximum at 303 nm (Fig. 7B), which is consistent with the fluorescence emission of free or water-exposed Tyr residues. A single tyrosine residue (Tyr59 in mature protein) is found at the C-terminal end of AgB8/3 (see Fig. 1), indicating that this region of the monomer is exposed to the solvent in

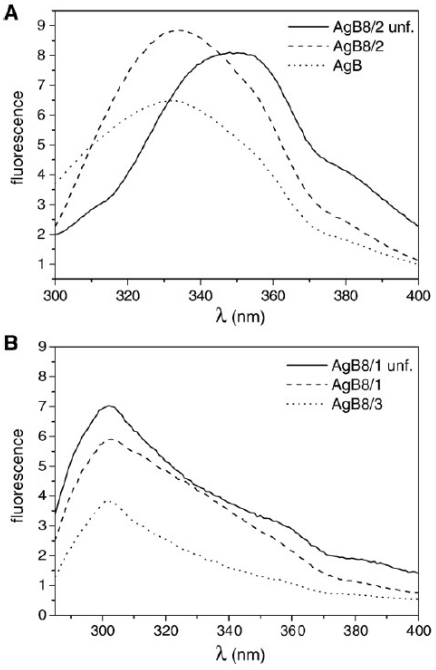

Fig. 7. Fluorescence analysis of AgB8 oligomers. (A) Intrinsic fluorescence of tryptophan excited at 280 nm was analyzed in the 300–400 nm range; AgB8/2 unf. (unfolded control, solid line); AgB8/2 (dashed line); AgB (dotted line). (B) Fluorescence emission spectra of AgB8/1 and AgB8/3, acquired at an excitation wavelength of 274 nm; AgB8/1 unf. (unfolded control, solid line); AgB8/1 (dashed line); AgB8/3 (dotted line). AgB8/2 or AgB8/1 oligomers denatured with 6 M guanidine–HCl were used as unfolded controls.

oligomer conformation. On the other hand, the exposed single AgB8/1 Tyr residue (Tyr20 in mature protein) occupies a position near the N-terminal end of the subunit, close to that of Trp16 in AgB8/2 (see Fig. 1), which, in contrast, was found to be buried in the oligomeric structure. To explain the solvent exposure of the immunogenic N-terminus of AgB8/1 (p38), González-Sapienza and Cachau [26] proposed a model in which there is an intramolecular bent of an α-helix over itself, due to the distribution of hydrophobic residues. In such conformation, the predicted epitope containing the Tyr20 residue (EVKYFFER, residues 17–24) would be fully solvent exposed. Based on our results with recombinant AgB8 oligomers, we now propose that intermolecular in addition to or instead of intramolecular hydrophobic interactions would be the determinants of the AgB8/1 subunit conformation in which its N-terminus, including Tyr20, is solvent exposed.

3.4. Possible electrostatic and hydrophobic interactions in oligomers formation

To help us identify the possible interactions involved in oligomer formation, we constructed structural models of AgB8/1,

AgB8/2, and AgB8/3 subunits and calculated the electrostatic potential of their surfaces (Fig. 8). In our models, AgB8 subunits consist of an elongated α-helix with a short coil region determined by the Pro-X-Gly pair (DPLG region), as proposed in the p38 model [26]. For AgB8/1 (Fig. 8A), the model predicted a somewhat scattered charge density distribution, although a predominantly hydrophobic face could be defined. AgB8/2 presented, in its central portion, a region of low polarity, approximately neutral, but also restricted to only one face of the helix (Fig. 8B). For AgB8/3 (Fig. 8C), a similar configuration was observed, but with a more pronounced charge separation. Therefore, the molecules would behave as a facial amphiphile, hydrophilic in one face and hydrophobic in the other. From these models, we can infer that the amphiphilic subunits could self-assembly into oligomers accommodating their hydrophilic and hydrophobic surfaces, so that non-polar regions would be segregated from the solvent in a hydrophobic core and polar regions would be exposed to solvent. This would form a multimeric structure thermodynamically more stable than individual monomers. The hydrophobic effect is considered the principal driving force for all protein–protein associations [34,35], and amphiphilic α-helices, such as those predicted for AgB subunits, seem to be involved in the oligomerization of some proteins, such as eye lens α-crystallin [36,37] and dynamin [38].

Due to a more diffuse charge distribution in AgB8/1 surface, we can suggest that its oligomerization tendency would not be so pronounced as that of AgB8/2 or AgB8/3, in which aggregation would be favored. This prediction is in agreement with our results from size exclusion chromatography, cross-linking and limited proteolysis experiments, which are indicative of a less aggregative character for AgB8/1 and a less compact structure for the corresponding oligomer.

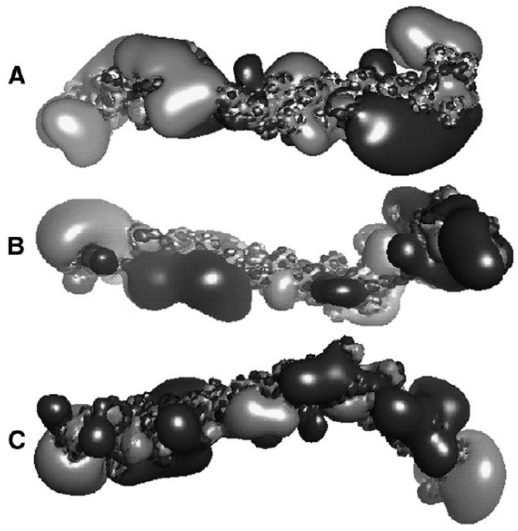

Fig. 8. Surface electrostatic potential calculations with the APBS software for AgB subunits (A) AgB8/1, (B) AgB8/2 and (C) AgB8/3. High (negative) and low (positive) electronic density surfaces are indicated by different tones, from light gray to black, respectively. Bubble volumes are proportional to electrostatic potential, with the smaller ones evidencing neutral (hydrophobic) regions. The models are oriented from their N to C-terminal ends from left to right, and in a view that favors the visualization of the hydrophobic face.

In conclusion, the results reported here demonstrated, for the first time, that AgB8 recombinant subunits self-assemble into high molecular mass homo-oligomers with structural features similar to those of the parasite-produced AgB. Besides, the molecular modeling of AgB8 subunits helped us to identify surfaces possibly involved in electrostatic and hydrophobic interactions responsible for homo-multimers formation. Although our results helped to shed some light on AgB structural properties, other experimental approaches, including the analysis of mutagenized subunits, will be necessary to confirm theoretical predictions and to explain how different subunits could interact with each other and with other molecules (such as hydrophobic ligands) in the context of AgB oligomer. Moreover, the co-expression of AgB8 subunits in the same host cell may allow the production of hetero-oligomers with a structure more similar to that of AgB. Studies on AgB oligomerization may help in the design of drugs that inhibit or interfere with protein–protein interactions [39,40], disturbing oligomer function and/or stability. The elucidation of the overall structure of an AgB8 oligomer is likely to require crystallographic studies, and, with that in mind, screenings to obtain crystals of the AgB8 recombinant oligomers have already been started by our group.

Acknowledgments

We thank Adriana C. Alves, for her assistance with the size exclusion chromatography, and Dr. Célia Carlini, for the kind gift of chymotrypsin. This work was supported by CNPq, FAPERGS, RTPD Network (SIDA/SAREC), and FAPESP SMolBNet (00/10266-8) and CEPID/CBME (98/14138-2) programs. K.M.M. is a recipient of a CNPq MSc fellowship. S.M.N.S. and M.B.C. are recipients of PhD fellowships from FAPESP and CAPES, respectively.

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ANEXO II

CURRICULUM VITAE

CURRICULUM VITAE resumido

Monteiro, K. M.

Dados Pessoais

Nome Karina Mariante Monteiro
Nascimento Porto Alegre, RS, Brasil - 13/07/1981
Endereço profissional
Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul
Av. Bento Gonçalves, 9500, prédio 43421, salas 204/206
Agronomia, Porto Alegre,
91501-970, RS – Brasil
Telefone 33086070 **email** karina@cbiot.ufrgs.br

Formação Acadêmica/Titulação

2006 Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Identificação e caracterização de proteínas expressas pelo metacestódeo de ***Echinococcus granulosus*** durante a infecção do seu hospedeiro intermediário
Orientador: Henrique Bunselmeyer Ferreira
Bolsista do: Conselho Nacional de Desenvolvimento Científico e Tecnológico

2005 - 2006 Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Caracterização estrutural de oligômeros de subunidades recombinantes do antígeno B de Echinococcus granulosus, Ano de obtenção: 2006
Orientador: Henrique Bunselmeyer Ferreira
Bolsista do: Conselho Nacional de Desenvolvimento Científico e Tecnológico

2000 - 2004 Graduação em Ciências Biológicas.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

Prêmios e títulos

2009 Medalha Samuel Pessoa de 3° lugar para trabalho apresentado do XXI Congresso Brasileiro de Parasitologia e II Encontro de Parasitologia do Mercosul, Sociedade Brasileira de Parasitologia

2005 Trabalho Menção Honrosa do XIX Congresso Brasileiro de Parasitologia, XIX Congresso Brasileiro de Parasitologia

2003 Trabalho Destaque do XV Salão de Iniciação Científica e XII Feira de Iniciação Científica da UFRGS, UFRGS

Produção bibliográfica

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1. MONTEIRO, K. M., CARVALHO, M. O., ZAHA, A., FERREIRA, H. B. Proteomic analysis of the ***Echinococcus granulosus*** metacestode during infection of its intermediate host. ***Proteomics***, 10: 1985-1999, 2010.

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