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“Caracterización de Inhibidores de Serino-proteasas (Serpinas) de *Fasciola hepatica* como fundamento para el desarrollo de nuevos métodos de control de la Fasciolosis”

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Abreviaturas

°C	Grados centígrados
AOX1	Proteína alcohol oxidasa 1
AOX2	Proteína alcohol oxidasa 2
BMGY	Acrónimo del inglés *Buffered glycerol-complex medium*, medio complejo tamponado con glicerol
BMMY	Acrónimo del inglés *Buffered methanol-complex medium*, medio complejo tamponado con metanol
BSA	Sero albúmina bovina
DO	Densidad óptica
DTT	Ditiotreitol
EST	Acrónimo del inglés *Expressed sequence tag*, marcador de secuencia expresada
FPLC	*fast protein liquid chromatography*
GAGs	Glicosoaminoglicanos o glicosaminoglucuronano
HCl	Ácido clorhídrico
IgG	Inmunoglobulina G
IL	Interleucinas
kDa	Kilo Dalton
Milli-Q	Agua desionizada
Mut^-	Fenotipo de *Pichia pastoris* que no utiliza metanol
Mut^+	Fenotipo de *Pichia pastoris* que utiliza metanol
Mut^s	Fenotipo de *Pichia pastoris* que utiliza methanol lentamente
µL	Microlitro
mL	Mililitro
mM	Milimolar
NaCl	Cloruro de sodio
NEJ	Acrónimo del inglés *Newly excysed juvenile*, juveniles recientemente desenquistado
NMR	Acrónimo del inglés *Nuclear magnetic resonance*, resonancia magnética nuclear

PBS	Tampón fosfato salino
PES	Productos de excreción/secreción
PCR	Reacción en cadena de la polimerasa
PDB	Acrónimo del inglés *Protein data bank*
pH	Potencial hidrogeniónico
pI	punto isoeléctrico
PMSF	Acrónimo del inglés *phenylmethylsulfonyl fluoride*, fluoruro de fenilmetilsulfonilo.
RCL	Acrónimo del inglés *Reactive center loop*, bucle del centro reactivo
R.P.M.	Revoluciones por minuto
SCM	Sitio de clonaje múltiple
TAE	Tampón Tris-Acetato-EDTA
TM	Temperatura de *melting*
Tris	Tris(hidroximetil)aminometano, $(HOCH_2)_3CNH_2$
v/v	Volumen/volumen
YNB	Acrónimo del inglés *Yeast nitrogen base*
YPD	Acrónimo del inglés *Yeast extract-peptone-dextrose meduim*, medio de extracto de levadura-peptona-dextrosa

Resumen

Las proteasas son enzimas notables que participan en innumerables procesos fisiológicos importantes en los animales. Sin embargo, la proteólisis no regulada puede llevar a la aparición de algunas enfermedades funcionales. Los inhibidores de proteasas desempeñan funciones cruciales en el desarrollo y la supervivencia de los parásitos. Estos inhibidores participan en la modulación de las respuestas inmunes de sus huéspedes vertebrados. Los miembros de la familia de las serpinas (inhibidores de serino-proteasas por sus siglas en inglés) son inhibidores irreversibles de las serino-proteasas y regulan los sistemas relacionados con la defensa contra los parásitos. Actualmente hay información limitada disponible sobre los inhibidores de la proteasa del trematodo hematófago *Fasciola hepatica*. En esta tesis, se caracterizaron cuatro serpinas de *F. hepatica* (FhS-1 - FhS-4). La caracterización bioquímica reveló que rFhS-2 inhibe la actividad de la catepsina G de neutrófilos humanos. Comparativamente, rFhS-4 inhibe la actividad de la quimotripsina pancreática bovina y la catepsina G. En consecuencia, el índice de estequiometría (SI) para rFhS-2 y catepsina G fue 2.6, mientras que para rFhS-4 fue 2.4 para quimotripsina y 1.3 para catepsina G. Además, se observó la formación de complejos irreversibles entre rFhS-2 y rFhS-4 con estas proteasas. El análisis bioquímico de rFhS-4 demuestra que tiene una constante de segundo orden (ka) de 3.8 x 10^3 M-1 s-1 para quimotripsina. De manera similar, con otras serpinas, ambas proteínas recombinantes se unen a la heparina con alta afinidad. La localización en tejidos demostró que estas serpinas no están localizadas en los mismos órganos, que FhS-2 se localiza en el ovario y FhS-4 en las células intestinales, y ambos se ubicaron conjuntamente en las espinas dentro del tegumento. Estos hallazgos proporcionan la base para los posibles roles funcionales de estas proteínas como parte de un mecanismo del gusano adulto para evadir el sistema inmunitario del huésped y proteger los huevos para continuar el ciclo de vida del parásito. Una mayor comprensión del papel funcional de estas serpinas podría conducir al descubrimiento de nuevas intervenciones de control antiparasitario.

Palabras claves: inhibidores de catepsina G, inhibidores de quimiotripsina, helmintos, relación parásito-hospedero.

Summary

Protease inhibitors play crucial roles in parasite development and survival, modulating immune responses of their vertebrate hosts. Members of the serpin (serine protease inhibitors) family are irreversible inhibitors of serine proteases and regulate systems related to defense against parasites. Limited information is currently available on protease inhibitors from the blood fluke *Fasciola hepatica*. In this study, we characterized four serpins from the liver fluke *Fasciola hepatica* (FhS-1 – FhS-4). Biochemical characterization reveals rFhS-2 inhibited the activity of human neutrophil cathepsin G. Comparatively, rFhS-4 inhibited the activity of bovine pancreatic chymotrypsin and cathepsin G. Accordingly, the stoichiometry index (SI) for rFhS-2 and cathepsin G was 2.6, while for rFhS-4 was 2.4 for chymotrypsin and 1.3 for cathepsin G. Additionally, formation of irreversible complexes between rFhS-2 and rFhS-4 with these proteases were observed. Biochemical analysis of rFhS-4 demonstrates it has a second-order constant (ka) of 3.8 x 103 M-1 s-1 for chymotrypsin. Similarly, with other serpins, both recombinant proteins binds to heparin with high affinity. Tissue localization demonstrated that these serpins are not in the same organs, FhS-2 is localized in the ovary and FhS-4 in the gut cells, and both of them co-localized in the spines within the tegument. These findings provide the basis for possible functional roles of these proteins as part of a mechanism in the adult fluke to evade the host immune system and to protect the eggs to continue the parasite life cycle. Further understanding of serpins from the blood fluke may lead to the discovery of novel anti-parasitic control interventions.

Keywords: helminths, cathepsin G inhibitor, chymotrypsin inhibitor, parasite-host relationship

1. INTRODUCCION

1.1 *Fasciola hepatica*

F. hepatica es un endoparásito que pertenece al filo Platyhelminthes (del griego *platy*: aplanado y *helminthes*: gusanos) algunos tienen vida libre y otras especies parasitan vertebrados e invertebrados. Las características principales de este filo son animales triblásticos, simetría bilateral, acelomados, y cuerpo achatado dorsoventralmente. El intestino termina en fondo ciego y la única abertura del tracto digestivo es la boca. El sistema excretor es protonefridial, y su función es principalmente de regulación osmótica, siendo que los productos de excreción son eliminados principalmente por la superficie del cuerpo (Walker, 2001). Algunos de los parásitos de este filo se incluyen en la clase trematoda. Los gusanos de esta clase presentan adaptaciones para la vida parasitaria, algunas de las cuales facilitan la supervivencia e infestación dentro del huésped, tales como: ventosas, tegumento con cutícula protectora, ausencia de órganos sensoriales y producción de grandes cantidades de huevos. *F. hepatica* pertenece a la subclase digenea (*di*: dos, *genea*: vidas), el nombre hace referencia a un ciclo de vida heteróxeno con dos hospederos: intermedio y definitivo (Olson et al., 2003). En el hospedero intermediario, generalmente un molusco del género *Lymnaea*, habitan las etapas larvas del parásito y en el huésped definitivo, generalmente mamíferos, habitan los estadios reproductores (Andrews, 1999).

La fase adulta del parásito *F. hepatica* es un gusano plano con forma de hoja que mide de 1,5 a 5 cm de largo y 1,5 a 5 cm de ancho en la parte más gruesa del cuerpo. El cuerpo es de color rosa, mientras que el intestino presenta color marrón por la presencia de bilis y sangre. En la parte anterior del cuerpo hay dos ventosas: la oral que se encuentra alrededor de la boca y se utiliza para la ingestión de alimento y adhesión, y la ventosa ventral que tiene función de adhesión. El cuerpo está recubierto por el tegumento, que está formado por un sincitio delimitado por una membrana doble y posee microvellosidades. Bajo el

sincitio hay una membrana basal y una capa de fibras musculares, que aseguran la locomoción del helminto, y el parénquima. En el parénquima se incluyen los sistemas digestivo, excretor y nervioso. El sistema digestivo es altamente desarrollado y ramificado, y como *F. hepatica* no tiene sistema circulatorio, este sistema es responsable de la distribución de nutrientes para las células. En la etapa adulta, *F. hepatica* es un parásito hematófago. Los huevos eliminados en las heces del huésped definitivo poseen 150 x 90 μm, color amarillo-marrón, y tienen un opérculo por donde emerge el miracidio. Están compuestos por células vitelinas alrededor de un óvulo fertilizado (Andrews, 1999). Del huevo eclosiona una larva llamada de miracidio (130x28 μm), que tiene el cuerpo en forma de cono y cubierto de cilios (Malek, 1980; Valero et al., 2009). El NEJ (Acrónimo del inglés *Newly excysed juvenile*, juveniles recientemente desenquistado) es la etapa invasiva de *F. hepatica* en el hospedero definitivo. La migración del NEJ hasta el hígado y los canales biliares lleva aproximadamente 8 semanas, en las cuales los NEJ crecen y se desarrollan en adultos alcanzando el tamaño completo en 12-14 semanas después de la infección (Boray, 1969).

1.1.1 **Ciclo biológico**

Los hospederos definitivos de *F. hepatica* son infestados cuando ingieren vegetación conteniendo metacercarias, larvas enquistadas, en terrenos inundados o cerca de cursos de agua (Mas-Coma et al., 2014). Los seres humanos son infestados al ingerir plantas acuáticas o agua contaminada con metacercarias (Figura 1). Los NEJ eclosionan en el duodeno, atraviesan rápidamente la pared intestinal, entran en la cavidad peritoneal y migran hasta alcanzar el parénquima hepático. Después de un período de alimentación y crecimiento de aproximadamente 8 semanas dependiendo del hospedero, se desplazan a su destino final dentro de los canales biliares, donde maduran y producen huevos. Los huevos se liberan de los canales biliares hasta el intestino grueso por los fluidos biliares y son

eliminados por las heces, contaminando los pastizales. Cuando las condiciones ambientales de luz y temperatura son adecuadas, comienza el período de maduración y eclosionan los miracideos. Esta larva tiene capacidad natatoria e infecta al hospedador intermediario, gastrópodos pertenecientes a la familia Lymnaeidae en el caso de la *F. hepatica*. En el gastrópodo, el miracidio pierde las cilias y forma un esporocisto. El esporocisto es una masa celular germinativa donde cada célula se multiplica y produce una redia. Las redias crecen hasta estallar la pared del esporocisto y son liberadas en el caracol. Las redias se multiplican y producen cercarias (fase larvaria final), que emerge del caracol para encistarse en la vegetación que está cerca o en la superficie del agua, empezando la etapa de metacercaria. Como consecuencia, durante la migración y el desarrollo en los hospedadores, los parásitos encuentran diferentes tejidos, macromoléculas, microambientes fisiológicos dinámicos y respuestas inmunes del huésped.

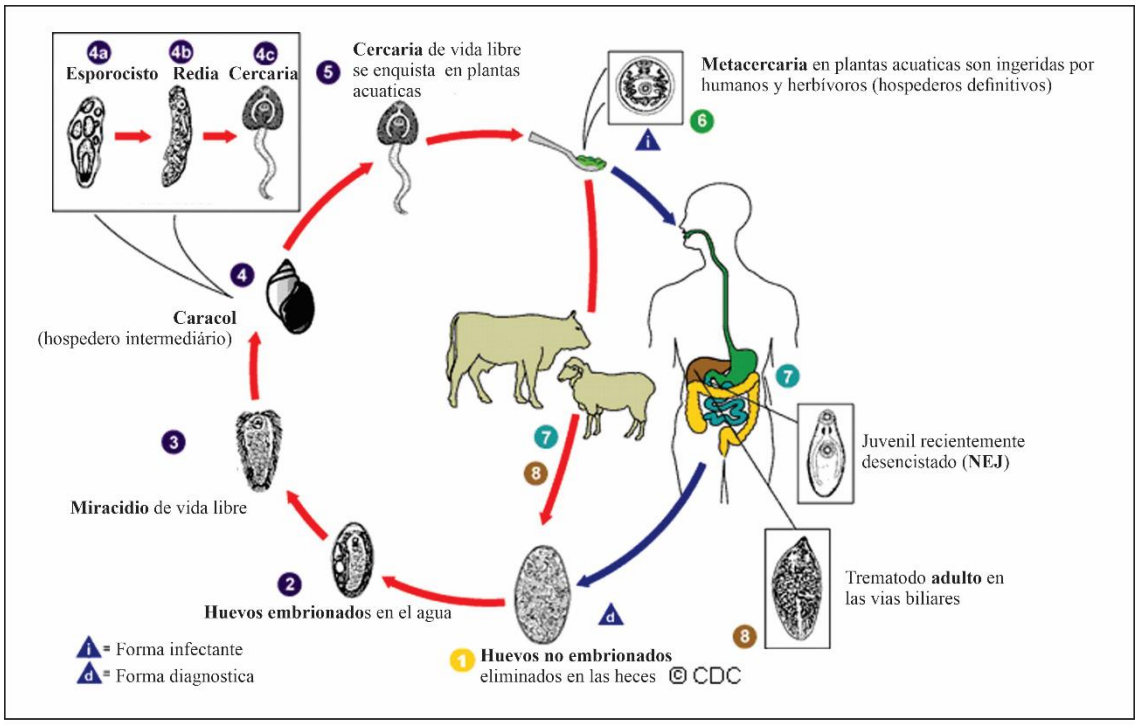

Figura 1. Ciclo biológico del parásito *Fasciola hepatica*. Adaptado de la plataforma DPDx – CDC (Division of Parasitic Diseases, Centers for Disease Control and Prevention)

1.1.2 **Patología, diagnóstico y prevención de la fasciolosis**

La fasciolosis es una enfermedad zoonótica que afecta a herbívoros y seres humanos y es causada por los parásitos trematodos *Fasciola hepatica* y *Fasciola gigantica*. *F. hepatica* es una especie que posee distribución mundial mientras que *F. gigantica* posee distribución restringida a las regiones tropicales de África y Asia (Mas-Coma et al., 2005). Esta enfermedad causa grandes pérdidas económicas en la ganadería, estimadas en más de 3.000 billones de dólares en todo el mundo, que incluyen reducción en la ganancia de peso, disminución de la fertilidad, producción de leche en bovinos y lana en los ovinos (Spithill et al., 2012). El principal método utilizado para el control de la fasciolosis es el uso de anti-helmínticos (Armour, 1975; Mitchell, 2003). Sin embargo, el tratamiento es de alto costo y existe la ocurrencia de reinfecciones y resistencia (Aguilera-Luiz MM, 2012; Molina-Hernandez et al., 2015; Olaechea et al., 2011). Por esto, existe la necesidad de desarrollar estrategias de control alternativas, que sean más efectivas y de menor costo. Para realizar esto, es importante mejorar el conocimiento sobre la biología del parásito y de su relación con el hospedero, y en particular determinar a nivel molecular cuales son las moléculas y/o sistemas que participan.

Entre 8 y 10 semanas después de la ingestión de metacercarias, los gusanos adultos se instalan en los canales biliares del hígado del huésped definitivo, donde se fijan a las paredes y se alimentan de sangre. El patrón de alimentación multi-sitio combinado con las espinas del tegumento de la *F. hepatica* irritan y lastiman los canales biliares, lo que provoca espesamiento en las paredes de los canales. La irritación crónica puede llevar a la calcificación de las paredes de los conductos biliares y el deterioro de la función hepática. Cuando la carga parasitaria es grande, puede causar anemia debido a la ingestión de sangre (Isseroff et al., 1979; Torgerson, 1999).

El diagnóstico de la parásitosis es desafiante, ya que gran parte de los animales infestados no presentan síntomas. Cuando sintomática existe fiebre, temblor, dolores en la zona del hígado, hepatomegalia y/o eosinofilia. En los casos de fasciolosis aguda, puede ocurrir ictericia, necrosis en el hígado y anemia. En general, los signos clínicos de fasciolosis pueden ser fácilmente confundidos con infecciones de nematodos, y estos no necesitan manifestarse para que la productividad del huésped se vea afectada. El diagnóstico es posible desde los 3 meses de infección y las técnicas generalmente utilizadas son exámenes coproparasitólogicos o serológicos (Alvarez Rojas et al., 2014; Braun et al., 1995; Mezo et al., 2004).

Los métodos de control de este parásito están enfocados en la reducción de la enfermedad en animales, para evitar pérdidas económicas. El método de control más utilizado es el tratamiento del rumiante con anti-helmínticos, o el control biológico del caracol (Torgerson, 1999). Con el tratamiento con molusquicidas es posible controlar la población de hospederos intermediarios con éxito y buena relación costo-beneficio. Sin embargo, este enfoque no es muy aceptado debido al riesgo de contaminación ambiental, particularmente de poblaciones económicas y biológicamente importantes, como peces o cangrejos. En el caso de los antihelmínticos, diferentes compuestos se utilizan dependiendo de la especie infestada, del costo y de la seguridad (Sanyal, 1995). Un problema que limita el uso de drogas en el tratamiento de la enfermedad es el aumento de resistencia de los parásitos frente a los antihelmínticos y la presencia de residuos xenobióticos en alimentos y en el medio ambiente (Tsiboukis et al., 2013; Wolstenholme and Martin, 2014). Además, existe una demanda de varios mercados económicos internacionales que estimulan la búsqueda de nuevos métodos de control. Una alternativa al uso de antihelmínticos y molusquicidas son las vacunas. Y para buscar blancos biológicos es necesario incrementar el conocimiento de la relación parásito-huésped.

1.2 Serino-proteasas

Las proteasas, también denominadas peptidasas, son proteínas fundamentales en los procesos biológicos de los parásitos. Son enzimas proteolíticas que catalizan la escisión hidrolítica de los enlaces peptídicos. Las serino-proteasas (EC 3.4.21) son endopeptidasas que poseen un residuo de serina en su sitio activo (Patel, 2017). Estas poseen un estrecho espectro de funciones que si no están bajo estricta regulación pueden causar graves daños tanto al parásito como al hospedero (Knox, 2007). Muchas de estas proteasas se encuentran en los PES (productos de excreción/secreción) al mismo tiempo que su inhibidor, en *F. hepatica* esto es particularmente visibles para catepsinas y sus inhibidores que son la clase de endoproteasas más estudiadas en este parásito hasta el momento (Cancela et al., 2008; Di Maggio et al., 2016; Robinson et al., 2009). En un principio se especuló con que la función de estos inhibidores era interaccionar con las proteasas del parásito, pero existen evidencias de otros roles como por ejemplo modular la respuesta inmune del hospedero (Hill and Hastie, 1987; Knox, 2007; Zang and Maizels, 2001).

1.3 Inhibidores de serino-proteasas

Los inhibidores de serino-proteasas o serpinas han atraído la atención por su rol regulatorio en los procesos de coagulación sanguínea e inflamación (Knox, 2007). Muchos inhibidores forman moléculas tetraédricas que mimetizan el estado intermediario formado durante el mecanismo de catálisis, como es el caso del PMSF (Acrónimo del inglés *phenylmethylsulfonyl fluoride*, fluoruro de fenilmetilsulfonilo). Otros inhibidores forman compuestos estables intermediarios como las serpinas. Las serpinas son la superfamilia de inhibidores de proteasas más grande y más ampliamente distribuidas (Irving et al., 2000).

La mayoría actúan sobre serino-proteasas pero también han sido identificadas serpinas que actúan sobre caspasas y cisteíno-proteasas (Ray et al., 1992; Schick et al., 1998). Su principal función es inhibitoria pero se han encontrados serpinas que participan como transportadores de hormonas, chaperonas o supresores tumorales (Nagata, 1996; Pemberton et al., 1988; Zou et al., 1994). Las serpinas poseen un funcionamiento de tipo "suicida" o "uso único" en el cual ocurre un solo cambio conformacional que permite inhibir a su proteasa blanco (Law et al., 2006). Poseen una estructura terciaria característica y conservada está formada por tres hojas β, ocho o nueve α hélices y varios bucles que forman una estructura metaestable (Gettins, 2002). La región responsable de la interacción con la proteasa blanco es el centro reactivo (RCL, reactive center loop), un bucle que se encuentra expuesto en la estructura de la serpina (Gettins, 2002; Law et al., 2006).

1.3.1 Estructura y Clasificación

El nombre serpinas surge a partir de que las primeras serpinas caracterizadas eran inhibitorias, sin embargo existen varios miembros que no presentan actividad inhibitoria como son la angiotensina y la ovoalbúmina (Doolittle, 1983; Hunt and Dayhoff, 1980) o inhiben otras clases de proteasas (Annand et al., 1999; Schick et al., 1998). A pesar de la variabilidad de funciones encontradas entre las serpinas, estas poseen relaciones filogenéticas y de estructura de proteínas relacionadas. Hasta el momento existen 16 clados, que se identifican como clado A hasta el clado P aunque existen serpinas que no pertenecen a ninguno de ellos y son llamadas de "huérfanas" (Irving et al., 2000).

La estructura terciaria metaestable de las serpinas está altamente conservada entre los miembros de la superfamilia donde el tamaño promedio de las proteínas es de alrededor de 400 aminoácidos con un peso molecular de 40-50 kDa. La estructura terciaria está compuesta por tres hojas-β (A-C) y 7-9 α-hélices (A-I) y una región llamada de RCL. El RCL contiene los aminoácidos que se unen al sitio activo de la proteasa blanco

posibilitando la actividad inhibitoria. El RCL contiene aproximadamente 20 residuos de aminoácidos, conforme al sistema de nomenclatura la unión peptídica susceptible a hidrolisis es la que se encuentra en la posición P_1-$P_{1'}$ y determina la especificidad de la proteasa blanco (Khan et al., 2011). Las serpinas cuando no están en complejo con su proteasa blanco adoptan diferentes conformaciones que son críticas para su función inhibitoria. Inicialmente la serpina se une con su proteasa blanco a través de la formación de un complejo no covalente del tipo Michaelis por la interacción de los residuos P_1y $P_{1'}$ del inhibidor con los sitios blanco de la proteasa (Gettins, 2002).

1.3.2 Mecanismo de inhibición

El mecanismo empleado por las serpinas para inhibir proteasas es altamente conservado, presentando una inhibición de tipo suicida y esta ejemplificado en la Figura 2. En lugar de la inhibición típica utilizada por otros inhibidores de proteasas, las serpinas pasan por un cambio conformacional luego de su unión con la proteasa que previene la catálisis. Brevemente, el cambio conformacional incluye que el RCL sea insertando en la hoja-β A, formando una hoja-β extra antiparalela. Esto convierte a la serpinas de un estado estresado a un estado relajado de baja energía (transición de S a R, stressed and relaxed por sus siglas en inglés).

La serpina se une con la proteasa blanco a través de un complejo tipo Michaelis no covalente por la interacción con los residuos que flanquean al enlace escindible (P_1-P_1'), este enlace determina la especificidad de la proteasa (Gettins, 2002). El ataque de la serina del sitio activo del enlace escindible lleva a la creación de un enlace éster covalente entre la serina de la proteasa y el esqueleto carbonilo del residuo P_1 que resulta en la escisión del enlace peptídico. El RCL unido con la proteasa blanco se inserta en la hoja-β A, y es

translocado unos 70Å generando que su sitio activo se distorsione, previniendo su hidrolisis y generando la unión covalente irreversible. Esta transición estructural que ocurre en la serpina genera un aumento en la estabilidad conformacional (Horvath et al., 2005).

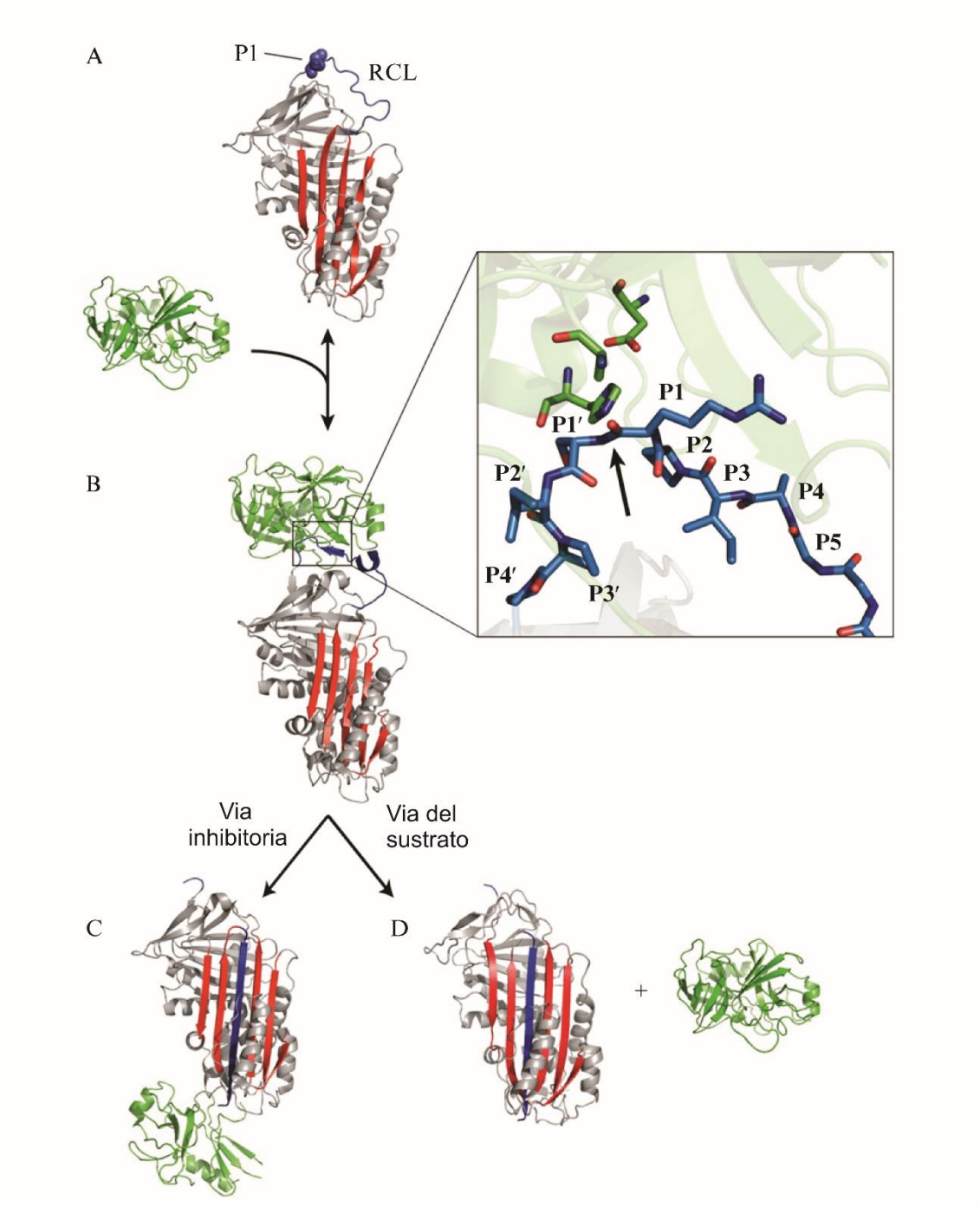

Figura 2. Representación del mecanismo de inhibición suicida de las serpinas. El esquema representa la interacción entre la serpina (en gris con la hoja-βA en rojo y el RCL en azul) y la proteasa (en verde). A: estructura de la forma nativa de la serpina α-1-antitripsina, donde la posición del residuo P_1 está marcado. B: complejo de Michaelis no covalente. El recuadro representa los residuos de aminoácidos que interactúan entre la proteasa y la serpina antes de

que ocurra el clivaje de los residuos P_1-P_1´ (indicado con una flecha, en verde: aminoácidos de la proteasa, en azul: residuos de aminoácidos del RCL). C: Via inhibitoria, se forma el complejo inhibitorio. D: Via del sustrato, la serpina es clivada y la proteasa liberada. (Modificado de Khan M.S., et al. 2011).

A partir de la formación del ultimo intermediario pueden ocurrir dos cosas que van a generar productos diferentes. Uno de ellos es la continuación de la reacción de proteólisis, donde el intermediario acil es hidrolizado, y resultando en la liberación de la serpina clivada y la enzima libre. El otro camino, termina en la inhibición de la actividad de la proteasa e implica que el RCL se inserta en la hoja-β. Esto genera un cambio conformacional dentro de la estructura de la serpina que provoca el dislocamiento de la proteasa desde la parte superior de la serpina hasta la inferior, resultando en la formación de un complejo covalente. Esto deja a la proteasa catalíticamente inactiva por las distorsiones que sufre el sitio activo (Gettins, 2002).

Además, los residuos de aminoácidos que se encuentran en los sitios P_4-P_4' están conservados en todas las serpinas inhibitorias y los residuos de la región bisagra tienen una pequeña cadena lateral que permite la flexibilidad necesaria para la formación del complejo. Se cree que la fuerza motriz para este cambio conformacional es la pérdida de energía asociada con la inserción del bucle en el complejo.

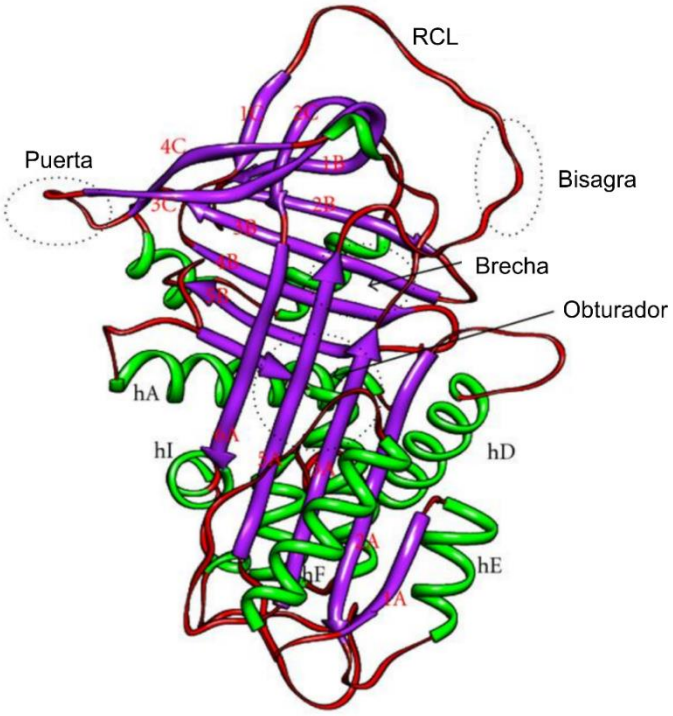

Figure 3: Dominios importantes para controlar los cambios conformacionales de las serpinas. El RCL participa en el reconocimiento de las proteasas e inicia el cambio conformacional. La región del RCL entre los residuos P_{15}–P_9 se conoce como región bisagra. Con algunas variaciones la mayoría de las serpinas poseen 3 hojas-β (llamada A, B y C, en rojo) y ocho o nueve α-hélices (llamadas hA–hI, en negro). Las cinco hebras plegadas de la hoja- β A contienen dos regiones funcionalmente importantes; la brecha y el obturador. (Modificado de Khan M.S., et al. 2011, en base a un PDB de la conformación nativa de la antitripsina).

Varias regiones son importantes para controlar y modular los cambios conformacionales que ocurren en las serpinas. La Figura 3 muestra las regiones estructuralmente importantes de la serpina (Whisstock et al., 2000). La región bisagra del RCL, que se encuentra entre los residuos P_{15}–P_9 proporciona movilidad en la transición S → R. Esta región contiene residuos conservados entre los residuos P_{15}-P_{10}, y el aminoácido en P_{14} es de importancia crítica, ya que su inserción en la hoja β A es un requisito previo para la actividad inhibitoria (Chang et al., 1996). En la caso de la serpina antitrombina, la sustitución en el residuo P_{12} de alanina a treonina provoca la pérdida de actividad inhibitoria (Carrell et al., 1994). Los residuos altamente conservados ubicados en la región del

obturador de la hoja-β A son serinas en las posiciones 53 y 56 que desempeñan un papel importante en las transiciones conformacionales de la serpina (Krem and Di Cera, 2003).

1.3.3 Serpinas de parásitos

En los helmintos parásitos, las serpinas suelen participar en la modulación de las respuestas inmunes y la supervivencia en el hospedero. En *Brugia malayi*, el componente principal de los productos secretados es una serpina (MB-SPN-2) que inhibe la actividad enzimática de las proteasas catepsina G y elastasa de neutrófilos (Zang et al., 1999). En Ascaris, las serpinas están presentes en la superficie del intestino, facilitando su supervivencia al inactivar proteasas del huésped (Martzen et al., 1985). Una serpina de *Echinococcus granulosus* (Shepherd et al., 1991) tiene la capacidad de inhibir el reclutamiento de neutrófilos. En las especies de *Schistosoma*, además de controlar la homeostasis de las serino-proteasas, podrían realizar otras funciones (Mebius et al., 2013). En *Schistosoma mansoni*, una serpina inhibe elastasa de neutrófilos, modulando su actividad frente a la degradación de tejido para permitir la migración del parásito (Ghendler et al., 1994; Quezada et al., 2012). Las serpinas conocidas de *Schistosoma japonicum* inhiben elastasa pancreática humana (SjB10) y quimotripsina, tripsina y trombina (SjSPI) (Molehin et al., 2014; Zhang et al., 2018). En *Schistosoma haematobium*, una serpina de membrana posee actividad anti-tripsina (Huang et al., 1999). Se han estudiado dos serpinas en *Clonorchis sinensis*, denominadas CsSERPIN y CsSERPIN3, ambas altamente expresadas en la etapa de metacercaria y que poseen diferencias en la región del RCL y su localización dentro del parásito (Yang et al., 2014).

Este conocimiento ha llevado a suponer que las serpinas secretadas por *F. hepatica* podrían participar en interrumpir el equilibrio homeostático del huésped, como una forma de prevenir, ralentizar y/o evadir las defensas del huésped. A pesar del creciente interés en

estudiar proteasas derivadas de parásitos en *F. hepatica* (Dalton et al., 2003; Jayaraj et al., 2009; Mokhtarian et al., 2016), existen pocos datos disponibles sobre los inhibidores de proteasas. En un estudio proteómico realizado por nuestro grupo de investigación, se encontraron secuencias que coinciden con esta superfamilia de inhibidores en las proteínas solubles somáticas de NEJ, PES de NEJ y PES de adultos, respectivamente (Di Maggio et al., 2016-Anexo xx).

1.4 Sistema de expresión para proteínas heterólogas

Existen varios sistemas de expresión para producción de proteínas heterólogas, procariotas, eucariotas, células de insectos y de mamíferos. La preferencia por un sistema se basa en las prioridades o necesidades de cada proteína heteróloga a ser expresada. Existen varios criterios básicos como son: nivel de expresión, estabilidad del material producido, aplicaciones futuras o preservación de la actividad proteica (Fickers, 2014)

Dentro de los microorganismos recomendados para expresión de proteínas heterólogas están las levaduras. Inicialmente, fue utilizada la levadura *Saccharomyces cerevisiae*, sin embargo presento inconvenientes como la inestabilidad plasmídica, los bajos rendimientos de producción y el fenómeno de la hiperglicosilación (Buckholz and Gleeson, 1991; Romanos, 1995). Estos inconvenientes llevaron al desarrollo de sistemas de expresión alternativos basados en otras levaduras como *Pichia pastoris*, *Hansenula polimorpha*, *Kluvero mycelactis*, *Schizosaccharomyces pombe*, *Arxula adeninivorans* y *Yarrowia lipolytica* (Fickers, 2014). Esto se justifica por ser organismos eucariotas, de fácil crecimiento y manipulación y con la capacidad de realizar modificaciones postraduccionales y ausencia de endotoxinas en los productos de la expresión (Idiris et al., 2010; Porro and Mattanovich, 2004).

1.4.1 *Pichia pastoris*

Desde hace algunos años esta levadura ha demostrado ser un sistema altamente eficaz de producción de proteínas y es la levadura más utilizada actualmente. Se debe principalmente al hecho de poseer un promotor fuerte y regulado como es el promotor que regula al gen de la enzima alcohol oxidasa y a que su metabolismo le permite alcanzar elevadas densidades celulares sin que los productos del metabolismo inhiban su crecimiento (Chen et al., 2012). En esta levadura, los genes AOX1 y AOX2 codifican para las enzimas alcohol oxidasa1 y 2 respectivamente (Vanz et al., 2012). La enzima Aox1 representa el 90 % del total de la actividad alcohol oxidasa en la célula y representa más del 30% del total de proteínas solubles (Cos et al., 2006). *P. pastoris* se caracteriza por poseer una rápida velocidad de crecimiento en medios de cultivos más económicos compuestos por una fuente de carbono (glicerol o metanol), biotina, sales y agua (Cereghino and Cregg, 2000; Macauley-Patrick et al., 2005), su manipulación genética es semejante a la realizada en *S. cerevisiae*, lleva a cabo plegamiento de proteínas y la generación de modificaciones pos-traduccionales (Li et al., 2007; Mellitzer et al., 2012). También, esta levadura secreta pequeñas cantidades de proteínas endógenas y por consiguiente, las proteínas heterólogas secretadas se encuentran parcialmente puras, lo que facilita su purificación (Cereghino and Cregg, 2000; Fickers, 2014).

Las cepas de *P. pastoris* se clasifican por su capacidad de metabolizar el metanol en: Mut^{+} estas cepas poseen un crecimiento en metanol igual al de la cepa silvestre, ya que portan los genes AOX1 y AOX2 funcionales. Las cepas Mut^{S} poseen un crecimiento lento en presencia de metanol respecto a la cepa silvestre, ya que apenas el gen AOX2 se encuentra funcional. Las cepas Mut^{-} no pueden emplear el metanol como fuente de carbono y energía, ya que poseen a los genes AOX1 y AOX2 inactivados (Fickers, 2014). Estos tres

grupos se distinguen por sus velocidades de crecimiento en presencia de metanol (Stratton et al., 1998).

La secreción de las proteínas heterólogas expresadas constituye una herramienta que facilita su purificación. Para esto, es necesario que la proteína heteróloga este fusionada a un péptido señal que la conduzca a través de la ruta de secreción hacia el medio extracelular.

1.4.2 Vectores de expresión

Los vectores de expresión desarrollados para la expresión de proteínas heterólogas en la levadura *P. pastoris* pueden ser mantenidos y replicados en *Escherichia coli*, poseen un marcador de selección que puede ser usado en uno o ambos microorganismos y son comercializados por la Corporación Invitrogene. Estos vectores están compuestos por un promotor, un sitio de clonaje múltiple (SCM) con uno o más sitios de restricción para la inserción del gen heterólogo, un terminador transcripcional, un marcador de selección que puede ser usado en *E. coli, P. pastoris* o en ambos y opcionalmente una secuencia que codifica para una péptido señal (FAT-α ó PHO5) entre el promotor y el SCM que garantiza la secreción de la proteína heteróloga al medio de cultivo (Cregg et al., 2000).

2. Justificativa del trabajo

La justificación básica del presente estudio es la evidente carencia de investigación relevante sobre los inhibidores de serino-proteasas en el parásito hematófago *Fasciola hepatica*, el cual es uno de los parásitos que causa perjuicios económicos a nivel nacional. Hasta ahora, como puede fundamentarse en la bibliografía, se han constatado resultados sobre la presencia de estas proteínas en varios parásitos digeneos pero aún no existe información en *F. hepatica*. Asimismo, en un análisis proteómico realizado por nuestro grupo de investigación se identificaron secuencias que poseen los aminoácidos conservados entre las serpinas y con esto se identificó su presencia en los PES de parásitos adultos así como también en la fase juvenil (NEJ).

3. Hipótesis

La hipótesis de esta tesis de doctorado es que las serpinas de *Fasciola hepatica* podrían estar cumpliendo un rol importante en la relación parásito-hospedero, así como también ser importantes en procesos que tienen que ver con la coagulación sanguínea para auxiliar al parásito en su supervivencia en el hospedero mamífero.

4. OBJETIVOS

4.1 Objetivo general

El objetivo general de esta tesis doctoral es la caracterización de serpinas de *Fasciola hepatica*.

4.2 Objetivos específicos

4.2.1 Utilizar herramientas bioinformáticas y bases de datos para buscar serpinas de organismos genéticamente relacionados para la generación de primers para rastrear nuevas serpinas en *F. hepatica*.

4.2.2 Determinación *in silico* de aminoácidos críticos en la estructura del centro reactivo (RCL) de las serpinas en *F. hepatica*.

4.2.3 Optimizar el sistema de expresión eucariota para serpinas de *F. hepatica*.

4.2.4 Realización de ensayos enzimáticos de inhibición con las serpinas para determinar actividad sobre serino-proteasas.

4.2.5 Determinar la variación del perfil de expresión de las serpinas encontradas en diferentes tejidos de *F. hepatica*.

5. MATERIALES Y METODOS

5.1 **Declaración de ética**

Este trabajo fue llevado a cabo de acuerdo con los aspectos éticos y metodológicos permitidos por las normas internacionales de legislación animal. Los protocolos de utilización de animales de laboratorio fueron aprobados por la comisión de ética del uso de animales (CEUA) de Rio Grande do Sul, Brasil (número de protocolo 27369). Hígados de bovino fueron recolectados de un matadero en la región de Porto Alegre, Rio Grande del Sur, Brasil inmediatamente después del sacrificio. Infecciones por *F. hepatica* fueron diagnosticadas en el matadero por inspectores de carne independientes y el material biológico desechado por el protocolo local de matadero. El matadero autorizado por el Ministerio de Agricultura y Pesca de Uruguay (MGAP), cumple con la Ley Nacional de Bienestar Animal nº 18471 de 2009, ley de protección, bienestar y posesión de animales, regulada por: Decreto nº 62.

5.2 **Obtención de material biológico de *F. hepatica* para inmunohistoquímica**

Los parásitos adultos de *F. hepatica* fueron obtenidos en un matadero local de la región de Porto Alegre, Rio Grande del Sur, Brasil. Los adultos de *F. hepatica* fueron colectados a partir de los conductos biliares de hígados de vacunos y transportados al laboratorio en bilis de animal sano donde fueron procesados con un protocolo previamente establecido (Cancela et al., 2008). Brevemente, los gusanos adultos fueron lavados en PBS estéril al menos 4 veces o hasta que el contenido intestinal fuera visiblemente eliminado, a continuación, fueron colocados en un tubo de 1.5 ml, conteniendo formaldehido 4% y fueron almacenados hasta su uso.

5.3 **Identificación de secuencias codificantes para serpinas en *Fasciola hepatica*.**

Secuencias codificantes de serpinas de *Fasciola hepatica* fueron recuperadas a partir de una base de datos pública online (www.gasserlab.org) formada por EST (Expressed sequence tag). Estas secuencias fueron obtenidas desde las bases de datos y clonadas en un experimento previo del que la disertante no formó parte (Berasain et al., 2013). Para confirmar la identidad de las secuencias se realizó una búsqueda contra una base de datos no redundante dentro de Genebank (compilada en abril de 2014) utilizando el algoritmo BLAST, que permite comparar informaciones de las secuencias biológicas primarias sean de aminoácidos, proteínas o nucleótidos. En este caso fue utilizado el algoritmo BLASTp que compara una secuencia de aminoácidos contra una base de datos de proteínas. Para validar la identificación de las secuencia como serpinas, las mismas se inspeccionaron en busca de codón inicio (representado con el triplete de nucleótidos ATG) y stop (que puede ser uno de los siguientes tripletes de nucleótidos: TAG, TAA o TGA), longitud de la secuencia en amino ácidos (de unos 400-500 aminoácidos) y la presencia de secuencias de aminoácidos conservadas dentro de las serpinas conocidas como NAVYFKG y DVNEEG (Gettins, 2002; Tirloni et al., 2014).

5.4 **Análisis *in silico* de las secuencias de serpinas**

Las secuencias de nucleótidos obtenidas como fue descrito en la sección 4.3 fueron analizadas para obtener las secuencias deducidas de aminoácidos. La presencia de secuencia de péptido señal en las serpinas de *F. hepatica* fue predicha utilizando el servidor SignalP 4.0 (Petersen et al., 2011). Los sitios putativos de N-glicosilación fueron encontraron utilizando el servidor NetNGly4.0 (Steentoft et al., 2013). El peso molecular teórico y los puntos isoeléctricos de las serpinas fueron calculados utilizando la

herramienta Compute pI/Mw a través del sitio web ExPASy (http://web.expasy.org/compute_pi/pi_tool-Ref.html).

5.5 **Modelado tridimensional de las proteínas.**

Para realizar el modelaje de la estructura terciaria fue utilizando un enfoque de modelado comparativo. Fueron realizadas búsquedas en la base de datos Genebank utilizando el algoritmo BLASTp contra una base de datos de proteínas (pdb del inglés Protein Data Bank, http://www.rcsb.org). Esta base de datos es un archivo de estructuras tridimensionales de proteínas obtenidas por cristalografía de rayos X o NMR. Con esta búsqueda se procuró determinar la mejor secuencia a utilizar como molde teniendo en cuenta: homología entre las secuencias molde y la serpina, un 30 % de cobertura y GAPs menores a 5%. Para esto fueron utilizados los modelos 1AZX (Antithrombin/Pentasaccharide Complex) para el modelado de la FhS-1 y el modelo 1DZG (Antithrombin-III) fue utilizado para el modelado de la FhS-2, FhS3 y FhS-4. El alineamiento de las secuencias realizado utilizando el software ClustalW (Larkin et al., 2007) fue utilizado como archivo de entrada para el programa Modeller 9v14 (Webb and Sali, 2014). El programa Modeller constituye un método de modelado comparativo diseñado para encontrar la estructura más probable de una proteína determinada, en función de su alineamiento con una proteína homóloga con estructura tridimensional conocida. El modelo tridimensional de la proteína problema se obtiene por satisfacción de restricciones espaciales. El programa utiliza la proteína molde para tomar las distancias entre residuos y esto se complementa con datos de distancias de enlaces, ángulos diedros, distancias de interacciones débiles, El mejor modelo es aquel que viola el menor número de restricciones espaciales. Los modelos generados se evaluaron utilizando QMEAN4 y PROCHECK para estimar la confiabilidad del modelo y predecir su calidad (Benkert et al., 2008; Morris et

al., 1992). El potencial electrostático para las estructuras de FhSs se calculó utilizando la herramienta Adaptive Poisson-Boltzmann Solver (APBS). Los estados protonados fueron asignados utilizando los parámetros para el campo de fuerza de solvatación (PARSE) por PDB2PQR para cada estructura (Unni et al., 2011). La ejecución de APBS y la visualización de los potenciales electrostáticos resultantes fueron realizaron mediante el uso del programa Visual Molecular Dynamics (VMD) (Humphrey et al., 1996) con ± 5 kT/e de campos de contorno positivo y negativo. Las secuencias de aminoácidos se inspeccionaron manualmente en busca de motivos de unión a glicosaminoglicanos (GAG) (Hileman et al., 1998). Las vueltas-β (o β-turns) en la región RCL de FhS-2 y FhS-3 se predijeron utilizando la herramienta NetTurnP-tweak a través del sitio web DTU Health Tech (http://www.cbs.dtu.dk/services/NetTurnP/) (Petersen et al., 2010).

5.6 **Clonado, expresión y purificación de las serpinas recombinantes**

5.6.1 **Clonación en el vector de expresión pPICZαC.**

Para expresar las 4 secuencias codificantes de serpinas de *F. hepatica* fueron diseñados primers específicos para clonar en *Pichia pastoris* (Tabla 1 y Anexo 1). Las secuencias de estas serpinas fueron clonadas en vector de clonación pGEM®-T Easy en un trabajo anterior (Berasain et al., 2013). Para el diseño de los primers para clonar en *P. pastoris* fueron utilizadas las secuencias de nucleótidos obtenidas previamente y fueron adicionados sitios de corte para las enzimas de restricción (*Cla*I and *Not*I, Invitrogen, EE.UU) además en el primer reverso (Rv) se adicionaron los nucleótidos necesario para 6 histidinas a ser utilizadas posteriormente para purificar a las proteínas recombinantes (Tabla 1, Figura 4 y Anexo 1).

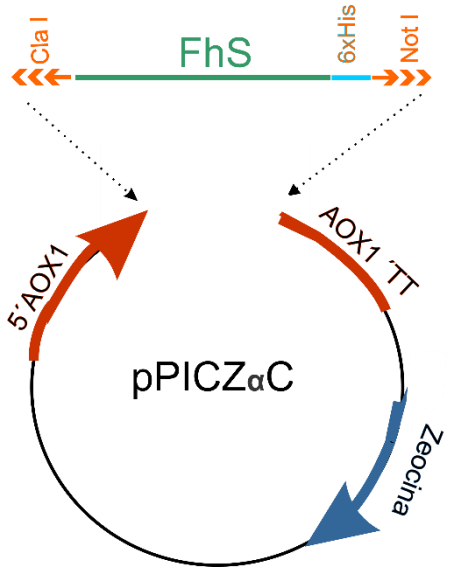

Figura 4. Esquema de la estrategia de clonado de las serpinas de *F.hepatica* en *P. pastoris*. En la figura se detalla el lugar de inserción de la secuencia de serpina (5´AOX y 3ÁOX), Las enzimas de restricción utilizadas (ClaI y NotI) y la secuencia que codifica para la resistencia al antibiótico Zeocina.

El ADN molde (50 ng) utilizado fueron las secuencias clonadas dentro del vector pGEMt® easy que llamaremos FhS-1, FhS-2.4, FhS-2.6 y FhS-4 de aquí en más. Para clonar las secuencias en el vector de expresión pPICZαC para *P. pastoris*, se realizó una reacción de PCR con la polimerasa Elongase®. Se realizó la siguiente mezcla de reacción por cada muestra: 35 µl de agua Mili-Q; 5 µl tampón A (Invitrogen); 5 µl tampón B (Invitrogen); 1 µl de los primers forward y reverse para cada serpina a una concentración stock 10 µM, 1 µl de Elongase® Enzyme Mix (Invitrogen) y 1 µl de DNA molde, consiguiendo así un volumen final de 50 µl. Las condiciones de la reacción de PCR fueron una desnaturalización inicial a 94 °C por 5 min, 34 ciclos de 30 seg a 94 °C, 30 seg a 59 °C, 1:30 min a TM °C y una extensión final a 68 °C por 5 min. Las mismas se llevaron a cabo en un termociclador Infinigen Thermal Cycler TC-XX/H. Para visualizar el resultado de la PCR, fue realizada una electroforesis en gel de agarosa 0,8% en tampón TAE con 5 µl de bromuro de etídio 10 mg/ml por cada 100 ml. Se sembraron 5 µl de los productos de PCR en cada pocillo con 1 µl de buffer de carga 6X (Promega). Además, en un pocillo se

sembró como referente de corrida un marcador de peso molecular Lambda HindIII DNA Markers (Invitrogen). La corrida fue realizada por 15 min a 150 voltios y visualizada en un transiluminador UV.

Luego vector e insertos fueron purificados utilizando una matriz de sílica utilizando el kit *Geneclean*^{TM} de la siguiente manera. Los fragmentos de gel de agarosa conteniendo los plásmidos se incubaron con la solución de NaI por 5 minutos a 55ºC. Luego se adicionaron 5 µl de la suspensión de sílica *Glassmilk*^{TM} y se incubó durante 15 minutos a temperatura ambiente para luego centrifugar por 5 minutos a 5.000 rpm. Se descartó el sobrenadante y el pellet fue resuspendido con el tampón *Newwash*^{TM} (etanol 50%, Tris pH 7.5, NaCl 0.1M, EDTA 1mM) y se centrifugo 5 minutos a 5.000 rpm, este procedimiento se repitió 3 veces. Para eluir el material genético se agregaron 5 µl de agua milliQ y se incubó 3 minutos a 55ºC, se centrifugo 5 minutos a 5.000 rpm y el material genético del sobrenadante fue cuantificado por gel de agarosa. Los vectores purificados fueron digeridos con las enzimas de restricción ClaI y NotI (Invitrogen) durante 4 horas a 37ºC para generar extremos cohesivos que fueron posteriormente ligados con la enzima T4 DNA ligase (Invitrogen, EE. UU) durante 16 horas a 4ºC. Se utilizó una relación molar plásmido-inserto 3:1. La identidad de las secuencias fue confirmada mediante secuenciación realizado en el Hospital de Clínicas de Porto Alegre, Brasil y fue confirmada como clonado positivo cuando la secuencia obtenida concuerda con la existente en los clones realizado anteriormente en el vector pGEMt® easy.

Secuencia (5’ – 3’)	Nombre	Tamaño	GC%	TM
AAAAAA**TCGATG**GGAGAAGTCACTTTTG	FhS-1 Fw	28	39,3	56,4
TTT*GCGGCCGC*CTAGTGGTGGTGGTGGTGGTGCTCTTCAGCCGATGTTACG	FhS-1 Rv	51	50	55
AAAAAA**TCGAT**GACCTCATCTATGGAACATTCCTTGAAGAG	FhS-2 Fw	41	41,4	57,9
TTT*GCGGCCGC*TTAGTGGTGGTGGTGGTGGTGGTTCACCTCCGGATCAGTAACG	FhS-2 Rv	54	48	58
AAAAAA**TCGAT**GTGCAAGTCGAAGGTGCCGGAC	FhS-4 Fw	33	61,9	62,4
TTT*GCGGCCGC*TTAGTGGTGGTGGTGGTGGTGCAAGCGTTCACGTGGATTGGTCAC	FhS-4 Rv	56	61,9	62,4

Tabla 1. Primers específicos generados para el clonado de las serpinas de *Fasciola hepatica* en el vector de expresión pPICzαC. Tamaño: tamaño del iniciador en nucleótidos. GC (%): porcentaje de guaninas y citosinas; TM: temperatura de alineamiento teórica en grados centígrados. En **negrito**: sitio de corte para la enzima de restricción ClaI. En *itálico*: sitio de corte para la enzima de restricción NotI. Subrayado: nucleótidos que codifican para el aminoácido histidina. (Anexo I)

5.6.2 **Transformación de la cepa *E. coli* TOP10 por electroporación**

Una vez realizada la ligación de las secuencias de FhSs con el vector pPICZαC, se procedió a realizar la transformación por electroporación. Para dicho procedimiento se prepararon células competentemente (protocolo descrito en el Anexo 2) de la cepa bacteriana *E. coli* TOP10 (Invitrogen). Una vez que se obtuvo la cepa bacteriana competente, se llevó a cabo la transformación para lo cual se realizó el siguiente procedimiento: Se retiraron las células competentes del freezer (-70 ºC) e inmediatamente se colocaron en hielo. Se les agregó 2 µl de DNA plasmídico, y se transfirió las células competentes y los plásmidos a una cubeta de electroporación de 90 µl de capacidad con 0,1 cm de largura del arco. El material fue electroporado con un pulso de 2500 volts y el

material fue transferido para un tubo de 1,5 mL conteniendo 1 mL de medio SOB (Anexo 2) incubándose a 37°C por una hora de forma tal de permitir a la bacteria recuperarse y expresar la resistencia al antibiótico que lleva el plásmido. Posteriormente se centrifugó a 5.000 rpm por 5 min, se descartó parte del sobrenandante y 100 µl de pellet fueron plaqueados en condiciones de esterilidad utilizando esferas de vidrio estériles en placas conteniendo medio Low Salt LB (Anexo 2) con el antibiótico Zeocina (25 µg/ml).

5.6.3 **Extracción de ADN a pequeña escala (miniprep)**

A partir de una placa Low Salt LB con células transformadas con el plásmido a extraer (TOP10-FhS-pPICZαC), se seleccionaron varias colonias aisladas y se inoculó en 2,5 ml de Low Salt LB con Zeocina. Se dejó incubar durante la noche a 37 ºC en agitación (240 rpm). Las células fueron centrifugadas a 12.000 rpm durante 30 segundos para obtener un precipitado, descartando el sobrenadante. Los precipitados fueron resuspendidos en 100 µl de la solución I (Anexo 2), agitando vigorosamente en vortex e incubados por 10 min en hielo. Se adicionaron 200 µl de Solución II (Anexo 1), (la cual fue preparada en el momento) y se homogeneizó el contenido invirtiendo el tubo rápidamente por 5 veces y colocando en hielo por 5 minutos como máximo. Luego fue adicionado 150 µl de solución III (Anexo 2), homogeneizó en vortex levemente y colocó en hielo por 20 min. Se centrifugó a 12.000 g por 5 minutos a 4 ºC y se transfirió el sobrenadante a un tubo nuevo. Se adicionó 1 volumen de fenol (450 µl), se agitó levemente y se centrifugó 12.000 g por 5 min. Luego se transfirió el sobrenadante a un nuevo tubo y se adicionó 1 volumen de fenol-cloroformo. Luego se agitó, centrifugo y colectó nuevamente. Los pasos del protocolo que involucraron la adición de fenol y/o cloroformo a la muestra fueron realizados en campana de extracción. Se adicionó 1 volumen de cloroformo y se volvió a

agitar, centrifugar y colectar de igual forma que antes. Se precipitó el DNA con 1 ml de etanol helado 100% y 40 µl de acetato de sodio 3 M (pH 5,2). Se incubó a -70 ºC por 1 hora o por 16 horas a -20 °C. Se centrifugó a 12.000 rpm por 20 min a 4 ºC. Se descartó el sobrenadante y se invirtió el tubo en papel absorbente para secar. Luego se lavó el pellets con 1 ml de etanol 70%. Se descartó el etanol y dejó secar bien sobre papel por 10 min. Por último, se resuspendió el material en 20 µl de solución conteniendo RNAsa (20 µg/ml) y se incubó por 30 min a 37 ºC.

5.6.4 **Digestión con enzimas de restricción ClaI and NotI**

Se realizó la digestión de los productos obtenidos de la fase anterior utilizando las enzimas de restricción ClaI (10 U/µL, Fermentas) y NotI (10 U/µL, Fermentas). Se realizó una reacción conjunta para las dos enzimas que contuvo aproximadamente 150 ng/µl de productos, 1 µl de 10X Buffer M, 1 unidad de cada una de las enzimas en un volumen final de 10 µl. Luego se incubó a 37 °C durante 2 horas. Previo a la digestión con las enzimas de restricción se realizaron corridas en geles de agarosa con el fin de estimar la concentración de DNA de las muestras, para lo cual se las comparó con el marcador de peso molecular Lambda HindIII DNA Markers (Invitrogen). El producto de la digestión con enzimas de restricción fue visualizado en una electroforesis en geles de agarosa 0,8% en un transiluminador UV.

5.6.5 **Secuenciación del material clonado**

Además de la digestión por enzimas de restricción fue realizada la secuenciación de los plásmidos para verificar la presencia del inserto. Para la secuenciación del plásmido pPICZαC se utilizaron los primers AOX3’ y AOX5’, específicos del vector.

Posteriormente las secuencias obtenidas fueron comparadas en la base de datos GenBank. Para la visualización y edición de secuencias se utilizó el software BioEdit (Hall, 1999).

5.6.6 **Inclusión del vector pPICαC dentro de la cepa X-33.**

Luego de confirmar la presencia del inserto dentro del vector de expresión mediante secuenciación se procedió a digerir 100 ng del vector con inserto con la enzima de restricción SacI (Invitrogen, EE.UU) durante 24 horas. El protocolo utilizado fue el de preparación de células competentes condensado (Lin-Cereghino et al., 2005). Brevemente, se dejan crecer células de levadura cepa X-33 durante la noche, a 28°C y 240 rpm en medio de cultivo YPD (Anexo 1). Al día siguiente se inocularon estas células hasta obtener una DO_{600} = 0,16-0,20 en 50 ml de medio de cultivo YPD y se dejó crecer hasta una DO_{600} = 0,8-1,0. El medio fue centrifugado a 5.000g por 5 minutos a temperatura ambiente y el sobrenadante fue descartado. Las células fueron resuspendidas en 9ml de la solución BEDS fría (Anexo 1) suplementado con 1ml de DTT 1M. Las levaduras en la solución BEDS fueron incubadas en agitación por 5 minutos, 100 rpm a 30°C. Las células fueron centrifugadas a 5.000g por 5 minutos a temperatura ambiente y el sobrenadante fue descartado y las células suspendidas en 500 µL de solución BEDS. Para transformar estas células X-33 competentes se mezclaron con 100ng del plasmido con inserto digerido con la enzima de restricción SacI y fueron electroporadas utilizando un electroporador Gene Pulser Xcell™ (Bio-Rad, EE.UU.). Con parámetros establecidos a 1,5 kV, 25 µF. Estas levaduras transformadas se plaquearon en placas con medio YPB y dejaron crecer por 5 días a 28°C. El screening de colonias positivas fue realizado mediante PCR de colonia con los primers específicos de cada serpinas y primers específicos del vector de clonación utilizando la enzima Taq polimerasa (Ludwig, Brasil) con el siguiente ciclado: 5 minutos

a 94ºC seguido por 34 ciclos de 30 segundos a 94ºC, TM por 30 segundos y 1:30 minutos por 72ºC y una elongación final de 5 minutos a 72ºC.

5.6.7 **Expresión y purificación de serpinas recombinantes expresadas en *P. pastoris***.

Las colonias transformadas fueron seleccionadas en medio de cultivo solido YPDS (conteniendo extracto de levadura 1%, peptona 2%, dextrosa 2%, agar 2%, Anexo 1) selectivo con el antibiótico Zeocina (100 µg/µl). Los transformantes positivos fueron cultivados en un erlenmeyer de vidrio de 2L en medio complejo tamponado con glicerol (BMGY, Anexo 1) a 29°C durante 24 horas, cuando este cultivo alcanzó una DO_{600nm} de 1.0 fue utilizado para inocular un medio de cultivo mínimo tamponado (BMMY, Anexo 1) conteniendo 0,5% (v/v) de metanol para inducir la expresión de las proteínas recombinantes a 240 rpm, 28°C durante 5 días y con una concentración constante de metanol (0,5%). 1 mL del sobrenadante fue recogido cada 24 horas y precipitado con sulfato de amonio (525 g/l) durante la noche a 4ºC con agitación. El precipitado fue centrifugado a 11.200 rpm durante 1 hora a 4ºC, y el pellet resuspendido en PBS 1x para ser posteriormente visualizado en un SDS-PAGE 12% para determinar el día con mayor expresión de proteína heteróloga. Con esto, se procedió a determinar que clon sería utilizado para expresar cada inhibidor basándose en: días de expresión y visualización de bandas en el gel. Para proceder con la caracterización, el producto de expresión fue dializado en tampón Tris-HCl 20 mM, pH 7,4 (Anexo 1). Las rFhSs fueron purificadas por cromatografía de afinidad en condiciones nativas utilizando columnas de sefarosa FF His-Trap® (GE healthcare, EE.UU) con concentraciones crecientes de imidazol y posteriormente, las proteínas purificadas por afinidad se dializaron con tampón Tris-HCl 20 mM, pH 7,4 y almacenaron a -20 °C. Brevemente, las muestras fueron filtradas con

filtros con membranas de poros de 0,45 μm y las muestras fueron aplicadas luego de que la columna de sefarosa con niquel fuera equilibrada con buffer de ligación (Tris-HCl 20 mM, pH 7,4). La columna HisTrap (General Electric, EEUU) de 5ml permite purificar las proteínas que poseen una región de histidinas en la secuencia de aminoácidos por cromatografía de afinidad de iones metálicos inmovilizados. La elución de las proteínas fue realizada con un gradiente de imidazol (100, 200, 300 y 500 mM). Para determinar en qué concentración de imidazol eluyen las serpinas fue realizado un SDS-PAGE 12% para cada una de las purificaciones. La concentración proteica fue determinada utilizando el kit BSA Protein Assay (Thermo Scientific) siguiendo las recomendaciones del fabricante.

5.6.8 **Western blot**

Esta técnica fue utilizada para confirmar la expresión de las serpinas durante los 5 días de expresión y determinar en qué día se finalizaría la expresión. También fue utilizada para confirmar la obtención de anticuerpos anti serpinas producidos en conejo. Para confirmar la expresión fueron utilizados 20 μL de las fracciones precipitadas en sulfato de amonio los cuales fueron corridos en geles de SDS-PAGE al 12%, seguido por transferencia a membrana de nitrocelulosa. Las membranas fueron bloqueadas con buffer de bloqueo (5% de leche descremada con 0,1% Tween en PBS). Las incubaciones con el anticuerpo anti-His (1:1000) fueron realizadas overnight a 4ºC. Una proteína conocida, calreticulina, que fue expresa en el laboratorio y posee cola de histidinas fue utilizada como control positivo. Al día siguiente, las membranas fueron lavadas 3 veces con PBS, incubadas por 10 minutos en TRF (Buffer de revelación para fosfatasa alcalina) y se reveló con el sustrato cromogénico NBT/BCIP (Nitro-blue tetrazolium chloride / 5-bromo-4-chloro-3'-Indolyphosphate p-toluidine salt, Promega) en buffer de reacción.

Para confirmar la obtención de anticuerpos anti serpinas, 200 µg de serpina pura fueron corridos en geles de SDS-PAGE al 12%, seguido por transferencia a membrana de nitrocelulosa. Las membranas fueron bloqueadas con buffer de bloqueo (5% de leche descremada con 0,1% Tween en PBS). Los sueros anti-rFhS fueron diluidos en buffer de bloqueo y absorbido por 1 hora con lisado de *P. pastoris* expresando vector pPICZαC.). Suero pre-inmune fue utilizado como control negativo (1:1.000). Las tiras incubadas en las distintas diluciones de suero de conejo se colocaron overnight a 4 °C. Al día siguiente, se lavaron 3 veces con solución de bloqueo por 5 min cada uno. Luego fueron incubadas con un anticuerpo secundario, anti-IgG de conejo fosfatasa alcalina (Invitrogen) (1:5.000) por 1 hora a temperatura ambiente. Posteriormente se procedió a realizar lavados con PBS, incubar con TRF (Buffer de revelado para Fosfatasa alcalina) por 10 min y fueron revelados con el sustrato cromogénico NBT/BCIP (Nitro-blue tetrazolium chloride / 5-bromo-4-chloro-3'-Indolyphosphate p-toluidine salt, Promega) en buffer de reacción.

5.6.9 **Análisis proteómico de rFhS-2 y rFhs-3**

Fue observado que existen dos serpinas recombinantes que poseen similitud en su secuencia de aminoácidos, pero diferencias a nivel de actividad (esto será analizado en el punto 4.7 donde se aborde la caracterización bioquímica de los clones). Como todo material preparado para ser enviado a un espectrómetro de masas, cada equipamiento o material utilizado en estos ensayos fue preparado nuevo y estéril y las piezas de equipo esterilizadas para garantizar la viabilidad de la muestra durante el proceso. Para confirmar las diferencias entre rFhS-2 y rFhS-3, las proteínas purificadas se resolvieron en un gel SDS-PAGE 12%, con posterior tinción con Coomassie G-250 (CBB-G250). Las áreas del gel que presentaban bandas de proteínas fueron cortadas y colocadas en un eppendorf con 50% de

metanol y 5% de ácido acético y fueron digeridas con tripsina (Shevchenko et al., 2006). Las mezclas de péptidos trípticos, producto de esta digestión, fueron analizados por LC-MS/MS, utilizando cromatografía líquida de nanoflujo y espectrómetro de masas Easy NanoLC II y un espectrómetro de masas Q Exactive (Thermo Scientific, EE. UU.). Los péptidos eluídos de la columna analítica se pulverizaron directamente en el espectrómetro de masas. Los espectros de masas resultantes se buscaron en una base de datos no redundante que comprende secuencias del genoma de *F. hepatica* y nuestras secuencias de serpinas (Cwiklinski et al., 2015a).

5.7 **Caracterización bioquímica de las serpinas recombinantes**.

5.7.1 **Ensayos de deglicosilación**

Para determinar si las serpinas de *F. hepatica* expresadas en levadura son glicosiladas, las proteínas purificadas por afinidad se trataron con enzimas que permiten la desglicosilación de proteínas de acuerdo con las instrucciones del fabricante (New England Biolabs, EE. UU, Anexo 1). Brevemente, fueron utilizados 100 µg de cada una de las serpinas recombinantes que se incluyeron en un tubo con 18 µL de agua. Se agregaron 1 µL del tampón de desnaturalización de glicoproteínas y la mezcla se incubó a 100°C por 10 minutos. Las muestras se dejaron enfriar en hielo por 10 segundo y se agregaron 10 µL del tampón de reacción G7 y 5 µL de NP40 10% y 15 µL de agua. La desglicosilación fue confirmada mediante SDS-PAGE al 12% teñido con azul brillante de Coomassie.

5.7.2 **Ensayos de inhibición**.

La exploración para determinar la actividad inhibitoria de las serpinas de *F. hepatica* fue realizada con incubaciones de las serpinas recombinantes (rFhS2, rFhS3 y rFhS4) frente a un panel de serino-proteasas con concentraciones crecientes de serpinas y con la posterior adición de un sustrato sintético cromogénico o fluorogénico específico para cada proteasa blanco. La actividad enzimática remanente fue calculada y evaluada la actividad inhibitoria de las serpinas recombinantes de *F. hepatica* en relación a una mezcla de reacción enzima/sustrato sin inhibidor. Las proteasas catepsina G, tPA humano y uPA de ratón se adquirieron de Molecular Innovations (EE. UU.). Los factores de coagulación humano IXa, Xa, XIa, XIIa y la plasmina fueron compradas de Enzyme Research Laboratories (Indiana, EE. UU.). La quimotripsina pancreática bovina, la tripsina pancreática bovina y la elastasa pancreática porcina se adquirieron de Sigma (EE. UU.). Los substratos para tripsina bovina y plasmina humana (N-p-Tosyl-Gly-Pro-Lys pNa), y para elastasa de páncreas porcino (N-Succinyl-Ala-Ala-Ala-pNa), trombina (N-Benzoyl-Phe-Val-Arg-p-pNa-HCl) y quimotripsina bovina, catepsina G humana y quimasa humana y de rata (Succinyl-Ala-Ala-Pro-Phe-pNA) se adquirieron de Sigma. Sustratos para tPA y uPA de ratón (pyroGlu-Pro-Arg-pNA-HCl); para el factor de coagulación humano Xa (Bz-IIe-Glu(γ-OR)-Gly-Arg-pNA-HC) y para los factores de coagulación humano XIa y XIIa (H-D-Pro-Phe-Arg-pNA-2HCl) se adquirieron de Chromogenix (Massachusetts, EE.UU). El sustrato para el factor de coagulación humano IXa (CH_3SO_2-D-CHG-Gly-Arg-pNA) se adquirió de Pentapharm (Basel-Landschaft, Suiza).

Como prueba inicial de la actividad del inhibidor, se incubaron proteasa y exceso molar (de 2 a 800 veces) de la serpina recombinante durante 15 minutos a 37 °C, en el tampón de reacción apropiado (200 mM Tris-HCl, 150 mM NaCl, 0.1 % BSA, pH 7.4). La actividad residual de la proteasa fue medida mediante la adición de sustrato a una

concentración final de 200 µM. La actividad enzimática se midió a DO_{405nm} cada 11 segundos durante 15 min a 30 °C utilizando un lector de placas y se comparó con la actividad de proteasa residual en ausencia de serpina (control positivo). Los datos adquiridos se sometieron a análisis utilizando el software Prism 7 (GraphPad Software, California, EE. UU.) para determinar los valores de velocidad inicial de hidrólisis del sustrato. Los porcentajes de inhibición de la actividad de la proteasa se determinaron mediante la fórmula: $100 - (V_i/V_0) \times 100$ donde, V_i = actividad en presencia de serpinas recombinantes, y V_0 = actividad en ausencia de serpinas recombinantes. Los datos se presentan como porcentajes de inhibición de lecturas duplicadas y al menos ensayos duplicados de cada experimento.

5.7.3 **Formación de complejos**.

Las rFhS-2 y rFhS-4 activas fueron incubadas con las proteasas para las que mostraron inhibición en la sección 3.9.2 (quimotripsina y/o catepsina G) con diferentes relaciones molar de proteasa/serpina (1: 0.625, 1: 1.25, 1: 2.5, 1: 5 y 1:10 para catepsina G y 1: 2.5, 1: 5, 1:10 y 1:20 para quimotripsina) a 37 °C durante 1 hora en un volumen final de 10 µl. Las reacciones fueron ajustadas a este volumen para garantizar que toda la muestra fuera analizada por SDS-PAGE. Las mezclas de reacción se desnaturalizaron por calor y se resolvieron mediante electroforesis con geles de poliacrilamida SDS-PAGE al 12% con tinción con Coomassie Brilliant Blue R-250.

5.7.4 **Determinación de la constante de inhibición de proteasa (k_a).**

Para determinar la constante de inhibición (k_a) se incubaron proteasa con exceso de serpina en presencia de sustrato en una relación molar [serpina]/[enzima]>5. Esto, permitió el monitoreo constante de la tasa de hidrólisis del sustrato y, mediante la comparación de la tasa de pérdida de actividad proteolítica, permitió calcular la constante de inhibición. El método discontinuo se utiliza cuando la tasa de inhibición es baja y no pueden utilizarse curvas progresivas a menos que se utilicen altas concentraciones de serpina y proteasa; pero estas pueden no ser factibles. Los parámetros cinéticos para la inhibición de proteasas, como constante de velocidad observada (k_{obs}) y la constante de asociación de segundo orden (k_a), se determinaron utilizando métodos discontinuo (Horvath et al., 2011). La concentración final de sustrato (succinil-ala-ala-pro-phe-pNA) fue de 0,2 mM para cada ensayo. La curva de progreso se obtuvo al controlar el aumento de la absorción a 410 nm, que resultó de la liberación de p-nitroanilina.

5.7.5 **Eficiencia de la reacción de inhibición de proteasas**

Para esto se procedió a calcular la Estequiometría de la Inhibición (SI) Esta, es una medida del equilibrio entre la reacción inhibitoria y la reacción del sustrato y describe el número de moles de serpina requeridas para inhibir 1 mol de proteasa. Si la vía inhibitoria procede más rápido que la vía del sustrato entonces la SI se aproxima a 1 pero si la vía del sustrato prevalece sobre la inhibitoria, la SI es mayor a 1. Los valores de estequiometría de inhibición (SI) de cada rFhS para cada proteasas se determinaron incubando cantidades constantes de proteasas con concentraciones crecientes de serpina (rango de concentraciones usadas) y midiendo la actividad de residual, como ya fue descrito (Horvath et al., 2011). Brevemente, las proteasas que mostraron inhibición con las rFhS

(catepsina G y quimotripsina) se incubaron con concentraciones crecientes de rFhS en una placa de 96 pocillos para producir relaciones molares de serpina: proteasa (S / P) que van de 0 a 40 para quimotripsina y de 0 a 20 para catepsina G en el tampón de reacción apropiado (200 mM Tris-HCl, 150 mM NaCl, 0.1 % BSA, pH 7.5 respectivamente). Los tiempos de incubación fueron de 60 minutos a 37 °C, las actividades residuales de las proteasas se determinaron utilizando el substrato succinil-Ala-Ala-Pro-Phe-pNA, 200 µM para ambas proteasas. Una gráfica de la actividad residual de la proteasa en función de la relación molar de serpina a proteasa produce una línea recta y el valor donde la línea cruza el eje X se consideró el valor SI (Kantyka and Potempa, 2011).

5.7.6 **Interacción con heparina**

500 µg de las rFhS purificadas por afinidad en el punto 3.8 fueron utilizadas en una columna HiTrap Heparin HP de 5 ml (GE Healthcare Bio-Sciences) de acuerdo con las instrucciones del fabricante en un Akta FPLC (GE Healthcare, USA). La columna fue equilibrada con tampón de ligación (fosfato de sodio 10 mM, pH 7,0) y la elución fue realizada utilizando un gradiente continuo de tampón de elución (fosfato de sodio 10 mM, cloruro de sodio 2 M, pH 7,0). Las fracciones eluídas así como las muestras de rFhS pre-columna fueron analizadas en un SDS-PAGE al 12% y se detectadas con tinción con Coomassie Brilliant Blue R-250.

5.8 **Estudio de la expresión y/o localización de serpinas en diferentes tejidos y estadios de *F. hepatica*.**

5.8.1 **Producción de anticuerpos específicos**.

Para la producción de anticuerpos anti rFhS-2 y rFhS-4, un conejo por cada serpina fue inmunizado subcutáneamente tres veces con intervalos de 14 días con 200 μg de rFhS emulsificada con adyuvante Montanide 888/Marcol 52 (Exxon Mobil Corporation). A los 14 días del último refuerzo (*booster*), la sangre de los conejos fue colectada y el suero fue separado por centrifugación a 10.000 rpm por 5 min a 4 ºC. Alícuotas de suero fueron conservadas a -20 ºC hasta su uso. Se realizó purificación de IgG utilizando columnas de HiTrap™ Protein G HP (GE Healthcare) siguiendo las recomendaciones del fabricante.

5.8.2 **Localización de serpinas en adultos de *F. hepatica***.

Los parásitos adultos de *F. hepatica* recuperados a partir de bovinos diagnosticados en el frigorífico con *F. hepatica* fueron transportados al laboratorio en bilis a 37 °C. Los parásitos se enjuagaron en solución salina y se les permitió regurgitar el contenido intestinal para ser fijados de forma plana en paraformaldehído al 4% (PFA) en PBS (pH 7,2). Los parásitos fueron cortados longitudinalmente con bisturí generando dos mitades (ventral y dorsal) y lavados en PBS durante 24 horas a 4 °C, luego se incubaron con el anticuerpo anti rFhS purificado durante 24 horas a 4 °C (diluido 1:200) en solución de lavado. Después del período de incubación, las secciones del parásito se lavaron con PBS con seroalbúmina bovina 1%; Triton X-100 0,3% y azida sódica 0,1% durante 24 horas a 4 °C. La sección de parásito fue incubada con un anticuerpo secundario anti-conejo marcado con Alexa 555 (1: 1000, Cell Signaling Technology, EE. UU.) Durante 24 horas

a 4 ° C y con DAPI (1: 1000) durante 30 minutos a temperatura ambiente, se realizaron 3 lavados con PBS y las secciones del parásito fueron montadas en portaobjetos. Como controles fueron utilizados para incubar las secciones de *F. hepatica* suero preinmune e anticuerpo secundario. Los resultados fueron observados utilizando un microscopio de barrido láser confocal LSM 710 ZEISS y las imágenes se procesaron con el software Zen 2.3 SP1 y Adobe Photoshop CS4 (Adobe System Inc., EE. UU.).

6 RESULTADOS

Los resultados de esta tesis fueron publicados en la revista *International Journal for Parasitology* bajo el título "Serpins in *Fasciola hepatica*: insights into host–parasite interactions". El articulo científico contiene la descripción del trabajo experimental realizado durante el período de doctorado asi como los resultados obtenidos.

Declaración de contribuciones de los autores:

Idea y diseñó de los experimentos: LSDM, LT, MU, CC, CL, PB, ISV

Realización de los experimentos: LSDM, LT, MU, PB

Reactivos/materiales/herramientas de análisis aportados: CL, AM, PB, ISV

Redacción del manuscrito: LSDM, LT, ISV, PB

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Serpins in *Fasciola hepatica*: insights into host–parasite interactions

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ABSTRACT

Protease inhibitors play crucial roles in parasite development and survival, modulating the immune responses of their vertebrate hosts. Members of the serpin family are irreversible inhibitors of serine proteases and regulate systems related to defence against parasites. Limited information is currently available on protease inhibitors from the liver fluke *Fasciola hepatica*. In this study, we characterised four serpins from *F. hepatica* (FhS-1–FhS-4). Biochemical characterisation revealed that recombinant FhS-2 (rFhS) inhibits the activity of human neutrophil cathepsin G, while rFhS-4 inhibits the activity of bovine pancreatic chymotrypsin and cathepsin G. Consistent with inhibitor function profiling data, rFhS-4 inhibited cathepsin G-activated platelet aggregation in a dose-responsive manner. Similar to other serpins, rFhS2 and rFhS-4 bind to heparin with high affinity. Tissue localisation demonstrated that these serpins have different spatial distributions. FhS-2 is localised in the ovary, while FhS-4 was found in gut cells. Both of them co-localised in the spines within the tegument. These findings provide the basis for study of functional roles of these proteins as part of an immune evasion mechanism in the adult fluke, and in protection of eggs to ensure parasite life cycle continuity. Further understanding of serpins from the liver fluke may lead to the discovery of novel anti-parasitic interventions.

1. Introduction

Fasciolosis is a zoonotic foodborne disease caused mostly by the liver flukes *Fasciola hepatica* and *Fasciola gigantica*. It is a chronic disease that causes significant economic losses in livestock production worldwide, as well as an emerging human disease (Mas-Coma et al., 2014; Nyindo and Lukambagire, 2015). The definitive hosts of *F. hepatica* become infected when they ingest vegetation containing metacercariae, which are encysted dormant larvae. Newly excysted juveniles (NEJs) emerge in the duodenum and migrate up to the liver parenchyma. Following a period of blood feeding and growth, they move to the bile ducts where they obtain blood by puncturing the duct wall, and eggs are released into the large intestine with the bile fluids (Mas-Coma et al., 2014).

During migration and development, parasites are exposed to host immune responses. Serine proteases such as pro-coagulant (thrombin, factor Xa and factor XIa), pro-inflammatory (as neutrophil elastase, neutrophil cathepsin G, protease-3, trypsin-like, and chymotrypsin-like proteases), and complement proteases have a role in these host defence responses during liver fluke parasitism (Korkmaz et al., 2008; Cattaruzza et al., 2014). *Fasciola hepatica* has developed mechanisms to modulate the host immune response during NEJ migration and adult establishment in the liver. Proteomic analysis of *F. hepatica* tegument (Wilson et al., 2011) and excretory/secretory (ES) products (Robinson et al., 2009; Cwiklinski et al., 2015b; Di Maggio et al., 2016) revealed that they contain a variety of proteins with anti-haemostatic and immunomodulatory properties (Joachim et al., 2003; Serradell et al., 2007; Harnett, 2014; Martin et al., 2015), among which were protease inhibitors including serpins, Kunitz-type inhibitors, stefins and cystatins. Thus, the objective of the present study was to characterise serpins from *F. hepatica* to gain insight into their role(s) in fluke biology and the host-parasite relationship.

Serine protease inhibitors (serpins) are a large and broadly distributed superfamily of proteins with a variety of biological functions (Irving et al., 2000). In contrast to the canonical lock-and-key mechanism of inhibition (Farady and Craik, 2010), serpins are suicide inhibitors, meaning that they are cleaved by the target protease at the scissile bond within the reactive centre loop (RCL), which then leads to conformational changes and covalent linkage that cause irreversible inactivation of the protease (Huntington et al., 2000; Gettins, 2002; Law

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et al., 2006). Specifically in helminths, serpins are involved in the modulation of immune responses. In *Brugia malayi,* the major component of the secreted products is a serpin (MB-SPN-2) that inhibits the proteolytic activity of human neutrophil elastase and cathepsin G (Zang et al., 1999). In *Ascaris*, serpins are present on the gut surface, where they facilitate survival of the parasite by inactivating host proteases (Martzen et al., 1985). One serpin from *Echinococcus granulosus* (Shepherd et al., 1991) has the ability to inhibit recruitment of neutrophils. In *Schistosoma* spp., serpins control homeostasis of serine proteases on both parasite and host (Mebius et al., 2013). In *Schistosoma mansoni, a* serpin inhibits neutrophil elastase, modulating its tissue degradation activity to allow parasite migration (Ghendler et al., 1994; Quezada et al., 2012). *Schistosoma japonicum* serpins inhibit human pancreatic elastase (SjB10), chymotrypsin, trypsin, and thrombin (SjSPI) (Molehin et al., 2014; Zhang et al., 2018). In *Schistosoma haematobium,* a membrane-anchored serpin presents anti-trypsin activity (Huang et al., 1999). Two serpins with different RCL sequences have been studied in *Clonorchis sinensis*, namely CsSERPIN and CsSERPIN3, both being highly expressed in different metacercaria tissues (Yang et al., 2014). These facts suggest that serpins secreted by *F. hepatica* could participate in modulation of the host homeostatic balance. Despite the increasing interest in studying *F. hepatica* proteases (Dalton et al., 2003; Jayaraj et al., 2009; Mokhtarian et al., 2016), there is still comparatively limited data available on *F. hepatica* protease inhibitors. In a previous proteomic study, five serpin sequences were found in adult ES products, one sequence in NEJ ES products, and another five in NEJ somatic soluble proteins (Di Maggio et al., 2016). Further understanding of protease inhibitors from parasitic flukes broadens our knowledge of parasite biology and immunomodulatory mechanisms, and may lead to the discovery of novel anti-parasitic interventions. This study builds on the knowledge of helminth serpins. We provide evidence that two *F. hepatica* serpins are functional inhibitors of chymotrypsin and neutrophil cathepsin G, and we discuss potential implications for the pathogenesis of this parasite.

2. Materials and methods

2.1. Ethics statement

This study was conducted in accordance with the ethical and methodological aspects preconised by the International and National Directives and Norms of the Animal Experimentation Ethics Committee from Universidade do Rio Grande do Sul, Brazil (UFRGS). The protocols were approved by the Comissão de Ética no Uso de Animais (CEUA) from UFRGS (protocol numbers 27,369 and 38,748). Cattle livers were collected from a local abattoir immediately after slaughter. Natural liver fluke infections were diagnosed at the abattoir by independent meat inspectors, and the biological material was discarded as per the local abattoir protocol. The abattoir is authorised by the Ministry of Agriculture and Fisheries of Uruguay (MGAP), and complies with the National Animal Welfare Act n° 18471 of 2009 law of protection, welfare and possession of animals (regulated by Decree n° 62/014 14.03.2014) as well as with the good animal welfare practices concerning transport and slaughter of cattle and sheep, prepared by the Technical Group of the Directorate General of Livestock Services (DGSG-MGAP) (Uruguay) in 2005, according to the recommendations of the 73rd General Session of the World Organisation for Animal Health (OIE) (France) on 27 May 2005.

2.2. Fasciola hepatica collection, RNA and cDNA synthesis

NEJs were obtained from *F. hepatica* metacercariae (Oregon strain) which were purchased from Baldwin Aquatics Inc. (USA). Adult worms and eggs were obtained from the bile ducts and gallbladder of infected cattle from a local abattoir in Montevideo, Uruguay. Total RNA was extracted from eggs, newly excysted juvenile (NEJ), and adult tissues by placing those in Trizol® reagent (Invitrogen, USA) and treating according to the manufacturer's recommendations. The RNA samples were resuspended in diethylpyrocarbonate (DEPC)-treated water. RNA concentration and purity were determined spectrophotometrically. OligodT primed cDNA was synthesised from 5 μg of total RNA using the SuperScript III kit (Invitrogen, USA).

2.3. Identification of F. hepatica serpin coding sequences

Serpins from other helminths (all sequences deposited in GenBank) were used as queries and sequences encoding *F. hepatica* serpins (FhS) were searched against *F. hepatica* transcriptomic databases (available at http://parasite.wormbase.org/index.html) using the Basic Local Alignment and Search Tool (BLAST) with the BLASTP, BLASTN, BLASTX, and FASTY algorithms (Altschul et al., 1990; Pearson, 2000). Partial sequences were used as input for clustering using the contig assembly programme (CAP) (Huang, 1992). To validate the accuracy of serpin-encoding sequence identification, assembled sequences were inspected for the presence of start and stop codons, amino acid length (350–450 amino acids of translated protein sequences), and the presence of two amino acid motifs described as conserved in known serpins: [N]-[A]-[V]-[Y]-[F]-[K]-[G] and [D]-[V]-[N]-[E]-[E]-[G] (Miura et al., 1995; Han et al., 2000). To confirm the identities of the assembled sequences, the deduced amino acid sequences were scanned against GenBank using the BLASTP homology search programme against the non-redundant protein sequence Database from National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/), and amino acid motifs using RPS-BLAST against the MEROPS database (Rawlings et al., 2016), as well as the NCBI conserved domains database containing the KOG, CDD, PFAM, and SMART motifs (Marchler-Bauer et al., 2017).

2.4. Cloning F. hepatica serpin coding sequences from cDNA

Five hundred nanograms of cDNA were used as template for real-time (RT)-PCR using Taq DNA Polymerase (Ludwig Biotecnologia, Brazil) and a set of primers described in Supplementary Table S1. The amplification reactions were carried out using cDNA from eggs, NEJs and adults. The thermal cycling profile used was 94 °C for 5 min; 34 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s and a final extension for 5 min at 72 °C. PCR amplicons were gel-purified using a GeneClean® II Kit (MP Biomedicals, USA) and cloned into a pGEM-T easy vector (Promega, USA) according to the manufacturer's recommendations. Positive clones were screened for the presence of plasmid with the appropriate insert. The nucleotide sequences of the inserts were determined by automated sequencing.

2.5. In silico analysis

Nucleotide sequences obtained as described in section 2.4 were analysed and the deduced amino acid sequences were obtained. The presence of secretion signal sequence was predicted using the SignalP 4.0 server (Petersen et al., 2011). Putative N-glycosylation sites were found using the NetNGly4.0 server (Steentoft et al., 2013). Theoretical molecular weight and pIs of the mature serpins were calculated using the Compute pI/Mw tool via the ExPASy website (http://web.expasy.org/compute_pi/pi_tool-Ref.html). To gain insight into the relationship of *F. hepatica* serpins with serpins from other helminth species, amino acid sequences were aligned with the Muscle algorithm (Edgar, 2004) in the MEGA6 programme (Tamura et al., 2013), using the best 10 blast matches from the NCBI non-redundant database for each *F. hepatica* serpin. Phylogenetic analyses were performed using the neighbour joining method (Kumar et al., 2008). Gapped positions

were deleted. Poisson correction was used as a substitution model to determine pairwise distances. Confidence was determined using bootstrap values at 1000 replicates. The alignment sequences were subsequently viewed using GeneDoc (Nicholas et al., 1997).

The three-dimensional (3D) structures of FhSs were predicted using a comparative modelling approach. Serpin 3D templates were retrieved from the Protein Data Bank (PDB) (http://www.rcsb.org) and used as follows: 1AZX (for FhS-1) and 1DZG (for FhS-2, FhS-3 and FhS-4). Sequence alignments were generated using the ClustalW algorithm (Larkin et al., 2007) and used as input in the Modeller 9v14 programme (Webb and Sali, 2014). The templates were chosen from the aligned sequences by the following criteria: presenting 30% similarity with no more than a five amino acid gap compared with the FhS sequence, and having an associated PDB file. Models generated were evaluated using QMEAN4 and PROCHECK to estimate model reliability and predict quality (Morris et al., 1992; Benkert et al., 2008). The electrostatic potential for FhS and antithrombin III (2B4X, positive control) structures were calculated using the Adaptive Poisson–Boltzmann Solver (APBS). Protonation states were assigned using the parameters for solvation energy (PARSE) force field for each structure by PDB2PQR (Unni et al., 2011). Execution of APBS and visualisation of resulting electrostatic potentials were performed by using the Visual Molecular Dynamics (VMD) programme (Humphrey et al., 1996) at ±5 kT/e of positive and negative contour fields. Additionally, amino acid sequences were manually inspected for annotated glycosaminoglycan (GAG) binding motifs as previously reviewed (Hileman et al., 1998). β-turns in the RCL region of FhS-2 and FhS-3 were predicted using the NetTurnP-tweak tool via the Technical University of Denmark (DTU) Health Tech website (http://www.cbs.dtu.dk/services/NetTurnP/) (Petersen et al., 2010).

2.6. Recombinant expression and affinity purification of F. hepatica serpins

FhS coding sequences were cloned into the *Pichia pastoris* expression vector pPICZαC (*Cla*I and *Not*I restriction sites) using a specific set of primers (Supplementary Table S1). Plasmids were linearised with *Sac*I and electroporated into *P. pastoris* X-33 strain according to the manufacturer's recommendations (Invitrogen, USA) using a Gene Pulser Xcell™ Electroporation System (Bio-Rad, USA) with parameters set to 1.5 kV, 25 μF. Transformed colonies were selected on yeast extract peptone dextrose medium with sorbitol (YPDS) agar plates with the antibiotic zeocin (100 μg/mL). Positive transformants were grown in buffered minimal glycerol complex medium (BMGY) at 29 °C for 1 day, harvested and suspended to OD_{600nm} of 1.0 in buffered minimal methanol-complex medium (BMMY) containing 0.5% (v/v) methanol to induce recombinant protein expression. The supernatant was collected after 2–4 days of growth at 29 °C with a constant methanol concentration (0.5%), then precipitated with ammonium sulphate saturation (525 g/L) and stirring overnight at 4 °C. The precipitate was pelleted at 11,200 *g* for 1 h at 4 °C, re-suspended in PBS pH 7.4 and dialysed against 20 mM Tris–HCl buffer pH 7.4. Subsequently, rFhSs were affinity-purified under native conditions using His-Trap FF Columns (GE Healthcare, USA). Purified proteins were dialysed against 20 mM Tris–HCl buffer pH 7.4 for subsequent assays.

2.7. Mass spectrometry analysis of rFhS

Purified proteins were resolved in a 12% SDS-PAGE stained with Coomassie brilliant blue G-250 solution (CBB-G250). Areas of the gel containing protein bands were cut and de-stained in 50% methanol/5% acetic acid and digested by trypsin (Shevchenko et al., 2006). Tryptic peptide mixtures were analysed by LC–MS/MS, using nanoflow liquid chromatography and mass spectrometry in an Easy NanoLC II and a Q Exactive mass spectrometer (Thermo Scientific, USA). Peptides eluted from the analytical column were electrosprayed directly into the mass spectrometer. Resulting mass spectra were searched against a non-redundant database comprising sequences from the *F. hepatica* genome and our serpin sequences (Cwiklinski et al., 2015a).

2.8. Deglycosylation assay

To determine if yeast-expressed serpins were glycosylated, affinity-purified proteins were treated with a Protein Deglycosylation Mix according to the manufacturer's instructions (New England Biolabs, USA). The Protein Deglycosylation Mix contains PNGase F, O-Glycosidase, Neuraminidase (sialidase), β1-4 Galactosidase, and β-N-Acetylglucosaminidase, and it is used to simultaneously remove N-glycans and O-glycans. Deglycosylation was confirmed by 12% SDS-PAGE stained with CBB-G250.

2.9. Production of anti-rFhS serum and IgG purification

Antiserum was raised against the purified recombinant serpins by s.c. immunisation of New Zealand rabbits with 200 μg of protein emulsified in oil adjuvant (Montanide 888/ Marcol 52). Serpin and adjuvant solution were mixed in a 1:1 ratio v/v. Three boosters of 200 μg of recombinant serpin in the same adjuvant were applied at 15-day intervals. Total IgG from sera was purified by affinity chromatography on a protein G Sepharose resin according to the manufacturer's instructions (GE Healthcare, USA).

2.10. Protease inhibition profile

Cathepsin G, human tPA and mouse uPA were purchased from Molecular Innovations (USA). Human factor IXa, factor Xa, factor XIa, factor XIIa and plasmin were purchased from Enzyme Research Laboratories (USA). Bovine pancreatic chymotrypsin, bovine pancreatic trypsin and porcine pancreatic elastase were purchased from Sigma (USA). Substrates for bovine trypsin and human plasmin (N-p-Tosyl-Gly-Pro-Lys pNa), and substrates for elastase from porcine pancreas (N-Succinyl-Ala-Ala-Ala-pNa), thrombin (N-Benzoyl-Phe-Val-Arg-p-pNa-HCl) and bovine chymotrypsin, human cathepsin G and human and rat chymase (Succinyl-Ala-Ala-Pro-Phe-pNA) were purchased from Sigma (USA). Substrates for mouse tPA and uPA (pyroGlu-Pro-Arg-pNA·HCl); for human factor Xa (Bz-IIe-Glu(γ-OR)-Gly-Arg-pNA•HC) and human factors XIa and XIIa (H-D-Pro-Phe-Arg-pNA-2HCl) were purchased from Chromogenix (Italy). Substrate for human factor IXa (CH_3SO_2-D-CHG-Gly-Arg-pNA) was purchased from Pentapharm (Switzerland).

As an initial test for inhibitor activity, protease and molar excess of rFhS (from two to 800-fold) were incubated for 15 min at 37 °C, in the appropriate reaction buffer (200 mM Tris-HCl, 150 mM NaCl, 0.1 % BSA, pH 7.4). Residual protease activity was measured by the addition of substrate at a final concentration of 200 μM in a total volume of 100 μL. Enzymatic activity was measured at OD_{405nm} every 11 s for 15 min at 30 °C using a plate reader, and compared with residual protease activity in the absence of serpin. Acquired data were subjected to one phase decay analysis in Prism 7 software (GraphPad Software, USA) to determine plateau values for initial velocity of substrate hydrolysis. Percentages of protease activity inhibition were determined using the formula: $100 - (V_i/V_0) \times 100$ where V_i = activity in presence of, and V_0 = activity in absence of, recombinant serpins. Data were calculated as the mean as the mean percentage of inhibition from duplicate readings and at least duplicate assays.

2.11. Stoichiometry of inhibition (SI) assay

The stoichiometry of inhibition (SI) value for each rFhS-protease pair was determined by incubating constant amounts of proteases with

increasing concentrations of serpin and measuring the residual protease activity, as previously described (Horvath et al., 2011). Briefly, proteases that showed inhibition by the *F. hepatica* serpins (cathepsin G and chymotrypsin) were mixed with increasing concentrations of rFhS in a 96-well plate to yield molar ratios of serpin:protease (S/P) ranging from 0 to 40 for chymotrypsin and 0 to 20 for cathepsin G in the appropriate reaction buffer (200 mM Tris-HCl, 150 mM NaCl, 0.1 % BSA, pH 7.4). The incubation time was 60 min at 37 °C and samples were cooled at room temperature after incubation. Residual protease activity was determined using Succinyl-Ala-Ala-Pro-Phe-pNA as a substrate for both enzymes (at a final concentration of 200 μM in a total volume of 100 μL). The SI value was determined as the x-intercept of a linear function of residual protease activity of protease versus the molar ratio of S/P (Kantyka and Potempa, 2011).

2.12. Determination of the rate of stable complex formation

Kinetic parameters for inhibition of proteases, namely the pseudo-first order association rate constant (k_{obs}) and second order association rate constant (k_a), were determined using discontinuous assay methods as previously described (Horvath et al., 2011). The pseudo-first order rate constant was determined by incubation of serpin-protease pairs at indicated concentrations (fixed concentration of protease and increasing concentrations of serpin) for different periods of time (0–15 min), followed by measurement of residual protease activity. All reactions were performed in buffer (200 mM Tris-HCl, 150 mM NaCl, 0.1% BSA, pH 7.4). The pseudo-first order constant, k_{obs}, was determined from the slope of a semi-log plot of the residual protease activity against time. The k_{obs} values were then plotted against the serpin concentration and the slope of the line of best fit was recorded as the second order rate constant, k_a.

To check covalent complex formation, active rFhS-2 and rFhS-4 were incubated with proteases that showed inhibition in section 2.9 (chymotrypsin and/or cathepsin G) with different protease/serpin molar ratios (1:0.625, 1:1.25, 1:2.5, 1:5 and 1:10 for cathepsin G and 1:2.5, 1:5, 1:10 and 1:20 for chymotrypsin) in 200 mM Tris-HCl, 150 mM NaCl, pH 7.4, at 37 °C for 1 h. The reaction mixtures were heat-denatured and resolved by 12% SDS-PAGE following CBB-G250 staining.

2.13. Heparin-binding assay

Affinity-purified FhSs (500 μg) were applied to a 5 mL HiTrap Heparin HP column (GE Healthcare, USA), according to the manufacturer's instructions, in an Akta FPLC system (GE Healthcare, USA). The column was equilibrated in 10 mM sodium phosphate, pH 7.0 and elution was performed using a linear gradient (0–2 M NaCl) in the same buffer. Eluted fractions, as well as pre-column FhS samples, were analysed by 12% SDS–PAGE and CBB-G250 staining.

2.14. Platelet aggregation assay

Platelet-rich plasma (PRP) was obtained from *Bos taurus* blood collected in a 5 mL vacuum blood tube containing sodium citrate (3.2%) (Plastilab, Lebanon). Total blood was centrifuged for 15 min at room temperature at 150*g*. The supernatant (PRP) was transferred into a new tube and centrifuged at 800*g* for 20 min. The pellet containing the platelets was washed and resuspended in Tyrode buffer (137 mM NaCl, 27 mM KCl, 12 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.55 mM glucose, 0.25% BSA; pH 7.4) and adjusted to an OD_{650nm} of 0.15. To verify inhibition of platelet aggregation, different amounts of FhS-4 (0 μM, 3.44 μM, 1.72 μM, 0.35 μM and 0.175 μM) were pre-incubated with cathepsin G (0.7 μM) at 37 °C for 15 min on a 96-well plate. After that, 100 μL of the PRP were added in each well and platelet aggregation was monitored for 30 min with an OD_{650nm}. As a control, a recombinant active cathepsin L-like enzyme from *Rhipicephalus microplus,* also expressed in *P. pastoris* and devoid of inhibitory activity against cathepsin G, was used (Clara et al., 2011). The percentage of platelet aggregation inhibition was quantified by calculating the area under the curve and expressed relative to the negative and positive controls (PRP and buffer only, and absence of serpin, respectively).

2.15. Immunolocalisation of F. hepatica serpins in adult fluke

Adult *F. hepatica* parasites were recovered from infected cattle at a local abattoir in Rio de Janeiro, Brazil, and transported to the laboratory in bile at 37 °C. Parasites were rinsed in warm saline and allowed to regurgitate their gut contents before being flat-fixed in 4% paraformaldehyde in PBS (pH 7.2). Parasites were cut longitudinally and washed in PBS with 1% BSA, 0.3% Triton X-100 and 0.1% sodium azide (washing solution) for 24 h at 4 °C, and then incubated with rabbit antiserum raised against purified rFhS for 24 h at 4 °C (diluted 1:200) in washing solution. After the incubation period, the sections were washed in washing solution for 24 h at 4 °C. Tissues were incubated with anti-rabbit secondary antibody tagged with Alexa 555 (1:1000, Cell Signalling Technology, USA) for 24 h at 4 °C, and then with DAPI for nuclear staining (1:1000, Sigma, USA) in washing solution for 30 min at room temperature. Finally, after washing three times with PBS, specimens were mounted on glass microscope slides in PBS. Incubations with pre-immune rabbit serum or with secondary antibodies only were used as controls. Specimens were viewed using a LSM 710 ZEISS confocal laser scanning microscope and images were processed with Zen 2.3 SP1 and Adobe Photoshop CS4 software (Adobe System Inc., USA).

3. Results

3.1. Fasciola hepatica contains at least 4 serpin-encoding sequences

Four full-length sequences encoding putative serpins were identified in the *F. hepatica* transcriptome, and were named FhS-1, FhS-2, FhS-3 and FhS-4 (Table 1). The serpin-coding sequences were aligned with alpha-1-antitrypsin retrieved from GenBank (Supplementary Fig. S1). Predicted amino acid sequences showed the presence of the two consensus sequences found in all serpins: [N]-[AV]-[VLF]-[YT]-[F]-[K]-[GE] and [EK]-[V]-[DN]-[E]-[EA]-[G], corresponding to the conserved serpin amino acid motifs [N]-[A]-[V]-[Y]-[F]-[K]-[G] and [D]-[V]-[N]-[E]-[E]-[G] (Miura et al., 1995; Han et al., 2000) (Supplementary Fig. S1). The RCL of each *F. hepatica* serpin was identified based on consensus residues (Hopkins et al., 1993; Gettins, 2002). The scissile bonds [P_1–P_1'] were predicted based on a conserved serpin feature of approximately 17 amino acid residues (named

Table 1
Accession numbers of *Fasciola hepatica* serpin-encoding sequences.

Serpin ID [a]	Database 01 [b]	Database 02 [c]	NCBI [d]
FhS-1	Fh_Contig10346, Fh_Contig14546	BN1106_s3864B000104	MN906751
FhS-2	Fh_Contig3253	BN1106_s122B000261	MN906752
FhS-3	Fh_Contig3253	BN1106_s122B000261	MN906753
FhS-4	Fh_Contig439, Fh_Contig11204	BN1106_s4565B000033	MN906754

[a] Sequences encoding *Fasciola hepatica* serpins were named FhS, in an acronym representing *Fasciola hepatica* serpin.
[b] Nucleotide sequences retrieved from the *Fasciola hepatica* databases used to assemble complete FhS coding sequences (Young et al., 2010).
[c] Sequences retrieved from the *Fasciola hepatica* databases (Cwiklinsk et al., 2015).
[d] Genebank accession number sof FhS sequences characterised in this article.

P_{17} to P_1) between the start of the hinge region of the RCL and the scissile bond (Supplementary Fig. S1, boxed). RCL alignment predicted that the scissile bond is located between Arg-Ala for FhS-1, Met-Cys for FhS-2 and FhS-3, and Met-Ser for FhS-4 (Fig. 1 and Supplementary Fig. S1, boxed and marked with an asterisk).

FhS amino acid residues, protein length, and predicted molecular weight are consistent with data from other members of the serpin superfamily (Gettins, 2002). Deduced FhS amino acid sequences are in accordance with typical serpin size, ranging from 374 to 408 amino acid residues (Supplementary Fig. S1) with molecular weight and theoretical pI ranging from 41.4 to 46 kDa and 5.97 to 9.04, respectively (Table 2). All FhSs are predicted to have one potential N-glycosylation site ([N]-[X]-[T/S]). Interestingly, scanning for signal peptides revealed that none of the FhSs possess a leader sequence (Table 2). According to BLASTP results, the top 10 matches retrieved from this analysis were serpins from other trematode species. The best matches were: leukocyte elastase inhibitor from *C. sinensis* for FhS-1, serpin B from *C. sinensis* for FhS-2 and FhS-3, and EP-45 precursor from *S. japonicum* for FhS-4 (Table 2). According to 3D modelling, secondary and tertiary structure prediction analysis showed that these serpins contain eight α-helices and 15 β-strands, consistent with 3D structures described for serpins, and the RCLs of native inhibitory serpins are always exposed and accessible to target proteases (Supplementary Fig. S2).

Interestingly, FhS-2 and FhS-3 amino acid sequences are 96% identical (Fig. 1 and Supplementary Fig. S3). These two serpins were amplified with the same set of primers (Section 2.4, Supplementary Table S1); colonies were sequenced in duplicate to confirm the presence of these distinct sequences. The main difference between FhS-2 and FhS-3 was found in the RCL region between P_{11} and $P_{3'}$ residues, where they present 50% of conserved amino acids (7/14 amino acids). The differences were, in FhS-2 and FhS-3, respectively: P_{11} alanine or valine, P_7 threonine or alanine, P_5 threonine or isoleucine, P_4 valine or alanine, P_2 phenylalanine or proline, $P_{2'}$ alanine or leucine, and $P_{3'}$ alanine or valine. The scissile bond (P_1 and $P_{1'}$) is conserved between rFhS-2 and rFhS-3 (Fig. 1, Supplementary Fig. S3). To investigate whether the secondary structure was also different between FhS-2 and FhS-3, we analysed the presence of β -turns. Interestingly, this structure appears exclusively in FhS-3, which has a type VIb β-turn between proline (P_2) and methionine (P_1) (Fig. 1, marked with an asterisk in FhS-3). Mass spectrometry analysis of tryptic peptides generated from these samples confirmed these differences (Supplementary Fig. S4). Protein coverage was more than 60% including the RCL in both rFhS-2 and rFhS-3. The results also confirm that the two protein bands recovered after rFhS-2 purification and deglycosylation assays are serpins (Supplementary Fig. S4 and Fig. 3).

3.2. Recombinant F. hepatica serpins are glycoproteins

Data about expression in *P. pastoris* and affinity purification of rFhS-2, rFhS-3 and rFhS-4 are summarised in Supplementary Fig. S5. Efforts to express rFhS-1, from several clones, were unsuccessful. Daily samples of yeast-expressed recombinant proteins were subjected to western blotting analysis using a specific antibody against the C-terminal hexa histidine tag. Western-blot analysis showed that the recombinant proteins had a molecular mass of approximately 45 kDa, as predicted from the amino acid sequences (Table 2). Recombinant expression of *F. hepatica* serpins in the *P. pastoris* expression system produced a final yield of 200 μg of rFhS-2, 1 mg of rFhS-3, and 3 mg of rFhS-4 pure recombinant protein per litre of culture. When treated with deglycosylation enzymes, downward molecular mass shifts were observed, demonstrating that rFhS-2, rFhS-3 and rFhS-4 are glycosylated upon *P. pastoris* expression (Fig. 2). This observation is consistent with in silico analysis, which predicted *F. hepatica* serpins contain motifs for N-glycosylation (Table 2).

3.3. rFhS-2 and rFhS-4 target chymotrypsin-like proteases

To experimentally verify whether rFhS-2, rFhS-3 and rFhS-4 are inhibitory serpins as suggested by their primary sequence analysis, inhibitory activity against a selection of 14 host-derived serine proteases was tested. The enzymes were incubated with molar excess of rFhS, and the residual protease activity was determined (Fig. 3). The results show inhibition of chymotrypsin-like proteases by rFhS-2 (Fig. 3A) and rFhS-4 (Fig. 3C). rFhS-2 (1 μM) inhibited the activity of cathepsin G (86 nM) by 74% (Fig. 3A), while rFhS-4 (1 μM) inhibited the activity of chymotrypsin (7.2 nM) by 97%, and the activity of cathepsin G (86 nM) by 99% (Fig. 3C), the strongest inhibition of cathepsin G among the tested serpins. Despite a high sequence identity between rFhS-2 and rFhS-3, the latter did not present significant inhibitory activity against any of the tested proteases (Fig. 3B and Supplementary Fig. S3).

rFhS-2 inhibited cathepsin G with an stoichiometry index of 4.9 (Fig. 4A). For rFhS-4, the SI index against chymotrypsin was 2.4, and 1.3 against cathepsin G (Fig. 4B and C). rFhS-2 and rFhS-4 kinetics assays confirmed that they behave similarly to typical inhibitory serpins (Fig. 4). Second order association constants (k_a) were determined by the discontinuous curve method (Fig. 4D, E). The k_a value for FhS-2 with cathepsin G was 0.47 m^{-1} s^{-1}. The measured rate of rFhS4-chymotrypsin association was $k_a = 3.8 \times 10^3$ m^{-1} s^{-1} and for rFhS4-cathepsin G the k_a value was 2.55×10^3 m^{-1} s^{-1}.

The mechanism of action of rFhS-4 as a typical inhibitory serpin was confirmed by the formation of a covalent complex with target protease (Fig. 5). After incubation of chymotrypsin (25 kDa) or cathepsin G (30 kDa) with rFhS-4 (46 kDa), high molecular weight complexes were formed (as indicated by asterisks in Fig. 5). After rFhS-4 cleavage by protease, the C-terminal portion of serpin is released (4.8 kDa). The formation of covalent complexes was observed on 12% SDS-PAGE. Complexes are visualised as bands migrating between 55 kDa and 72 kDa, at approximately the same position as the sum of the B and C chains of chymotrypsin (23 kDa) or cathepsin G (30 kDa) and the cleaved rFhS-4 (41.2 kDa). As expected, more than one band appeared at higher molecular weight values compared with the purified rFhS-4 alone. Some low molecular weight proteins are present as well, corresponding to products of serpin hydrolysis during incubation. There was not apparent degradation when rFhS-4 was incubated with cathepsin G, probably due to its narrow extended cleavage specificities.

P_{17}[E]-P_{16}[E/K/R]-P_{15}[G]-P_{14}[T/S]-P_{13}[X]-$P_{12\text{-}9}$[A/G/S]-$P_{8\text{-}1}$[X]-P_1'-P_4[X]

ID	P19	P_{18}	P_{17}	P_{16}	P_{15}	P_{14}	P_{13}	P_{12}	P_{11}	P_{10}	P_9	P_8	P_7	P_6	P_5	P_4	P_3	P_2	**P_1**	$P_{1'}$	$P_{2'}$	$P_{3'}$	$P_{4'}$
A1AT	T	D	E	K	G	T	E	A	A	G	A	M	F	L	E	A	I	P	**M**	S	I	P	P
FhS-1	E	N	E	A	G	A	V	A	S	A	A	S	G	V	C	V	S	N	**R**	A	M	L	Q
FhS-2	E	D	E	E	G	A	E	A	A	A	A	S	T	A	T	V	V	F	**M**	C	A	A	I
FhS-3	E	D	E	E	G	A	E	A	V	A	A	S	A	A	I	A	V	P	**M***	C	L	V	I
FhS-4	K	N	E	A	G	V	E	A	T	A	A	T	A	M	M	A	V	P	**M**	S	L	L	V

Fig. 1. Structure-based reactive center loop sequence alignment of *Fasciola hepatica* serpin (FhS) with human α1-antitrypsin (A1AT). The alignment was constructed by comparison with human α1-antitrypsin (Protein Data Bank (PDB 1HP7)) as a template, using Bioedit and Genedoc. Amino acids in the scissile bond are boxed, and predicted β-turns are marked with an asterisk. Amino acid differences between FhS-2 and FhS-3 are underlined.

Table 2
Polypeptide features of *Fasciola hepatica* serpin (FhS) sequences.

ID	SP [a]	N-gly sites [b]	MW (kDa) [c]	pI [d]	Best match/accession number (% identity) [e]
FhS-1	–	1	41.4	5.97	CsSRP/GAA35588.2 (36)
FhS-2	–	1	42.3	6.73	OvSRP/XP_009172751.1 (43)
FhS-3	–	1	42.1	6.12	OvSRP/XP_009172751.1 (43)
FhS-4	–	1	46.0	9.04	SjSRP/CAX76359.1 (40)

[a] Signal Peptide (SP) was predicted using the SignalP 4.1 server (Petersen et al., 2011). Symbols (+) and (–) represent the presence and absence of signal peptide sequence, respectively.

[b] Putative N-glycosylation sites were predicted using the NetNGlyc 1.0 server, based on the putative N-glycosylation site sequence N-X-T/S.

[c,d] Molecular weigth (MW) and theoretical pI were calculated using the Compute pI/Mw tool via the Expasy website.

[e] The best match identities were obtained using BLASTP against the non-redundant protein database in GenBank. The numbers represent the GenBank accession numbers. CsSRP (*Clonorchis sinensis* serpin), OvSRP (*Opisthorchis viverrini* serpin), and SjSRP (*Schistosoma japonicum* serpin).

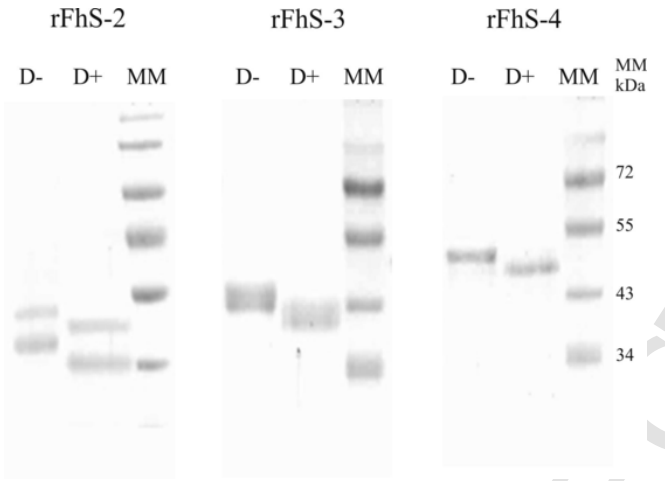

Fig. 2. Deglycosylation assays of recombinant *Fasciola hepatica* serpin (rFhS). N- and O-glycosylation assay for rFhSs. SDS-PAGE gel of FhSs samples either untreated (D–) or treated (D+) with a glycosidase cocktail.

3.4. Fasciola hepatica serpins FhS-2 and FhS-4 possess putative glycosaminoglycan-binding sites and bind to heparin

Glycosaminoglycans such as heparin can modulate the activity of several serpins (Rau et al., 2007). Fig. 6 shows that rFhS-2 and rFhS-4 bind to heparin, as the serpins were eluted from the heparin affinity column at high salt concentration: 0.6 M and 0.9 M NaCl for rFhS-2 and rFhS-4, respectively (Fig. 6A); no serpins were detected in the flow-through or wash fractions (Fig. 6B-C). These results are in accordance with the basic patches observed in silico in electrostatic surface potential predictions shown in Supplementary Fig. S2. The capacity of rFhS-3 to bind to heparin was not tested since this serpin did not show inhibitory activity against any of the tested proteases.

3.5. FhS-2 is localised in the ovary, and FhS-4 in the gut of adult F. hepatica

To determine the localisation of the serpins in adult fluke tissues, antibodies against rFhS-2 and rFhS-4 were used in confocal microscopy analyses (Fig. 7). In sections of adult worm samples, the anti-rFhS-2 and anti-rFhS-4 antibodies bound to cellular structures associated with ovary and gut, respectively (Fig. 7A–F and Supplementary Fig S6).

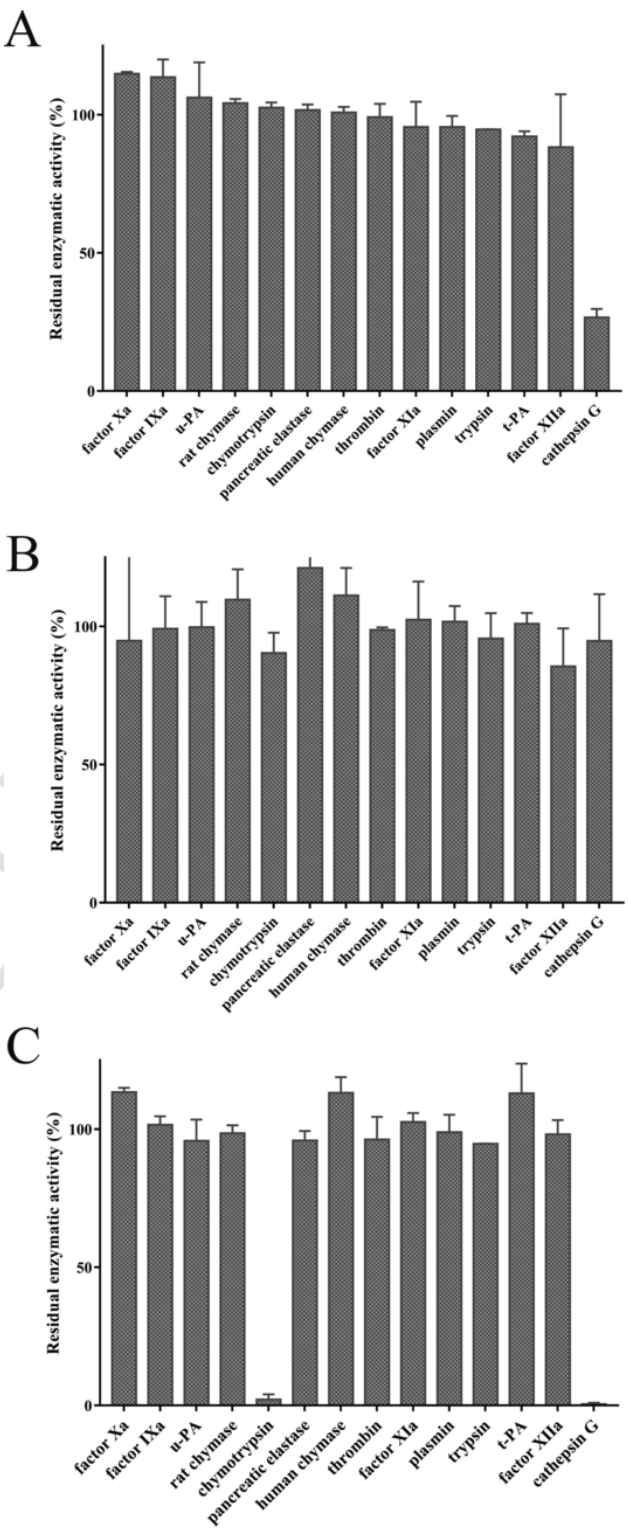

Fig. 3. Screening of *Fasciola hepatica* serpin (FhS) inhibitory activity against mammalian serine proteases. Graphs represent the residual activity of targeted serine proteases when treated with molar excess of recombinant serpins. (A) FhS-2; (B) FhS-3; (C) FhS-4. Error bars represent the mean ± S.E.M.

Furthermore, both antibodies bound differently in the tegument spines, where the signal for anti-rFhS-2 antibody localised in the outer region of the structure, and the signal for anti-rFhS-4 antibody concentrated in the apical region along the border (Fig. 7H and J). Noticeably, no la-

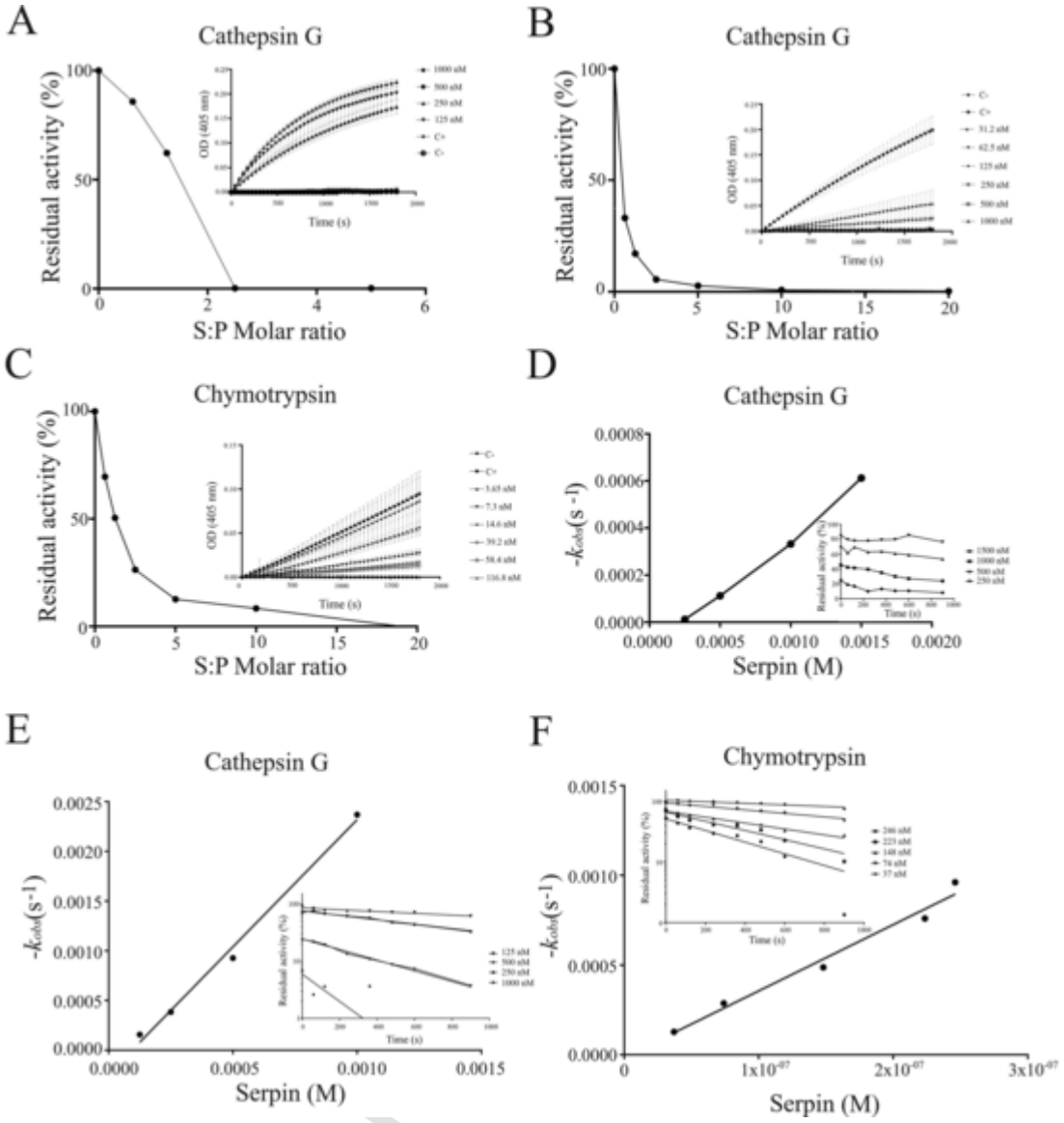

Fig. 4. Stoichiometry of inhibition assay for recombinant *Fasciola hepatica* serpin-2 (rFhS-2) and rFhS-4. Increasing amounts of rFhSs were pre-incubated for 1 h at 37 °C with a constant molar concentration of cathepsin G or chymotrypsin, resulting in increasing inhibitor:protease (I:E) molar ratios. Residual enzymatic activity was measured using a specific colorimetric substrate for each enzyme. The stoichiometry of inhibition was determined by linear regression. Three independent assays were performed in triplicate for each protease. (A) rFhS-2 against cathepsin G. (B) rFhS-4 against cathepsin G. (C) rFhS-4 against chymotrypsin. Error bars represent the mean ± S.E.M. Discontinuous assay of the inhibitory activity of rFhS-2 against cathepsin G (D), rFhS-4 against cathepsin G (E), and rFhS-4 against chymotrypsin (F). Semi-logarithmic plots of residual protease activity versus reaction time at different serpin concentrations. k_{obs} is plotted as a function of serpin concentration. Linear regression was used to calculate the second-order rate constant k_a for each inhibition reaction.

belling was detected on other parasitic organs or in the controls (Supplementary Fig. S6).

3.6. rFhS-4 inhibits platelet aggregation

Given that rFhS-4 strongly inhibited cathepsin G, the possibility that this serpin could inhibit platelet aggregation by cathepsin G was investigated. Platelet activation can be induced by different agonists including cathepsin G (LaRosa et al., 1994). Fig. 8 shows that platelet aggregation in the presence of cathepsin G was inhibited after incubation with various concentrations of rFhS-4. At a 1:0.25 (enzyme:inhibitor) ratio, platelet aggregation was inhibited by 40.5%, and when the ratio was 1:5, platelet aggregation was completely abolished (Fig. 8).

4. Discussion

Despite the extensive knowledge about serpins in higher eukaryotes, little is known about their function in parasites. Research on serpins in parasites has to date mostly focused on parasite vectors such as ticks (Tirloni et al., 2016; Chmelar et al., 2017) and mosquitoes (Gulley et al., 2013), and parasites that affects humans, such as *B. malayi* (Zang et al., 1999) or schistosomes (Quezada and McKerrow, 2011). Little is known about the function of serpins in the liver fluke *F. hepatica* (Ranasinghe and McManus, 2017). In this study, the identification and partial characterisation of four serpins in the transcriptome of the helminth parasite *F. hepatica* are described (FhS-1 to -4). These serpins belong to the inhibitor family I4 according to the MEROPS database for inhibitors and peptidases (Rawlings et al., 2018); members of this family could inhibit proteases from the S1 (Silverman et al., 2001), S8 (Dufour et al., 1998), C1 (Al-Khunaizi et al., 2002;

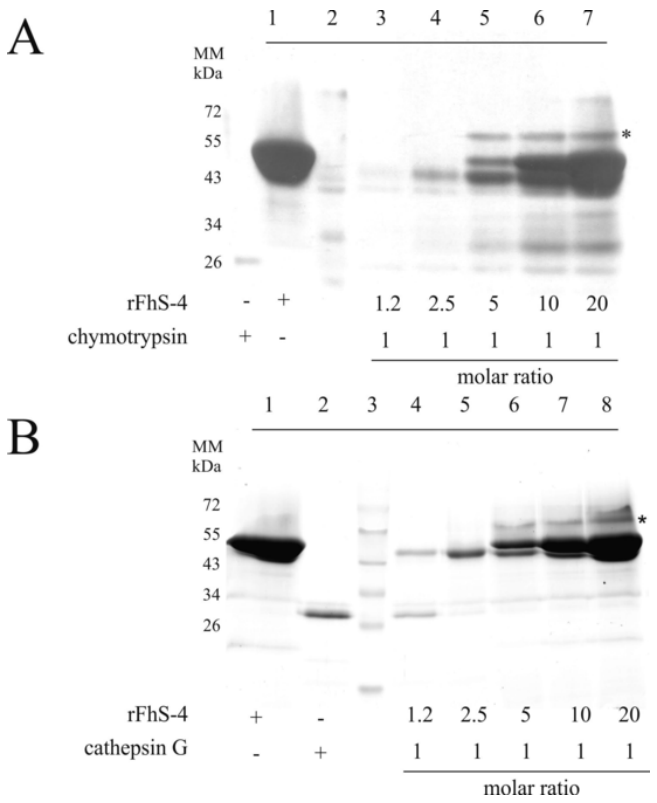

Fig. 5. SDS-PAGE analysis of stable covalent recombinant *Fasciola hepatica* serpin-4 (rFhS-4): target complexes. (A) Covalent complex formation between chymotrypsin and rFhS-4 at increasing concentrations. Lane 1, purified rFhS-4 alone; lane 2, chymotrypsin alone; lane 3, molecular mass marker; lane 4, 1:2.5 rFhS-4/chymotrypsin; lane 5, 1:5 rFhS-4/chymotrypsin; lane 6, 1:10 rFhS-4/chymotrypsin; lane 7, 1:20 rFhS-4/chymotrypsin. (B) Covalent complex formation between cathepsin G and rFhS-4 at increasing concentrations. Lane 1, purified rFhS-4 alone; lane 2, cathepsin G alone; lane 3, molecular mass marker; lane 4, 1:0.625 rFhS-4/cathepsin G; lane 5, 1:1.25 rFhS-4/cathepsin G; lane 6, 1:2.5 rFhS-4/cathepsin G; lane 7, 1:5 rFhS-4/cathepsin G; lane 8, 1:10 rFhS-4/cathepsin G. Bands corresponding to the covalent complex are indicated with an asterisk.

Irving et al., 2002) and C14 (Komiyama et al., 1994) protease families.

The *F. hepatica* genome has seven putative serpin sequences (BN1106_s3864B000104, BN1106_s122B000261, BN1106_s1727B000096, BN1106_s284B000286, BN1106_s4565B000032, BN1106_s4565B000033, BN1106_s4618B000050) (Cwiklinski et al., 2015b). Of these, the sequence BN1106_s3864B000104 corresponds to FhS-1, BN1106_s122B000261 corresponds to FhS-2 and FhS-3, and BN1106_s4565B000033 corresponds to FhS-4. The predicted amino acid sequences have low similarity with other serpins from parasitic helminths (less than 30%), a common feature in the serpin superfamily of proteins (Gettins, 2002). Additionally, the identities of sequences were established based on common features of the serpin superfamily such as the RCL, serpin motifs, similar lengths and predicted molecular masses (Fig. 1, Supplementary Fig. S2 and Table 1). Sequence analysis showed that none of the four *F. hepatica* serpins contain signal peptide or transmembrane domains, suggesting that they are cytosolic serpins. In our previously published proteomic analysis of the ES products from the intra-mammalian stages of these parasite (Di Maggio et al., 2016), FhS-1 was found in adult ES products, NEJ ES products, and among somatic soluble NEJ proteins. FhS-2/FhS-3 (which are not distinguishable by proteomic analysis because RCL regions are not present in the database) were found in the adult ES products, and in the somatic soluble NEJ proteins. As these serpins do not have signal peptides and were not found in exosomes (Cwiklinski et al., 2015b),

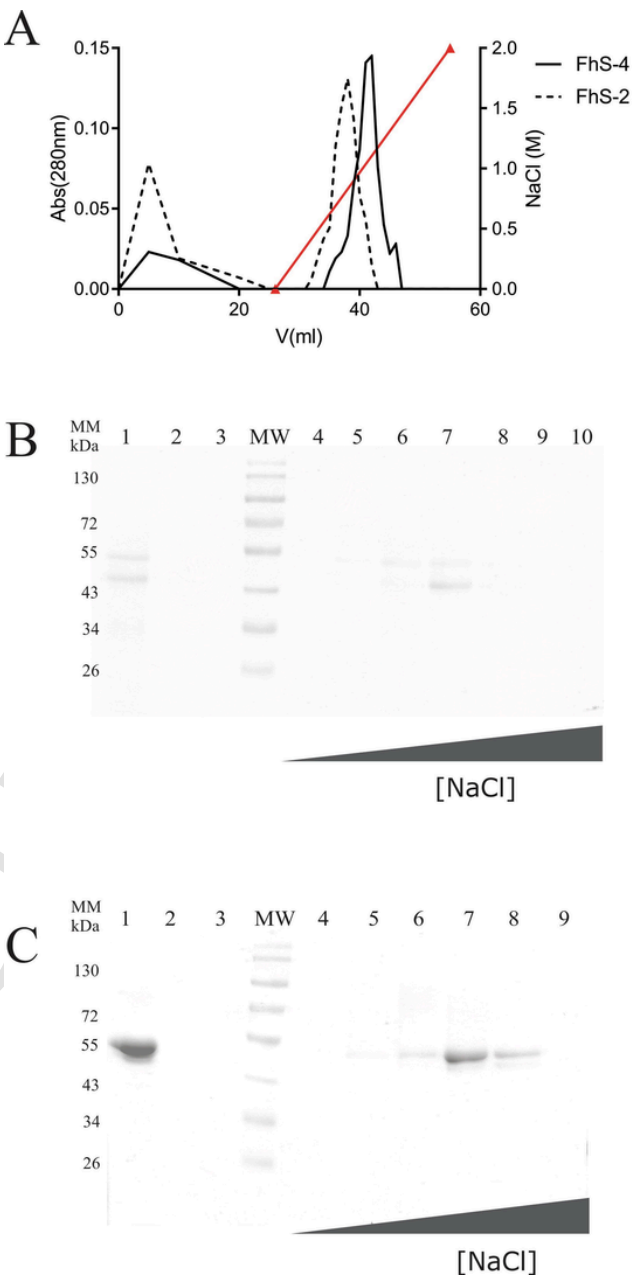

Fig. 6. Analysis of recombinant *Fasciola hepatica* serpin (rFhS) interaction with heparin. Purified FhS-2 or FhS-4 was applied to a heparin affinity column in 10 mM sodium phosphate, pH 7.0, chromatographed and eluted with a linear gradient of NaCl in buffer. Eluted proteins were subjected to 12% SDS-PAGE and stained with CBB-G250. (A) rFhS-2 and rFhS-4 elution pattern; red (grey) line represents the NaCl gradient. (B) rFhS-2. Lane 1, purified rFhS-2; lane 2, flow-through fraction; lane 3, wash fraction; MW, molecular weight marker; lane 4, fraction 8; lane 5, fraction 9; lane 6, fraction 10; lane 7, fraction 11; lane 8, fraction 12; lane 9, fraction 13; lane 10: fraction: 14. (C) rFhS-4. Lane 1, purified rFhS-4; lane 2, flow-through fraction; lane 3, wash fraction; MW molecular weight marker; lane 4, fraction 10; lane 5, fraction 11; lane 6, fraction 12; lane 7, fraction 13; lane 8, fraction 14; lane 9, fraction 15.

their secretion into the ES products might depend on another type of vesicle or a non-classical secretion pathway. Although none of the published proteomic studies identify the presence of FhS-4, we demonstrate that it is expressed at the gut surface in the adult fluke (Fig. 7D–F).

One of the characteristics of inhibitory serpins is that the RCL is accessible to target proteases (van Gent et al., 2003), and this was observed as well in the *F. hepatica* serpins (Supplementary Fig. S2). Three of the four serpins were successfully expressed in the *P. pastoris*

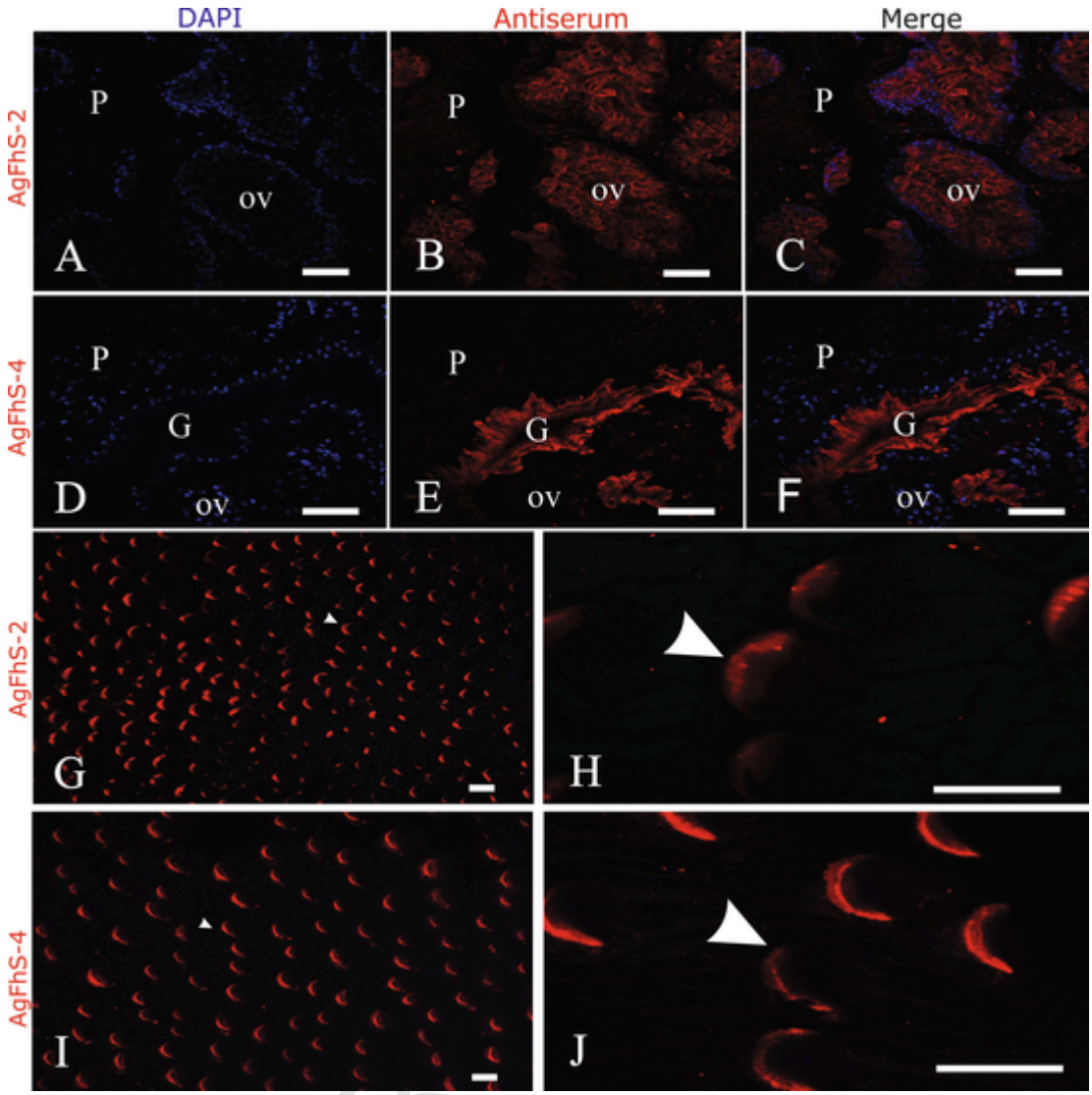

Fig. 7. Subcellular localisation of recombinant *Fasciola hepatica* serpin-2 (FhS-2) and FhS-4 in *Fasciola hepatica* adults. Immunolocalisation of FhS-2 and FhS-4 on longitudinal sections of adult flukes using rabbit anti-rFhS-2 and anti-rFhS-4 antibodies. Images were captured using 10× and 40× oil immersion objectives. (A–C) Sections of adult *F. hepatica* parasites showing localisation of FhS-2 in the cytoplasmic region of the ovary. (D–F) Localisation of FhS-4 inside gut cells but not in the lumen. (G–H) FhS-2 antibody signal in tegument spicules under 10x and 40× objective, respectively. (I–J) FhS-4 antibody signal in tegument spicules under 10× and 40× objective, respectively. Arrowheads indicate the antibody stain in the spicules. Scale bars = 50 μm. G, gut; P, parenchyma; OV, ovary.

expression system for biochemistry characterisation. The kinetic assays demonstrate that rFhS-2 inhibits cathepsin G, while rFhS-4 inhibits chymotrypsin-like serine proteases, namely cathepsin G and chymotrypsin (Fig. 4). rFhS-3 does not show any inhibitory activity against the serine proteases tested in this study.

FhS-2 and FhS-3 were initially amplified with the same pair of primers (Supplementary Table S1), and the nearly identical sequences were manually checked after sequencing. Nevertheless, we found different biochemical behaviour against the tested proteases, suggesting different biological functions. One interesting speculation is that they could be generated by alternative splicing of RCL encoding exons. Indeed, despite an overall sequence similarity of 96%, the two serpins share only 50% identity within the RCL region (Fig. 1 and Supplementary Fig. S1 and S3), and therefore differences in their inhibitory activity were expected. The RCL is crucial for the inhibitory function of serpins, undergoing a conformational change when the scissile bond is cleaved by the protease, and thus altering the topology of the complex (Irving et al., 2000). It is known that changes in amino acids within this region could affect activity either by changing the serpin to be non-inhibitory, or by changing the inhibition profile. This phenomenon is well studied in alpha-1-antitrypsin, where substitutions in the P_2 residue change the inhibition efficiency and could change the specificity of the serine protease (Irving et al., 2002; Chung et al., 2017), as well as in kallistatin, where substitutions in P_1 to P_3 residues were also shown to affect inhibition specificity (Chen et al., 2000). Additionally, changes in sequence or length of the RCL could change the tertiary structure of the serpin loop, which could alter the angle of insertion of the RCL into the α-helix after being hydrolysed (Zou et al., 1994; Irving et al., 2000), as was evident for FhS-3 (Fig. 1). Studies with mutant serpins showed that RCL modifications reassign the targets and change the serpin–protease interaction kinetics, as observed in plasminogen activator inhibitor-1 (PAI-1) and alpha-1-antitrypsin (Lawrence et al., 2000; Dufour et al., 2005). Differences in the rate of association constants and SI between FhS-2 and FhS-3 could be due to differences in the regions around the scissile bond, particularly P_{14}-P_9 and P_7-P_1, respectively. For example, in position P_7, FhS-2 has a threonine residue (hydrophilic), while FhS-3 has an alanine (aliphatic). In position P_2, FhS-2 has a phenylalanine (aromatic), while FhS-3 has a

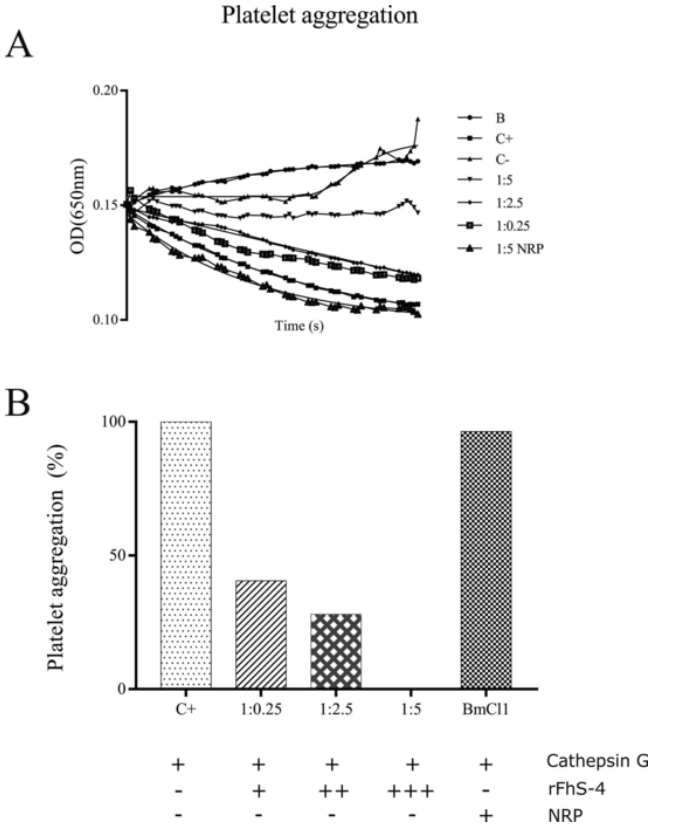

Fig. 8. Recombinant *Fasciola hepatica* serpin-4 (rFhS-4) inhibits cathepsin G-induced platelet aggregation. A platelet aggregation assay was performed using bovine platelet-rich plasma. (A) Kinetic assays. Tyrode solution with varying amounts of rFhS-4 was preincubated with cathepsin G (enzyme:serpin (w/w), 1:5, 1:2.5, 1:0.25) in a 50 μL reaction at pH 7.4, for 15 min at 37 °C. Platelet aggregation was initiated by addition of 100 μL of pre-warmed PRP, and monitored at 20 s intervals for 30 min at O.D. 650 nm. (B) Platelet aggregation percentage. Increasing amounts of rFhS-4 inhibit cathepsin G-induced platelet aggregation. B, tyrode solution; C+, without rFhS-4; C-, without cathepsin G; +, presence; −, absence; NRP, non-related protease, an active recombinant cathepsin L-like from *Rhipicephalus microplus* also expressed in *Pichia pastoris* and without inhibitory activity against cathepsin G was used as a control (Clara et al., 2011).

proline (aliphatic). The importance of proline in the secondary structure of proteins is well known. The presence of proline in a polypeptide chain acts as a structural disruptor of secondary structural elements such as α-helix, β-sheets and β-turns due to the exceptional conformational rigidity of proline (Morris et al., 1992; Petersen et al., 2010). FhS-3 proline in the P_2 site generates a predictive β-turn and that could explain the absence of inhibitory activity against proteases tested in this work (Fig. 1, predicted β-turn site is marked with an asterisk). All these amino acids have different spatial locations, which could not only reduce the bond/association between the inhibitor and the protease, but also impede the conformational changes that are crucial for inhibition (Im et al., 2004).

It is known that the hinge region, P_{15} to P_9 residues, provides mobility essential for the conformational change of the RCL in the stressed to relaxed (S → R) transition (Irving et al., 2000; Gettins, 2002). This transition is important to the function of inhibitory serpins, where the formation of a stable complex between the cleaved form of the inhibitor and the protease occurs, analogous to an enzyme-product complex (Gettins, 2002). Accordingly, the comparison between the hinge regions of FhSs and A1AT (Fig. 1) reveals conserved amino acids, suggesting that the expressed serpins are capable of forming stable complexes with their target proteases, as shown for rFhS-4 (Fig. 6) and rFhS-2 (data not shown).

In silico analysis shows one putative N-glycosylation site in each serpin (Table 2), which was confirmed by a deglycosilation assay for the three recombinant serpins (Fig. 2). These results suggest the recombinant serpins undergo post-translational modifications upon expression in an eukaryotic system, as do many other known serpins (Pemberton and Bird, 2004). These post-translational modifications help the serpins to become more stable, and/or protect against degradation without interfering with the inhibitory activity, as was shown for α-1-antitrypsin (Sarkar and Wintrode, 2011). In the case of antithrombin, the two isoforms (α and β) differ in their glycosylation pattern at the Asn^{135} position, which contributes to their different substrate affinity, localisation and function (McCoy et al., 2003; Pol-Fachin et al., 2011).

Charge distribution, as shown by in silico analysis, was different among the four *F. hepatica* serpins, even between the highly similar FhS-2 and FhS-3. Only FhS-3 and FhS-4 showed a prominent basic patch (Supplementary Fig. S2). An important feature of serpins is having the ability to bind various ligands, and rFhS-2 and rFhS-4 were both shown to bind to heparin with high affinity (Fig. 3). The high pI of FhS-4 (Table 1) is uncommon in this superfamily, with only one serpin from germinal centre B-cells and one from vertebrate blood cells showing similar pIs (Grigoryev et al., 1999; Paterson et al., 2007). The basic patches present in the Fh-Ss suggested the possibility of binding to negatively charged entities such as GAGs and DNA. In this work we were able to demonstrate that rFhS-4 has the ability to bind heparin with strong affinity (Fig. 3 and Supplementary Fig. S2). It is known that heparin can act as a co-factor for serpins, accelerating and/or improving the inhibition of proteases. For instance, the affinity of α-1-antitrypsin for some serine proteases increases as much as 48-fold in the presence of heparin (Huang et al., 2011; Khan et al., 2011).

A typical inhibitory serpin forms a covalent complex with its cognate protease which is resistant to SDS and thermal denaturation, has a SI close to 1 and an association constant (k_a) of 10^5 M^{-1} s^{-1} (Horvath et al., 2011). An SI approaching 1 indicates that the inhibitory pathway proceeds faster than the substrate pathway, and physiological serpins have 1:1 (S:P) molar ratios. The SI values and second rate constants show that rFhS-4 interacts with its target proteases differently; it demonstrates a greater inhibitory effect on cathepsin G, with an SI of 1.3 compared with 2.4 for chymotrypsin. The discontinuous method was used to determine the second order association rate constant (k_a) for rFhs-2 and rFhS-4 with their respective proteases. The results show that rFhS-4 appears to be a better cathepsin G inhibitor than rFhS-2, with a k_a of 2.55×10^3 m^{-1} s^{-1} (Fig. 5D–F). The k_a value of 3.8×10^3 m^{-1} s^{-1} indicates a fast inhibition of chymotrypsin by rFhS-4s. Complexes between FhS-4 and cathepsin G are visible on an SDS-PAGE gel at a 2:1 ratio. Both complex formation assays (for chymotrypsin and cathepsin G) revealed bands with molecular weights between that expected for the serpin and the serine protease individually, which could be a result of serpin degradation during the assay.

The localisation of the serpins in tissues of *F. hepatica* adults provides insights into the putative biological functions of these proteins in the adult fluke. FhS-2 was detected in the ovary and in the tegument spines (Fig. 7, Supplementary Figs. S6 and S7), and it has been previously found in intra-mammalian stage ES products, suggesting that it is secreted as part of the ES products (Di Maggio et al., 2016). The ovary in this parasite has ramifications, presenting smaller cells on the outside and larger cells inside (oocytes). Anti-FhS-2 antibody clearly localises in the cytoplasmic region of the oocytes (Fig. 7A–C). Mature oocytes are rich in electron-dense granules that are in contact with the cytoplasmic membrane and of which the function is unknown to date. Because rFhS-2 inhibits cathepsin G, a serine protease stored in neutrophil cytoplasmic granules, it is possible this serpin is involved in the host-parasite relationship, as a protection for the egg against the host immune system. Sequences identified as FhS-2 and/or FhS-3 were

found in various *F. hepatica* proteomic studies. In contrast, FhS-4 is located in cells that form the gut surface of the adult fluke, but apparently is not present in the lumen (Fig. 7D-F), and surprisingly, it was not found among the ES products (Robinson et al., 2009; Wilson et al., 2011; Di Maggio et al., 2016). This suggests FhS-4 could be involved in the assimilation of nutrients within the gut, or have other functions related to the regulation of parasitic proteases. rFhS-4 inhibits cathepsin G and chymotrypsin, and can inhibit platelet aggregation as well, therefore a role in the host-parasite relationship could not be excluded. Sera against both rFh-2 and rFhS-4 showed signals in the adult tegument: anti-FhS-2 antibody binds the apical region of the spines (Fig. 7G–H), while anti-FhS-4 antibodies appear all along the border in the apical region of the spicules in the tegument (Fig. 7I–J). Spines are rigid structures that assist the parasite in penetration and attachment to the host tissues. Their composition in *F. hepatica* is not well resolved, as opposed to other digenean parasites such as *S. mansoni,* where spines are known to be mainly composed of actin (Pearson et al., 1985).

As documented in *F. hepatica* and other haematophagous parasites, mechanical tissue damage is a consequence of the feeding process (Gajewska et al., 2005; Mihara, 2017). It induces tissue repair mechanisms mediated by host serine proteases such as inflammation, complement activation and platelet aggregation. Consequently, cathepsin G-induced platelet aggregation inhibition by rFhS-4 (Fig. 8) might be important during the feeding process, modulating host serine proteases.

In conclusion, the role of these *F. hepatica* serpins is not yet totally understood, but in silico and in vitro analyses indicate putative biological functions. rFhS-2 is active against cathepsin G, and rFhS-4 inhibits cathepsin G and chymotrypsin in vitro. Moreover, the tissue localisation suggests their participation in the parasite mechanisms of immune evasion.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2020.05.010.

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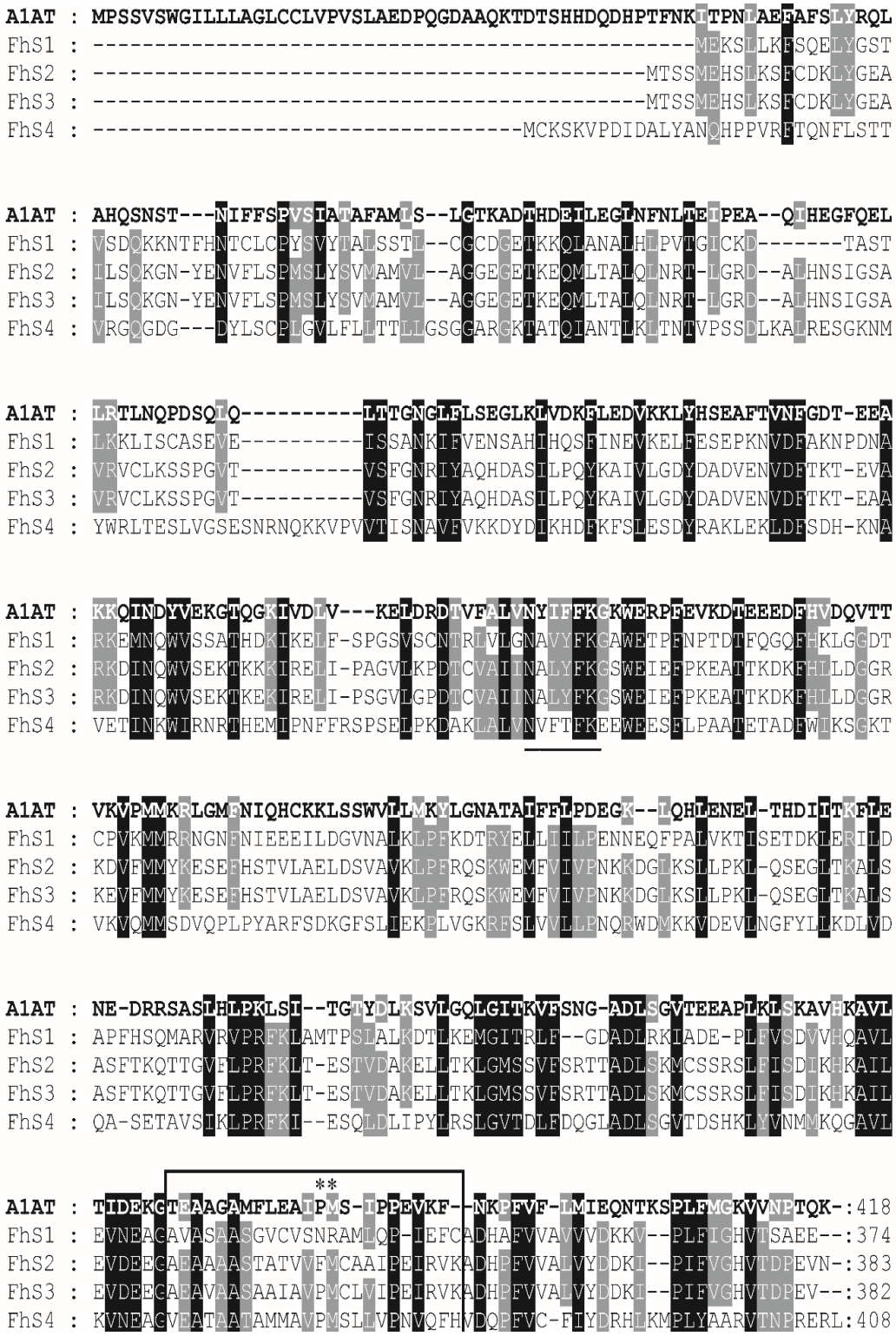
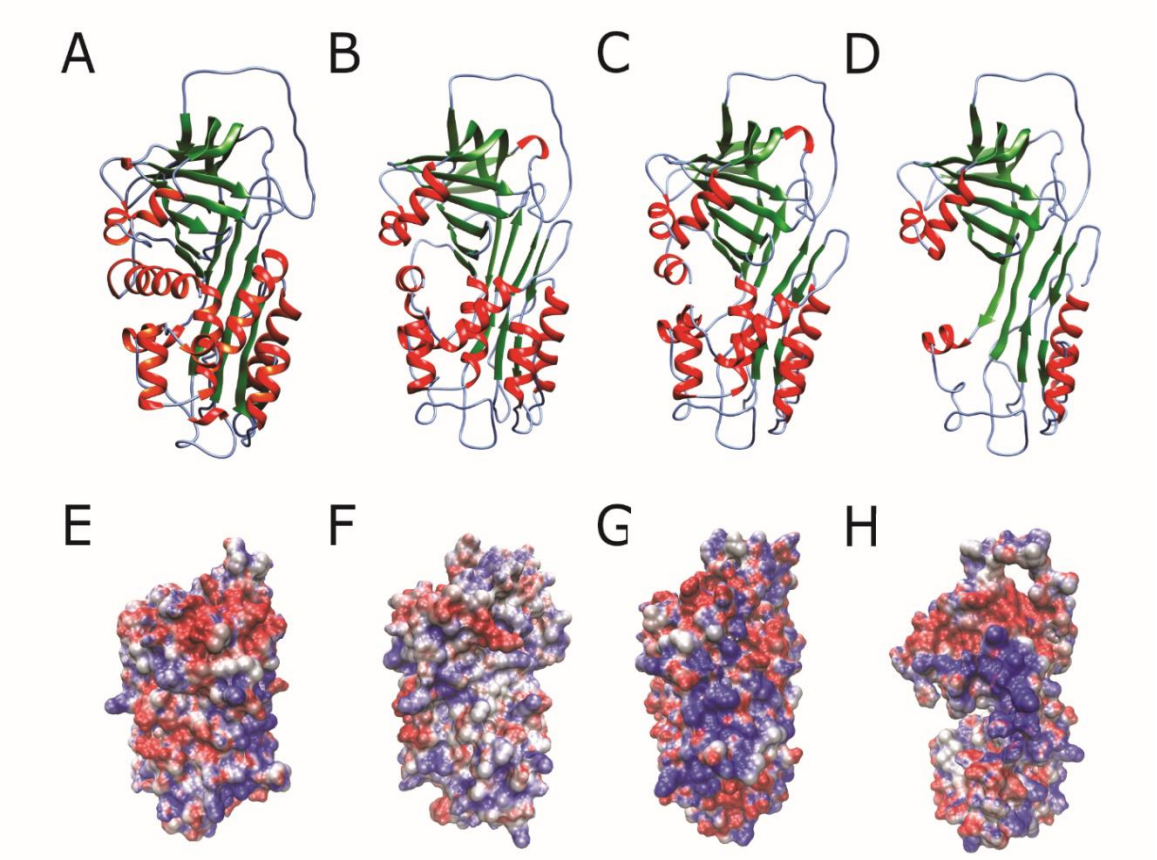
Supplementary Table S1. Primers for *F. hepatica* serpins cloning and expression. In bold, the restriction site sequences for ClaI and NotI enzymes. The sequence for the His-tag in the reverse primer is underlined. TM: melting temperature. %GC: guanine and cytosine content.

Sequence	Primer	TM	%GC
FhS-1	5'-AAAAA**ATCGAT**G GAGAAGTCACTTTTGAAGTTTTCACAGG-3'	56.4°	39.3
	3'-TTT**GCGGCCGC**CTAGTGGTGGTGGTGGTGGTGCTCTTCAGCCGATGTTACG-5'		
FhS-2	5'-AAAAA **ATCGAT**GACCTCATCTATGGAACATTCCTTGAAGAG-3'	57.9°	41.4
FhS-3	3'-TTT**GCGGCCGC**TTAGTGGTGGTGGTGGTGGTGGTTCACCTCCGGATCAGTAACG-5'	58°	48
FhS-4	5'-AAAAA**ATCGAT**GTGCAAGTCGAAGGTGCCGGAC-3'	62.4°	61.9
	3'-TTT**GCGGCCGC**TTAGTGGTGGTGGTGGTGGTGCAAGCGTTCACGTGGATTGGTCAC-5'	62.4°	61.9



Supplementary Figure S1. Structure-based sequence alignment of FhSs with human α1-antitrypsin (A1AT). The alignment was constructed by comparison with human α1-antitrypsin (PDB 1HP7, in bold) as a template, using Bioedit and Genedoc. Highly and moderately conserved residues are labelled in black and grey, respectively. RCL is boxed and the amino acids from the scissile bond are marked with an *. Conserved serpin sequences are underlined.



Supplementary Fig. S2. **Comparison of *Fasciola hepatica* serpin (FhS) predicted tertiary structure and predicted electrostatic surface potential.** (A-D) Serpins are shown in the native conformation, with the reactive center loop (RCL) being surface-accessible by target protease. The α-helices and β-sheets are represented in green and blue, respectively, and the amino acids in the scissile bond are marked in red. (E-H) Red surfaces indicate acidic regions with negative electrostatic potential. White regions are predicted neutral electrostatic potential regions. Blue surfaces are basic regions with positive electrostatic potential. (A, E) FhS-1; (B, F) FhS-2; (C, G) FhS-3; (D, H) FhS-4

Supplementary Figure S3. Structure-based sequence alignment of FhS-2 and FhS-3 showing the differences between both sequences. Highly and moderately conserved residues are labelled in black and grey, respectively. RCL is boxed and the amino acids from the scissile bond are marked with an *. Conserved serpin sequences are underlined.

Proteomic results for rFhS-2 and rFhS-3. In blue peptide identification for each serpin. Boxed: RCL region.

Identification peptides for upper band in purified **rFhS-2**: 43-55 kDa

```
001 MTSSMEHSLK SFCDKLYGEA ILSQKGNYEN VFLSPMSLYS VMAMVLAGGE GETKEQMLTA 060
061 LQLNRTLGRD ALHNSIGSAV RVCLKSSPGV TVSFGNRIYA QHDASILPQY KAIVLGDYDA 120
121 DVENVDFTKT EVARKDINQW VSEKTKKKIR ELIPAGVLKP DTCVAIINAL YFKGSWEIEF 180
181 PKEATTKDKF HLLDGGRKDV FMMYKESEFH STVLAELDSV AVKLPFRQSK WEMFVIVPNK 240
241 KDGLKSLLPK LQSEGLTKAL SASFTKQTTG VFLPRFKLTE STVDAKELLT KLGMSSVFSR 300
301 TTADLSKMCS SRSLFISDIK HKAILEVDEE GAEAAAASTA TVVFMCAAIP EIRVKADHPF 360
361 VVALVYDDKI PIFVGHVTDP EVN
```

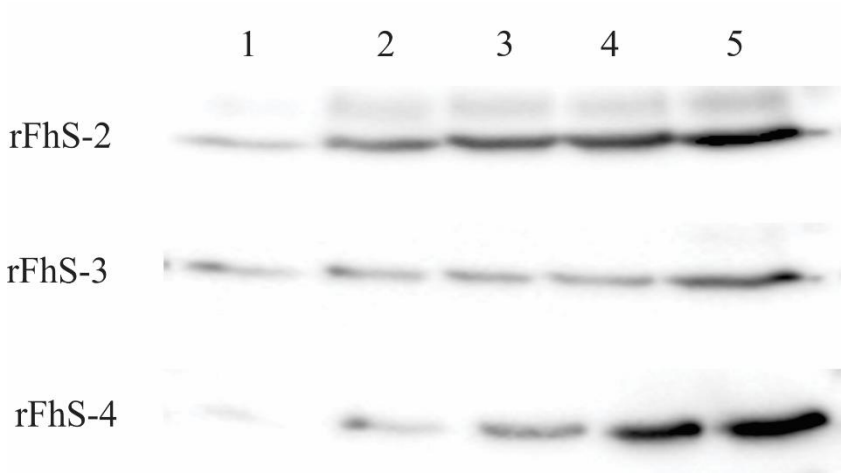
Identification peptides for lower band in purified **rFhS-2**: <43 kDa

```
001 MTSSMEHSLK SFCDKLYGEA ILSQKGNYEN VFLSPMSLYS VMAMVLAGGE GETKEQMLTA 060
061 LQLNRTLGRD ALHNSIGSAV RVCLKSSPGV TVSFGNRIYA QHDASILPQY KAIVLGDYDA 120
121 DVENVDFTKT EVARKDINQW VSEKTKKKIR ELIPAGVLKP DTCVAIINAL YFKGSWEIEF 180
181 PKEATTKDKF HLLDGGRKDV FMMYKESEFH STVLAELDSV AVKLPFRQSK WEMFVIVPNK 240
241 KDGLKSLLPK LQSEGLTKAL SASFTKQTTG VFLPRFKLTE STVDAKELLT KLGMSSVFSR 300
301 TTADLSKMCS SRSLFISDIK HKAILEVDEE GAEAAAASTA TVVFMCAAIP EIRVKADHPF 360
361 VVALVYDDKI PIFVGHVTDP EVN
```

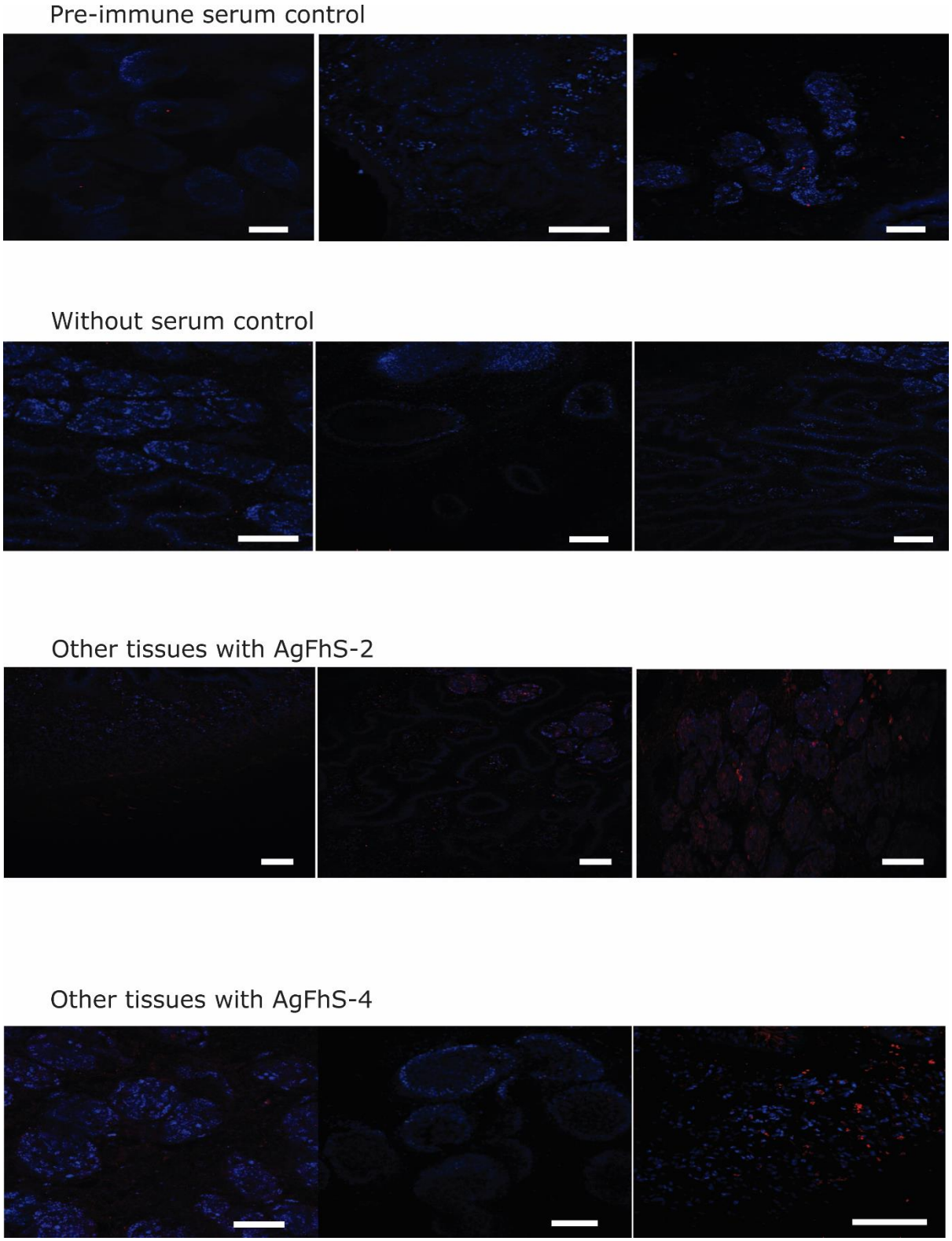
Identification peptides for **rFhS-3**:

```
001 MTSSMEHSLK SFCDKLYGEA ILSQKGNYEN VFLSPMSLYS VMAMVLAGGE GETKEQMLTA 060
061 LQLNRTLGRD ALHNSIGSAV RVCLKSSPGV TVSFGNRIYA QHDASILPQY KAIVLGDYDA 120
121 DVENVDFTKT EAARKDINQW VSEKTKEKIR ELIPSGVLGP DTCVAIINAL YFKGSWEIEF 180
181 PKEATTKDKF HLLDGGRKEV FMMYKESEFH STVLAELDSV AVKLPFRQSK WEMFVIVPNK 240
241 KDGLKSLLPK LQSEGLTKAL SASFTKQTTG VFLPRFKLTE STVDAKELLT KLGMSSVFSR 300
301 TTADLSKMCS SRSLFISDIK HKAILEVDEE GAEAVAASAA IAVPMCLVIP EIRVKADHPF 360
361 VVALVYDDKI PIFVGHVTDP EV
```

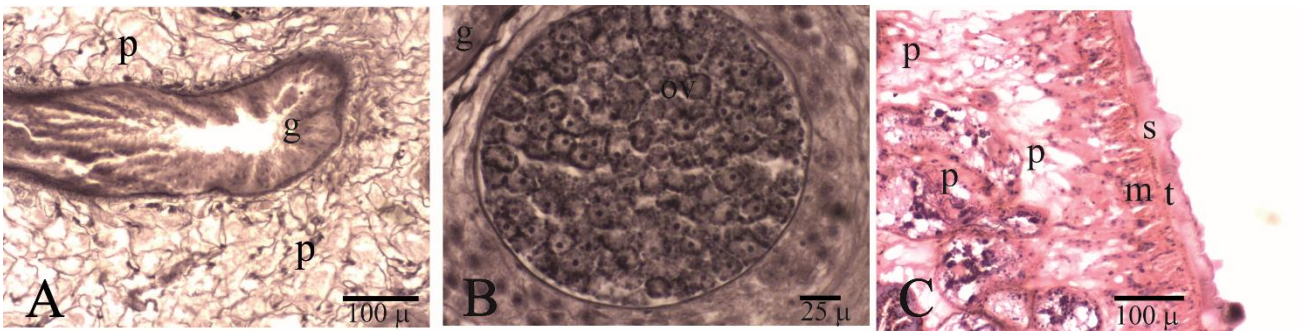
Supplementary Figure S4. FhS-2 and FhS-3 mass spectrometry analysis. Peptides identified within each serpin sequence are shown in blue. The RCL region of each serpin is boxed.



Supplementary Figure S5. Heterologous expression of recombinant *F. hepatica* serpins. Genes encoding FhS were cloned into pPICZαC and used to transform X-33 *P. pastoris* cells. ZeocinTM-resistant clones were selected and one clone was chosen for further experiments. To determine the optimal induction time for rFhS expression, cells were grown and induced with methanol for 1, 2, 3, 4 and 5 days (lanes 1 to 5). Cells were harvested and lysed. rFhSs were purified using Hi-Trap FF columns and eluted with increasing concentrations of imidazole. Samples were analyzed on a 12% SDS-PAGE and stained with Coomassie blue.



Supplementary Figure S6. Experimental controls for rFhS-2 and rFhS-4 subcellular localisation in *F. hepatica* adults. Immunolocalisation of rFhS-2 and rFhS-4 on longitudinal sections of adult flukes using rabbit anti-rFhS-2 and anti-rFhS-4 antibodies. DAPI-stained nuclei are shown in blue.



Supplementary Figure S7. Experimental controls for rFhS-2 and rFhS-4 subcellular localisation in *F. hepatica* adults. Hematoxylin-eosin (HE) preparation of adult *F. hepatica* tissues. Images were captured using a 10x and 20× objective. Sections of adult *F. hepatica* parasites showing (A) gut, (B) ovary and (C) tegument structures. g: gut, p: parenchyma, ov: ovary, t: tegument, m: muscle. Black bars indicate the scale bar for each panel.

6 DISCUSION

A pesar del extenso conocimiento sobre el papel de las serpinas en los eucariotas superiores, poco se sabe sobre su función en parásitos. El esfuerzo de investigación en serpinas de parásitos está centrada en los vectores de parásitos, como garrapatas (Tirloni et al., 2016) y mosquitos (Gulley et al., 2013), y parásitos que afectan a los humanos, como nematodos (Zang et al., 1999) o esquistosomas (Dvorak et al., 2008). En este trabajo, se describe, por primera vez, la identificación y caracterización parcial de cuatro serpinas del parásito helminto *Fasciola hepatica*. Estas serpinas pertenecen a la familia de inhibidores I4 de acuerdo con la base de datos MEROPS para inhibidores y peptidasas (Rawlings et al., 2016); los inhibidores de esta familia pueden inhibir a miembros de las familias de proteasas S1 (Silverman et al., 2001), S8 (Dufour et al., 2005), C1 (Al-Khunaizi et al., 2002; Irving et al., 2002) y C14 (Komiyama et al., 1994).

Del genoma publicado por Cwiklinsk *et al* en 2015 encontramos 7 secuencias con características de serpinas (número de secuencias: BN1106_s3864B000104, BN1106_s122B000261, BN1106_s1727B000096, BN1106_s284B000286, BN1106_s4565B000032, BN1106_s4565B000033, BN1106_s4618B000050). De estos, la secuencia BN1106_s3864B000104 corresponde con FhS-1, BN1106_s122B000261 se corresponde con FhS-2 y FhS-3 y BN1106_s4565B000033 con FhS-4. Una característica común en la superfamilia de las serpinas (Gettins, 2002) es que la secuencia de aminoácidos tiene baja identidad con otras serpinas de helmintos parásitos (alrededor de un 30% de identidad). La inferencia sobre la identidad de las secuencias se basa en las características comunes presentadas en los integrantes de la superfamilia de serpinas, como el RCL, los motivos serpina, la longitud del polipéptido y la masa molecular esperada (Figura suplementaria 1 y Tabla suplementaria 2). El análisis de las secuencias de serpina de *F. hepatica* mostró que ninguna de las cuatro secuencias contiene péptido señal o

dominios transmembrana, lo que sugiere que serían serpinas citosólicas (Tabla suplementaria 2). En un trabajo en paralelo realizado durante el doctorado que trata sobre el análisis proteómico de los productos de excreción/secreción (PES) en los estadios intra-mamífero de estos parásitos (Di Maggio et al., 2016), se encontraron péptidos que sugieren la presencia de FhS-1 y FhS-2/FhS-3 en los PES de adulto, FhS-1 se encontró en los PES de NEJ y FhS-1 y FhS-2/FhS-3 se encontraron en las proteínas solubles de los NEJ. Como estas serpinas no tienen péptido señal y no fueron encontradas dentro de los exosomas (Cwiklinski et al., 2015b), su secreción en los PES podría depender de otro tipo de vesículas o por una vía de secreción no clásica.

En todas las serpinas inhibitorias, el RCL está expuesto y es accesible a las proteasas blanco (van Gent et al., 2003) y, como pudimos ver a partir de la estructura terciaria de estas serpinas de *F. hepatica*, el RCL está expuesto, lo que las convierte en serpinas de clase inhibitoria (Figura suplementaria 2A-D). Para caracterizar mejor las 4 serpinas de *F. hepatica* encontradas a partir de las bases de datos, las serpinas recombinantes se expresaron en un sistema de expresión eucariota. De las 4 serpinas de *F. hepatica*, 3 de ellas se expresaron con éxito en el sistema de expresión de levadura (Figura suplementaria 5). rFhS-2 inhibe a la serino-proteasa catepsina G y rFhS-4 inhibe serino-proteasas de tipo quimotripsina como la catepsina G y la quimotripsina (Figuras 3 y 4 del manuscrito). En cuanto a rFhS-3, a pesar que esta serpina recombinante fue expresada con éxito en el sistema eucariota, no presentó actividad inhibitoria para las serino-proteasas utilizadas en este trabajo (Figura 3B del manuscrito). Teniendo en cuenta que FhS-2 y FhS-3 tienen una similitud del 96% en los aminoácidos de la región del RCL (Figura suplementaria 3 y Figura 1 del manuscrito), se esperaba observar diferencias en la actividad inhibitoria de ambos inhibidores. Como la región del RCL es crucial para la función inhibitoria de las serpinas, ya que experimenta un cambio conformacional cuando se cliva el enlace

escindible y esto altera la topología del complejo (Irving et al., 2000). Se sabe que los cambios en aminoácidos dentro de esta región pueden afectar la actividad no solo al hacer que la serpina no sea inhibitoria, sino que también puede modificar el perfil inhibitorio. Estos fenómenos ya fueron bien estudiados en la serpina alfa 1-antitripsina donde las sustituciones en el sitio P_2 cambia la eficiencia de la inhibición y hasta podrían cambiar la especificidad de la serino-proteasa blanco (Chung et al., 2017). En el caso de la serpina kallistatina, substituciones de aminoácidos en los sitios P_1 a P_3 podrían cambiar la especificidad de la inhibición (Chen et al., 2000). Aparte de eso, los cambios en los aminoácidos podrían alterar la conformación tridimensional del bucle y esto podría alterar el ángulo de inserción del RCL en la hélice α después de ser hidrolizado, modificando la inhibición (Irving et al., 2000). Estudiando en detalle la región del RCL (Figura 1 del manuscrito) se puede observar diferencias entre estas dos serpinas de *F. hepatica*. En la región del aminoácido P_{11}, vemos que en la FhS-3 hay una valina mientras que en la FhS-2 y en la alfa 1-antitripsina hay una alanina. Además, estas dos serpinas de *F. hepatica* presentan grandes diferencias en los aminoácidos de la región P_7-P_3´ (Figura 1 del manuscrito). Estas diferencias entre aminoácidos pueden generar que las secuencias se vuelvan rígidas, impidiendo que ocurra el cambio conformacional necesario para que la proteasa sea inhibida. Esto fue estudiado con la serpina alfa 1-antitripsina, donde fueron generados varios clones con el fin de observar que aminoácidos aumentan la estabilidad del inhibidor (Im et al., 2004). Este trabajo en alfa 1-antitripsina demostró que la existencia de cavidades y regiones saturadas de aminoácidos que permiten la movilidad necesaria en las serpinas inhibitorias. Siendo que las mudanzas en aminoácidos son más importantes en la región que están involucradas en la inhibición, como es la región del RCL y donde están concentradas la mayoría de las diferencias entre FhS-2 y FhS-3 (Figura suplementaria 3).

Se sabe que los cambios en los aminoácidos dentro de la región del RCL pueden afectar la actividad inhibitoria, ya sea cambiando el perfil inhibitorio o volviéndola no inhibitoria. Este fenómeno está bien estudiado en la alfa-1-antitripsina, donde las sustituciones en el P_2 cambian la eficacia de la inhibición y podrían cambiar la especificidad de la serina proteasa (Chung et al., 2017; Irving et al., 2002) y en la calistatina, las sustituciones en los residuos P_1 a P_3 también podrían cambiar la especificidad de inhibición (Chen et al., 2000). Aparte de eso, los cambios en los aminoácidos o en la longitud de RCL podrían alterar la estructura terciaria del bucle y alterar el ángulo en el que el RCL se inserta en la hélice α después de ser hidrolizado (Irving et al., 2000; Zou et al., 1994). Los estudios con serpinas mutantes han demostrado que las modificaciones en la región del RCL reasignan a las proteasas blanco y/o cambian la cinética de interacción serpina-proteasa. Esto se estudió bien con el inhibidor del activador del plasminógeno-1 (PAI-1) y la alfa-1-antitripsina (Dufour et al., 2005; Lawrence et al., 2000). Las diferencias de inhibición de proteasas entre FhS-2 y FhS-3 (Figura 4 del manuscrito) podrían deberse a los cambio de aminoácidos en las regiones alrededor del enlace escindible, particularmente en la región P_{14}-P_9 y la región P_7-P_1, respectivamente. Por ejemplo, en la posición P_7 FhS-2 tiene treonina (hidrofílica) mientras que FhS-3 tiene alanina (alifática) y en la posición P_2 FhS-2 tiene fenilalanina (aromática) mientras que FhS-3 tiene prolina (alifática). La importancia de la prolina en la estructura secundaria de las proteínas es bien conocida. La presencia de prolina en una cadena de polipéptidos actúa como un disruptor estructural de elementos de estructura secundaria como hélice α, hojas β y giros β debido a la excepcional rigidez conformacional de la prolina (Morris et al., 1992; Petersen et al., 2010). La prolina en el sitio P_2 de la FhS-3 genera un β-turn predictivo y eso podría explicar la ausencia de actividad inhibitoria contra las proteasas probadas en este trabajo (Figura 1 del manuscrito, el sitio de giro β predictivo está marcado con un asterisco). Todos estos aminoácidos tienen

una ubicación espacial diferente y eso no solo podría reducir el enlace/asociación entre el inhibidor y la proteasa, sino también impedir los cambios conformacionales que son cruciales para la inhibición (Im et al., 2004).

Se sabe que en la región bisagra, los residuos P_{15} a P_9, proporcionan movilidad que es esencial para el cambio conformacional del RCL en la transición S → R (Gettins, 2002; Irving et al., 2000). Esta transición es importante para realizar la función inhibitoria de las serpinas donde ocurre la formación de un complejo estable entre la forma escindida del inhibidor y la proteasa, análoga a un complejo enzima-producto (Gettins, 2002). De acuerdo con eso, la comparación entre la región bisagra de los FhSs con A1AT (Figura 1 del manuscrito) denota aminoácidos conservados, lo que sugiere que las serpinas expresadas son capaces de formar complejos estables con sus proteasas diana como se muestra para rFhS-4 (Figura 5 del manuscrito) y rFhS-2 (datos no mostrados).

El análisis *in silico* sugiere la presencia de sitios de glicosilación en los polipéptidos que fue confirmado experimentalmente (Figura 5B del manuscrito), lo que indica que las serpinas nativas sufren modificaciones postraduccionales cuando son expresadas en un sistema eucariota. Estas modificaciones postraduccionales ayudan a que las serpinas se vuelvan más estables y/o como protección contra la degradación, pero esto no significa cambios en la actividad inhibitoria, como se demostró para la alfa 1-antitpsina (Sarkar and Wintrode, 2011). En el caso de la antitrombina, las dos isoformas α y β difieren en su patrón de glicosilación en la posición Asn^{135}, y ambas isoformas presentan diferente afinidad, localización y función del sustrato (McCoy et al., 2003; Pol-Fachin et al., 2011).

La Figura suplementaria 2E-H muestra una representación de las cargas superficiales de FhS. Las cuatro proteínas muestran diferentes distribuciones de carga, incluso FhS-2 y FhS-3 que son altamente similares. Solo FhS-3 y FhS-4 muestran un parche básico prominente (Figura suplementar 2G y 2H). Una característica importante de las serpinas es

que tienen la capacidad de unirse a varios ligandos y rFhS-2 y rFhS-4 se unen a la heparina con alta afinidad (Figura 6 del manuscrito). El alto punto isoeléctrico del FhS-4 (Tabla suplementaria 2) no es una característica común en esta superfamilia, los únicos otros miembros que muestran pI similares son una serpina de células B y otra de células sanguíneas de vertebrados (Grigoryev et al., 1999; Paterson et al., 2007). Los parches básicos presentes en los Fh-Ss (Figura suplementaria 2E-H) sugieren la posibilidad de unión a entidades cargadas negativamente como GAG y ADN, en este trabajo pudimos demostrar que rFhS-4 tiene la capacidad de unir heparina con fuerte afinidad (Figura 6 del manuscrito). Se sabe que la heparina puede actuar como un cofactor de la función de las serpinas que puede acelerar y / o mejorar la inhibición de las proteasas. Como sucede con la alfa-1-antitripsina, en presencia de heparina, la afinidad por algunas serino-proteasas aumenta hasta 48 veces (Huang et al., 2011; Khan et al., 2011).

Una forma de determinar la eficacia de la inhibición de las serpinas con diferentes serino-proteasas es mediante las constantes de inhibiciones (Horvath et al., 2011). La serpina inhibitoria típica forma un complejo covalente con su proteasa blanco y este complejo que se torna resistente al SDS y a la desnaturalización térmica, tiene una estequiometría de inhibición cercana a 1 y una constante de asociación (k_a) de 10^5 M^{-1} s^{-1}. Cuando el valor de SI se aproxima a 1 significa que la ruta inhibitoria avanza más rápido que la ruta del sustrato y las serpinas tienen una relación molar de 1:1 con la proteasa. Los valores de SI y las constantes de segundo orden (K_{obs} y k_a) muestran que rFhS-4 interactúa con sus proteasas blanco (catepsina G y quimotripsina) con diferentes constantes de asociación; mostrando un mayor efecto inhibitorio sobre la catepsina G ya que el SI es de 1.3 y de 2.4 para la quimotripsina (Figura 4 del manuscrito). Los resultados muestran que rFhS-4 parece ser un mejor inhibidor de la catepsina G, en comparación con rFhS-2, con

una k_a de 2.55 x 10^3 $M^{-1}s^{-1}$ (Fig. 11D-F). El valor de ka para rFhS-4 y quimotripsina muestra que este es un inhibidor rápido con un k_a de 3.8 x 10^3 M^{-1} s^{-1}.

El experimento de formación de complejos de esta serpina, rFhS-4, con la quimotripsina muestra complejos de alto peso molecular a altas relaciones molares (2.5:1, 5:1 y 10:1, Figura 5 del manuscrito). Para la formación de complejos entre la catepsina G y rFhS-4, el complejo aparece visible en la relación molar de 2: 1 (Figura 5 del manuscrito). Ambos ensayos de formación de complejos (para quimotripsina y catepsina G) mostraron bandas en pesos moleculares entre la banda de serpina y de la proteasa, estas bandas podrían ser producto de la degradación de la serpina durante el ensayo.

La localización de las serpinas en los tejidos del adulto de *F. hepatica* proporciona algunas ideas sobre las posibles funciones biológicas de estas proteínas en la *F. hepatica* adulta. Se detectó señal del anticuerpo anti-FhS-2 en la región del ovario y las espinas del tegumento (Figura 7 del manuscrito). El ovario en estos parásitos posee ramificaciones y contiene células más pequeñas en el exterior y células grandes dentro (ovocitos); claramente el anticuerpo antiFhS-2 se localiza en la región citoplásmica de los ovocitos (Figura 7A-C del manuscrito). Los ovocitos maduros son células ricas en gránulos electrondensos que están en contacto con la membrana citoplásmica. La función de los gránulos es desconocida hasta la fecha. Como rFhS-2 inhibe la catepsina G, una serina proteasas almacenada en gránulos citoplásmicos de neutrófilos, podría estar involucrada en la relación parásito-huésped como protección del huevo contra el sistema inmunológico del huésped. Además, esta serpina forma parte de los PES de los estadíos intra-mamífero (NEJ y adulto) del parásito, lo que sugiere que se secreta o excreta del parásito adulto por alguna vía de secreción alternativa o por vesiculas (Di Maggio et al., 2016). La señal del anticuerpo anti-rFhS-4 fue encontrado en las células que forman el intestino de la fasciola adulta, pero aparentemente no está presente en el lumen (Figura. 7D-F del manuscrito).

Sorprendentemente, este inhibidor no ha sido encontrado en el análisis de los PES de estos parásitos (Di Maggio et al., 2016; Robinson et al., 2009; Wilson et al., 2011) lo que sugiere que podría estar involucrado en la asimilación de nutrientes dentro del intestino o poseer otras funciones relacionadas con la regulación de las proteasas parasitarias. Ambos anticuerpos, anti-rFhS-2 y anti-rFhS-4, señalizan en el tegumento de la fasciola adulta, el anticuerpo anti-FhS-2 presenta señal en la región apical de las espinas (Figura 7H del manuscrito) y el anticuerpo anti-FhS-4 se observa a lo largo del borde en la región apical (Figura 7I-J del manuscrito). Las espinas son estructuras rígidas que ayudan al parásito en la penetración y la unión a los tejidos del huésped. Su composición no está bien resuelta como en otros parásitos digeneos, como por ejemplo en *S. mansoni*, donde el componente principal de las espinas es la actina (Pearson et al., 1985).

8. CONCLUSIONES Y PERSPECTIVAS

Teniendo en cuenta todos los datos presentados en este trabajo, se identificaron cuatro serpinas del trematodo hepático *F. hepatica* y tres de ellas fueron caracterizadas bioquímicamente. El papel de estas proteínas en *F. hepatica* aún no se comprende, pero la predicción de especificidad basada en el RCL podría conducir a hipótesis comprobables sobre sus funciones. De las tres proteínas expresadas, una de ellas (rFhS-3) no presentó actividad inhibitoria hacia las serinas proteasas utilizadas. rFhS-2 inhibe catepsina G y rFhS-4 inhibe catepsina G y quimotripsina *in vitro*. Debido a la similitud en la secuencia de aminoácidos entre FhS-2 y FhS-3, estos dos inhibidores podrían generarse a partir de *splicing* alternativo de exones que codifican la región del RCL que es el lugar en donde están concentradas las diferencias y se conocen casos de serpinas en donde esto ocurre. La localización especifica en los tejidos del gusano adulto y las propiedades bioquímicas de FhS-2 y Fhs-4 podrían sugerir que forman parte de los mecanismos que utiliza el parásito para evadir el sistema inmunitario del huésped, no solo para que la supervivencia del gusano adulto sino también para que la entrega segura de los huevos y asegurar la continuidad del ciclo vital

De este trabajo a pesar de responder nuestros objetivos iniciales surgen nuevas preguntas que podrían ser respondidas en posibles futuros experimentos:

Confirmamos que de las 3 serpinas que conseguimos expresar 2 de ellas inhiben serino-proteasas, pero ¿podrían también inhibir catepsinas? Si tenemos en cuenta que FhS-2 es secretada en los PES donde hay muy pocas serino-proteasas y muchas catepsinas y sabemos que existen inhibidores de serino proteasa que inhiben catepsinas. Esto podría conformarse realizando los ensayos de inhibición con catepsinas en lugar de serino-proteasas.

Sabiendo que FhS-2 y FhS-4 se unen a heparina in vitro se podría confirmar si esto puede influir positiva o negativamente en la reacción de inhibición. Además, se podría analizar si

la presencia de un cofactor aumenta la inhibición de la rFhS-3 por alguna de las serino-proteasas usadas en este trabajo o por cisteino-protesasas. Nuevamente realizando ensayos de inihibicion in vitro pero agregando heparina como co-factor.

De los ensayos de inmunohistoquímica observamos que FhS-2 está presente en los ovarios de la *F.hepatica* adulta, y que estos poseen gránulos lo que nos lleva a pensar si es ahí donde se acumula el inhibidor. Para intentar determinas esto se podría realizar una inmuno microscopia electrónica ya que poseemos anticuerpos para nuestros inhibidores.

En *F. hepatica* no se ha demostrado que el bloqueo de la función de los neutrófilos pueda interferir con la trombosis in vivo, que normalmente está mediada por anticoagulantes, inhibidores de la agregación plaquetaria y vasodilatadores, que actúan directamente sobre el sistema hemostático. Con base en la bibliografía, las serpinas forman parte del control de la actividad de los neutrófilos (que expresan catepsina G y elastasa) en los mamíferos, y con la evidencia que rFhS-2 y rFhS-4 inhiben catepsina G de neutrófilos humanos, esto sugiere que estas proteínas podrían tener un papel en el proceso de modulación de neutrófilos, que actúan como mediadores anti-inflamatorios durante el parasitismo en el huésped bovino. Por lo tanto, sugerimos que las serpinas de *F. hepatica* podrían desempeñar un papel anticoagulante mediante la modulación de la actividad procoagulante de los neutrófilos. Para obtener el perfil completo del efecto anti-inflamatorio y anticoagulante/antitrombótico de la serpina rFhSRP4 se pueden realizar experimentos in vivo en ratas como: medición del edema de la pata por pletismometría; medición de la permeabilidad vascular y medición del tiempo de sangrado caudal.

9. ANEXOS

ANEXO 1 – Diseño de primers para clonar las 3 secuencias codificantes de serpinas en *Pichia pastoris.*

Primers para expresión de Fhserpin01 en el vector pPICZα C

Secuencia de cDNA:

5’ATGGAGAAGTCACTTTTGAAGTTTTCACAGGAACTGTATGGCTCGACAGTCAGTGA
CCAAAAAAAAAAATACCTTCCATAATACTTGTTTATGCCCATACAGTGTTTACACGGCT
CTGTCATCAACTCTCTGTGGCTGTGATGGGGAGACAAAGAAGCAGTTGGCGAATGCT
CTACACCTTCCTGTAACTGGTATCTGCAAAGACACTGCCAGTACTTTGAAAAAACTA
ATTTCATGCGCGTCTGAGGTTGAGATCTCGTCGGCTAACAAGATATTCGTGGAAAAC
AGTGCCCATATTCATCAAAGTTTCATCAACGAAGTCAAAGAGCTATTTGAGTCCGAA
CCAAAGAATGTTGATTTTGCTAAGAATCCAGATAACGCTCGAAAGGAAATGAATCAA
TGGGTCTCAAGCGCCACCCACGACAAAATAAAGGAGCTGTTCTCGCCGGGCTCGGTC
AGCTGCAACACTAGACTGGTGCTGGGAAACGCTGTGTACTTCAAAGGTGCTTGGGAA
ACTCCTTTCAATCCAACTGATACGTTTCAGGGTCAGTTTCACAAGCTTGGTGGTGATA
CTTGTCCCGTGAAAATGATGAGGCGGAATGGGAACTTTAACATTGAGGAGGAAATTT
TGGACGGGGTTAATGCCCTTAAGTTACCGTTCAAGGATACCAGATACGAACTATTGA
TCATATTGCCCGAAAACAATGAGCAATTTCCCGCTCTTGTCAAAACAATAAGTGAAA
CAGACAAGTTAGAAAGAATTTTGGATGCACCATTCCACAGCCAGATGGCACGTGTTA
GAGTACCTCGCTTCAAATTGGCCATGACTCCTAGCTTGGCGCTAAAAGATACACTGA
AAGAGATGGGCATCACCAGGCTTTTTGGTGATGCAGATTTGAGAAAAATTGCCGATG
AACCGCTTTTCGTTTCTGATGTGGTTCATCAAGCCGTGCTTGAGGTGAACGAGGCGG
GTGCTGTTGCTTCTGCCGCTTCGGGCGTGTGTGTTTCTAATCGGGCAATGCTTCAACC
GATCGAATTTTGCGCGGACCACGCTTTTGTAGTCGCGGTGGTCGTTGACAAGAAGGT
GCCACTGTTCATTGGTCACGTAACATCGGCTGAAGAGTAG -3

Enzimas que no cortan dentro de la secuencia:

AarI, AatII, AccI, Acc65I, AclI, AfeI, AgeI, AhdI, AleI, AloI, AloI, AlwI, AlwNI, ApaI, ApaLI, AscI, AseI, AsiSI, AvaI, AvrII, BaeI, BaeI, BamHI, BbeI, BbsI, BbvCI, BfrBI, BglI, BlpI, BmgBI, BmrI, BmtI, BpmI, Bpu10I, BsaHI, BsaWI, BsaXI, BsaXI, BsgI, BsiHKAI, BsiWI, BsmBI, BspEI, BspHI, BspMI, BsrFI, BsrGI, BssHII, BssSI, BstAPI, BstBI, BstEII, BstXI, BstZ17I, Bsu36I, BtgI, Cac8I, ClaI, DraI, DraIII, DrdI, Eco57I, EcoICRI, Eco57MI, EcoNI, EcoO109I, EcoRI, EcoRV, FseI, FspI, FspAI, HpaI, KasI, KpnI, MboII, MfeI, MluI, MmeI, MslI, NaeI, NarI, NcoI, NdeI, NgoMIV, NheI, NotI, NruI, NsiI, NspI, PacI, PciI, PflMI, PmeI, PpiI, PpiI, PpuMI, PshAI, PsiI, PspOMI, PsrI, PsrI, PstI, RsrII, SacI, SacII, SalI, SanDI, SapI, SbfI, SexAI, SfiI, SfoI, SgrAI, SmaI, SnaBI, SpeI, SphI, SrfI, SspI, StuI, StyI, SwaI, TspGWI,Tth111I, XbaI, XcmI, XhoI, XmaI, XmnI, ZraI

Primer Fw: en este caso NO incluyo el primer ATG → el AT esta incluido en la secuencia de la enzima de restricción ClaI y luego agrego un G para entrar en frame con la secuencia.

5' GAG AAG TCA CTT TTG AAG TTT TCA CAG G **3'** Tm = 56,4°C – GC: 39.3% →

5' **ATCGAT** GAGAAGTCACTT TTGAAGTTTTCACAGG **3'** → agrego sitio de clivado de ER (ClaI)

5' **ATCGAT G** GAGAAGTCACTTTTGAAGTTTTCACAGG **3'** → agrego una **G** para que la secuencia quede en marco de lectura

5' **AAAAA ATCGAT G** GAGAAGTCACTTTTGAAGTTTTCACAGG **3'** → agrego bases extras luego de la ER para reconocimiento.

AAAAAATCGATG GAGAAGTCACTTTTGAAGTTTTCACAGG → Primer Fw de Fhserpin01 para pPICZαC

Primer Rv

5' CGTAACATCGGCTGAAGAG**TAG** 3' → Tm = 55.0 – GC: 50%

5' CGTAACATCGGCTGAAGAG **cac cac cac cac cac cac TAG** **3'** → cola con 6 histidinas

5' CGTAACATCGGCTGAAGAG **cac cac cac cac cac cac TAG** GCGGCCGC **3'** → sitio de clivado de la ER (NotI)

5' CGTAACATCGGCTGAAGAG **cac cac cac cac cac cac TAG** GCGGCCGC **AAA** **3'** → agregado de bases

TTTGCGGCCGCCTAGTGGTGGTGGTGGTGGTGCTCTTCAGCCGATGTTACG → reversa y complementaria – Primer Rv de FhS-1 para pPICZαC

Sitios de clivado de las enzimas de restricción:

ClaI:

5′...AT▼CGAT...3′
3′...TAGC▲TA...5′

NotI:

5′...GC▼GGCCGC...3′
3′...CGCCGG▲CG...5′

Primers para expresión de FhS-2 y FhS-3 en el vector pPICZα C

FhS-2 – usada como molde ya que las secuencias N- y C-terminal de las FhS-2 y FhS-3 son idênticas.

ATGACCTCATCTATGGAACATTCCTTGAAGAGTTTTTGTGATAAATTGTATGGAGAAG
CTATATTGTCGCAGAAAGGAAATTATGAAAATGTTTTCCTGTCTCCGATGAGCTTGTA

CTCCGTGATGGCCATGGTTCTAGCTGGCGGTGAAGGAGAGACCAAAGAACAAATGCT
TACCGCATTACAGTTAAACCGTACACTTGGACGAGATGCACTTCACAATTCGATTGG
AAGTGCTGTTCGAGTTTGTTTGAAGTCATCGCCTGGAGTAACCGTGTCCTTTGGAAAT
CGGATATATGCACAGCACGATGCTAGTATACTCCCACAGTATAAAGCTATAGTTCTC
GGTGACTACGATGCGGATGTGGAAAATGTGGATTTCACTAAAACTGAAGTTGCACGT
AAAGATATCAATCAGTGGGTCAGCGAGAAAACAAAGAAAAAAATCCGGGAGTTAAT
TCCTGCTGGAGTTTTGAAGCCTGATACTTGTGTGGCCATCATTAACGCGTTGTATTTC
AAAGGTTCTTGGGAAATTGAATTCCCCAAAGAGGCCACAACAAAAGACAAATTTCAT
CTTCTTGATGGAGGTCGGAAGGACGTTTTCATGATGTATAAAGAAAGTGAATTTCAT
TCTACTGTTTTGGCTGAACTGGATTCAGTGGCCGTGAAGTTACCGTTCCGACAATCCA
AATGGGAAATGTTTGTCATTGTACCGAACAAGAAAGATGGTCTTAAGTCCTTGCTAC
CGAAACTACAATCAGAAGGACTTACAAAAGCACTAAGTGCATCGTTTACGAAGCAA
ACAACAGGTGTATTTCTACCACGCTTTAAGTTGACTGAAAGCACCGTGGATGCAAAG
GAATTGCTGACTAAGCTTGGTATGAGTTCGGTATTTTCGAGGACCACGGCGGATTTG
AGTAAGATGTGTTCTTCACGATCGTTGTTTATTTCGGATATAAAACACAAGGCAATCT
TGGAGGTTGACGAAGAAGGTGCTGAGGCTGCTGCTGCTTCGACTGCGACCGTGGTTT
TCATGTGTGCTGCTATTCCAGAGATTCGGGTGAAAGCAGATCACCCGTTCGTTGTGGC
TCTTGTTTATGATGACAAGATACCTATTTTCGTGGGACACGTTACTGATCCGGAGGTG
AACTAA

Enzimas que no cortan dentro de la secuencia:

AarI, AatII, Acc65I, AclI, AfeI, AgeI, AhdI, AleI, AlwNI, ApaI, ApaLI, AscI, AseI, AsiSI, AvaI, AvrII, BamHI, BanI, BanII, BbeI, BbsI, BciVI, BclI, BfrBI, BglI, BglII, BlpI, Bme1580I, BmgBI, BmrI, BmtI, BplI, BpuEI, BsaHI, BseRI, BseYI, BsgI, BsiHKAI, BsiWI, BsmI, BsmBI, Bsp1286I, BspMI, BsrBI, BsrDI, BsrFI, BsrGI, BssHII, BssSI, BstBI, BstEII, BstXI, BstYI, Bsu36I, BtsI, ClaI, DraI, DrdI, EagI, EcoICRI, EcoNI, EcoO109I, FauI, FseI, FspI, FspAI, HaeII, HgaI, Hin4I, Hin4I, HpaI, KasI, KpnI, MfeI, MlyI, MslI, MspA1I, NaeI, NarI, NdeI, NgoMIV, NheI, NlaIV, NotI, NruI, NsiI, NspI, PacI, PciI, PflMI, PleI, PmeI, PmlI, PpiI, PpiI, PpuMI, PshAI, PsiI, PspOMI, PstI, PvuII, RsrII, SacI, SacII, SalI, SanDI, SapI, SbfI, ScaI, SexAI, SfiI, SfoI, SgrAI, SmaI, SnaBI, SpeI, SphI, SrfI, SspI, StuI, SwaI, TaqII, TaqII, Tth111I, XbaI, XcmI, XhoI, XmaI, XmnI, ZraI

Primer Fw:

5’ ACC TCA TCT ATG GAA CAT TCC TTG AAG AG 3’ → Tm= 57,9°C , GC= 41,4%

5’ **ATCGAT** ACC TCA TCT ATG GAA CAT TCC TTG AAG AG 3’ → agrego sitio de clivado de ER (Clal)

5’ **ATCGAT G** ACC TCA TCT ATG GAA CAT TCC TTG AAG AG 3’ → agrego una **G** para que la secuencia quede en marco de lectura

5’ **AAAAA ATCGAT G** ACC TCA TCT ATG GAA CAT TCC TTG AAG AG 3’ → agrego bases extras luego de la ER para reconocimiento.

AAAAA ATCGATGACCTCATCTATGGAACATTCCTTGAAGAG → primer Fw FhS-2 y FhS-3 para pPICZαC

Primer Rv:

5’ CGT TAC TGA TCC GGA GGT GAA C TAA 3’ → Tm= 58,0 °C , GC=48 %

5’ CGT TAC TGA TCC GGA GGT GAA C **cac cac cac cac cac cac** TAA 3’ → cola de 6 His

5’ CGT TAC TGA TCC GGA GGT GAA C **cac cac cac cac cac cac** TAA GCGGCCGC 3’ → sitio de clivado de ER (Notl)

5’ CGT TAC TGA TCC GGA GGT GAA C **cac cac cac cac cac cac** TAA GCGGCCGC **AAA** 3’ → agregado de bases

TTTGCGGCCGCTTAGTGGTGGTGGTGGTGGTTCACCTCCGGATCAGTAACG
→reversa y complementaria - primer Rw de FhS-2 y FhS-3 para pPICZαC

Primers para expresión de FhS-4 en el vector pPICZα C

ATGTGCAAGTCGAAGGTGCCGGACATTGATGCGTTGTATGCGAACCAGCATCCACCG
GTTAGATTCACTCAGAATTTTTTGAGTACCACAGTCCGTGGACAAGGTGATGGCGAC
TATCTTTCTTGTCCCCTCGGAGTCCTATTTCTGCTCACCACTCTTTTGGGGTCTGGTGG
AGCACGCGGGAAAACTGCCACGCAGATCGCGAACACACTGAAGCTGACAAACACGG
TGCCCAGCTCGGATTTAAAGGCTTTACGGGAAAGCGGGAAAAATATGTATTGGCGTT
TGACTGAATCGTTGGTTGGCTCGGAATCAAATCGGAATCAGAAGAAAGTCCCGGTGG
TAACCATAAGTAACGCTGTTTTTGTGAAAAAGGATTATGATATCAAGCATGATTTCA
AGTTCAGTCTGGAGAGTGATTATCGTGCTAAACTGGAGAAGCTTGACTTCAGTGATC
ATAAGAATGCCGTGGAAACGATAAACAAGTGGATCAGAAATCGGACGCACGAAATG
ATTCCAAATTTCTTTCGCTCACCAAGTGAATTGCCGAAAGATGCAAAACTGGCCCTTG
TCAACGTGTTCACCTTCAAAGAGGAGTGGGAAGAGTCATTTCTGCCGGCTGCTACTG
AAACGGCCGATTTTTGGATCAAAAGCGGAAAGACCGTCAAAGTGCAAATGATGTCC
GATGTGCAACCATTGCCGTATGCTAGATTTTCGGATAAAGGATTTTCGTTGATCGAGA
AGCCTTTGGTGGGAAAGCGTTTCTCACTGGTTGTCCTTCTACCCAATCAAAGATGGGA
CATGAAAAAAGTGGACGAAGTCTTGAATGGTTTTTACCTTCTGAAAGATCTGGTTGA
TCAAGCAAGTGAGACTGCCGTGTCAATCAAGCTGCCACGATTTAAAATTGAAAGTCA
GTTGGATCTAATCCCATACTTGCGATCACTTGGGGTTACTGATTTGTTTGATCAAGGC
CTCGCAGATTTATCAGGTGTGACCGATTCCCATAAGTTATACGTTAACATGATGAAA
CAAGGCGCTGTGCTCAAAGTCAACGAGGCCGGAGTCGAGGCAACTGCCGCCACTGC
CATGATGGCTGTACCCATGTCCCTACTCGTTCCGAACGTGCAATTTCACGTGGATCAG
CCGTTTGTTTGTTTCATTTACGATCGCCATTTAAAAATGCCTTTGTATGCAGCACGTGT
GACCAATCCACGTGAACGCTTGTAA

Enzimas que no cortan dentro de la secuencia:

AarI, AatII, AccI, Acc65I, AclI, AfeI, AflII, AhdI, AleI, AloI, AloI, AlwNI, ApaI
ApaLI, AscI, AseI, AsiSI, AvaI, AvrII, BaeI, BaeI, BamHI, BanII, BbeI, BbsI, BbvCI, BciVI, BfrBI, BlpI, BmgBI, BmrI, BmtI, BplI, Bpu10I, BpuEI, BsaI, BsaBI, BsaXI, BsaXI, BsgI, BsiWI, BsmBI, BspEI, BspHI, BspMI, BsrBI, BsrGI, BssHII, BssSI, BstBI, BstZ17I, Bsu36I, ClaI, EciI, EcoICRI, EcoNI, EcoO109I, EcoRI, FseI, FspI, FspAI, KasI, KpnI, MfeI, MluI, MmeI, MscI, MspA1I, NarI, NcoI, NdeI, NheI, NotI, NsiI, NspI, PacI, PciI, PflMI, PmeI, PpiI, PpiI, PpuMI, PshAI, PsiI, PspOMI, PsrI, PsrI, PstI, PvuII, RsrII, SacI, SacII, SalI, SanDI, SapI, SbfI, ScaI, SexAI, SfcI, SfiI, SfoI, SgrAI, SmaI, SmlI, SnaBI, SpeI, SphI, SrfI, SspI, StyI, SwaI, TaqII, TatI, XbaI, XhoI, XmaI, ZraI

Primer Fw:

5’ TGC AAG TCG AAG GTG CCG GAC 3’ → Tm= 62.4°C , GC=61.9%

5’ **ATCGAT** TGC AAG TCG AAG GTG CCG GAC 3’ → agrego sitio de clivado de ER (ClaI)

5’ **ATCGAT G** TGC AAG TCG AAG GTG CCG GAC 3’ → agrego una **G** para que la secuencia quede en marco de lectura

5’ **AAAAA ATCGAT G** TGC AAG TCG AAG GTG CCG GAC 3’ → agrego bases extras luego de la ER para reconocimiento.

AAAAAATCGATGTGCAAGTCGAAGGTGCCGGAC → primer Fw FhS-4 para pPICZαC

Primer Rv:

5’ GTG ACC AAT CCA CGT GAA CGC TTG TAA 3’ → Tm= 62.4°C , GC=61.9%

5’ GTG ACC AAT CCA CGT GAA CGC TTG **cac cac cac cac cac cac** TAA 3’ → cola de 6 His

5’ GTG ACC AAT CCA CGT GAA CGC TTG **cac cac cac cac cac cac** TAA GCGGCCGC 3’ → sitio de clivado de ER (NotI)

5’ GTG ACC AAT CCA CGT GAA CGC TTG **cac cac cac cac cac cac** TAA GCGGCCGC **AAA** 3’ → agregado de bases

TTTGCGGCCGCTTAGTGGTGGTGGTGGTGGTGCAAGCGTTCACGTGGATTGGT CAC →reversa y complementaria - primer Rw de FhS-4 para pPICZαC

ANEXO 2 – Medios de cultivo, soluciones y protocolos generales

MEDIOS:

Low Salt LB (1 litro)

10 g	Triptona
5 g	NaCl
5 g	Extracto de levadura

Se llevó a un litro con agua destilada, se ajustó el pH a 7,5 y se esterilizó mediante autoclavado a 1 atm durante 20 min.
Para los medios solidos se agregó 1.5% agar.
Se utilizó Zeocina y se agregó a una concentración de 25 μg/ml.

YPD Yeast Extract Peptone Dextrose Medium (1 litro)

1%	Extrato de levadura
2%	Peptona
2%	Glucosa

Se llevó a un litro con agua destilada, se ajustó el pH a 7,5 y se esterilizó mediante autoclavado a 1 atm durante 20 min.
Para los medios solidos se agregó 2% agar.
Se utilizó Zeocina y se agregó a una concentración de 25 μg/ml.

BMGY (1 litro)

1%	Extracto de levadura
2%	Peptona

Se llevó a un litro con agua destilada y se esterilizó mediante autoclavado a 1 atm durante 20 min. Y se agregaron los siguientes compuestos autoclavados:

1%	Glicerol (10X)
1%	Fosfato de potasio 1M
1%	YNB (10)
0,1%	Biotina (500X)

BMMY (1 litro)

1%	Extracto de levadura
2%	Peptona

Se llevó a un litro con agua destilada y se esterilizó mediante autoclavado a 1 atm durante 20 min. Y se agregaron los siguientes compuestos autoclavados:

1%	Metanol (10X)
1%	Fosfato de potasio 1M
1%	YNB (10)
0,1%	Biotina (500X)

SOLUCIONES:

Soluciones utilizadas para la extracción y análisis de DNA

Solución I: Glucosa 50Mm, Cl 25mM (pH8); EDTA 10Mm (pH 8)

Solución II: NaOH 0,2N; SDS 1% (preparar en el momento)

Solución III: Acetato de K 5M; Acido Acético, H2O

Buffer Tris-acetato (TAE) 50X: 242 g de Tris base; 57,1 ml de ácido acético glacial; 10 ml EDTA 0,5M pH8 por litro.

Buffer de carga 6X para DNA: 30% glicerol; 0,25% azul de bromofenol; 0,25% azul de xilencianol

Soluciones utilizadas para la expresión y análisis de proteínas

SDS-PAGE:

Sample buffer (SB) 4X: 200 mM Tris-HCl pH 6,8; 400 mM DDT; 8% SDS; 0,4% Azul de bromofenol y 40% de glicerol.

Buffer de corrida Tris-glicina: 25 mM Tris, 250 mM glicina pH 8,3; 0,1% SDS.

Azul de Coomassie: 0,05% Coomasie brillant blue; 45% EtOH; 10% ácido acético.

Solución decolorante: 70% Ácido acético, 30% EtOH.

Western blot:

Buffer de transferencia: 25 mM Tris, 250 mM glicina pH 8,3; 0,1% SDS; 20% v/v EtOH.

PBS: 137 mM NaCl; 2,7 mM KCl; 10 mM Na_2HPO_4; 1,8 mM KH_2PO_4.

Solución de bloqueo: PBS, 5% leche en polvo descremada.

TRF (Buffer de revelación para Fosfatasa): 100 mM Tris- HCl pH 9,5; 100 mM NaCl; 5 mM $MgCl_2$.

Purificación por Columnas HisTrapTM FF®

Buffer de ligación: 100 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.4

Buffer de elusión (100 mM a 500 mM): 100 mM Tris-HCl, 0.5 M NaCl, 100 a 500 mM, imidazol pH 7.4

Purificación por Columnas HiTrap™ Protein G HP®

Buffer de ligación: $Na_2HPO_47H_2O$ 20 mM pH 7,0.

Buffer de elusión: Glicina-HCl 100mM pH 2,7.

Buffer de Neutralización: Tris-HCl 1M pH 9,0.

PROTOCOLOS COMPLEMENTARIOS Y DESCRIPCIONES GENERALES:

Cepa Bacteriana E. coli TOP10 (Invitrogen)

Esta cepa bacteriana es ideal para amplificar plásmido para su producción con alta eficiencia. Permite la replicación estable del plásmido con alto número de copias. El genotipo de las células TOP10 ofrece las siguientes características: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG. Debido a la mutación en el gen endA1 esta cepa es deficiente en endonucleasas lo que mejora la producción de DNA plasmídico. Además la mutación en el gen recA1 implica que son deficientes en recombinación, lo que aumenta la estabilidad del inserto en un plásmido. La mutación hsdR impide el clivaje del DNA por el sistema de endonucleasas EcoK. El gen lacZΔM15 en el epitoma F´permite la selección por color azul-blanco de los plásmidos recombinantes y el gen mcrA es importantes para la transformación eficiente del DNA metilado en los preparados genómicos. (One Shot® TOP10 Competent Cells, Invitrogen).

Protocolo de deglicosilacion:

Description of Enzymes Included in the Protein Deglycosylation Mix II

***O*-Glycosidase** (NEB #P0733), also known as Endo-α-N-Acetylgalactosaminidase, is a recombinant enzyme cloned from *Enterococcus faecalis* (1). It catalyzes the removal of core 1 and core 3 *O*-linked disaccharides from glycoproteins. The molecular weight is approximately 147 kDa.

PNGase F (Glycerol-free), Recombinant (NEB #P0709), also known as Peptide: N-glycosidase F, is cloned from *Elizabethkingia miricola* (formerly *Flavobacterium meningosepticum*) and expressed in *E. coli* (2). PNGase F (Glycerol-free), Recombinant is an amidase which cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins unless α(1-3) core fucosylated. The molecular weight is approximately 36 kDa.

α2-3,6,8,9 Neuraminidase A (NEB #P0722), also known as Sialidase A, is a recombinant enzyme cloned from *Arthrobacter ureafaciens* and expressed in *E. coli* (3). It catalyzes the hydrolysis of α2,3, α2,6, α2,8 and α2,9 linked N-acetylneuraminic acid residues from glycoproteins and oligosaccharides. The molecular weight is approximately 100 kDa.

β1-4 Galactosidase S (NEB #P0745), is a recombinant enzyme cloned from *Streptococcus pneumoniae* and expressed in *E. coli* (4). It is a highly specific exoglycosidase that catalyzes the hydrolysis of β1-4 linked galactose residues from oligosaccharides. The molecular weight is approximately 231 kDa.

β-N-Acetylhexosaminidase$_f$ (NEB# P0721), is a recombinant enzyme cloned from *Streptomyces plicatus* (5) and overexpressed in *E. coli* (6). It catalyzes the hydrolysis of terminal β-N-acetylgalactosamine and glucosamine residues from oligosaccharides. The molecular weight is approximately 100 kDa.

Protocol

1. **Non-Denaturing Reaction Conditions:**

When deglycosylating a native glycoprotein it is recommended that an aliquot of the glycoprotein is subjected to the denaturing protocol to provide a positive control for the fully deglycosylated protein. The non-denatured reaction can then be compared to the denatured reaction to determine the extent of reaction completion.

1. Dissolve 100 µg of glycoprotein into 40 µl H_2O.
2. To the native glycoprotein add 5 µl 10X Deglycosylation Mix Buffer 1.
3. Add 5 µl Protein Deglycosylation Mix II, mix gently.
4. Incubate reaction at 25°C (room temperature) for 30 minutes.
5. Transfer reaction to 37°C, incubate for 16 hours.
6. Analyze by method of choice.

Note: The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels.

Anexo 3 – Artículo Proteoma 1

SCIENTIFIC REPORTS

OPEN

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Across intra-mammalian stages of the liver fluke *Fasciola hepatica*: a proteomic study

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Fasciola hepatica **is the agent of fasciolosis, a foodborne zoonosis that affects livestock production and human health. Although flukicidal drugs are available, re-infection and expanding resistance to triclabendazole demand new control strategies. Understanding the molecular mechanisms underlying the complex interaction with the mammalian host could provide relevant clues, aiding the search for novel targets in diagnosis and control of fasciolosis. Parasite survival in the mammalian host is mediated by parasite compounds released during infection, known as excretory/secretory (E/S) products. E/S products are thought to protect parasites from host responses, allowing them to survive for a long period in the vertebrate host. This work provides in-depth proteomic analysis of *F. hepatica* intra-mammalian stages, and represents the largest number of proteins identified to date for this species. Functional classification revealed the presence of proteins involved in different biological processes, many of which represent original findings for this organism and are important for parasite survival within the host. These results could lead to a better comprehension of host-parasite relationships, and contribute to the development of drugs or vaccines against this parasite.**

Fasciolosis is a zoonotic foodborne disease caused mostly by the digenean trematode parasites *Fasciola hepatica* and *Fasciola gigantica*. *F. hepatica* has a worldwide distribution, while *F. gigantica* is found in tropical climates, with a much more focal distribution in parts of Africa and Asia, where these species overlap[1]. The disease causes significant economic losses in livestock production worldwide, also having increased relevance to human health in developing countries[1].

Current control relies mainly on the use of anthelmintic drugs, eradication of the intermediate host with molluscicides, as well as improving drainage systems to limit snails habitat[2]. Nevertheless, emerging resistance to anthelmintic drugs and the presence of xenobiotic residues in food and environment have stimulated the search for novel control methods. Immune control through the development of vaccines has emerged as a promising alternative; however, vaccines have to reach an appropriate level of efficacy to make them commercially viable[3]. Increasing efficacy is most likely to come through the discovery of additional and relevant vaccine antigens.

The definitive, mammalian host of *F. hepatica* is orally infected by metacercariae on plants. Newly excysted juveniles (NEJ) emerge in the duodenum and migrate to the liver. Following a period of blood feeding and growth in the liver, they move to the bile ducts, where they obtain blood by puncturing the duct wall, undergo maturation, and produce eggs[4]. Although adult flukes are reproductively active and the major responsible for the pathology in mammalian hosts, NEJ are the cause of significant damage to host tissues when migrating from the gut lumen to the bile ducts[4]. During migration and development, parasites encounter different host tissues and macromolecules, dynamic physicochemical microenvironments, and host responses such as blood coagulation, complement activation, in addition to other innate and acquired immune responses[5].

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A proteomic comparison of excretion/secretion products in *Fasciola hepatica* newly excysted juveniles (NEJ) derived from *Lymnaea viatrix* or *Pseudosuccinea columella*

Lucía Sánchez Di Maggio[a,b], Lucas Tirloni[a,c], Antônio F.M. Pinto[d], Jolene K. Diedrich[d], John R. Yates III[d], Carlos Carmona[b], Patricia Berasain[b,**], Itabajara da Silva Vaz Jr.[a,e,*]

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ARTICLE INFO

Keywords:
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ABSTRACT

The characteristics of parasitic infections are often tied to host behavior. Although most studies have investigated definitive hosts, intermediate hosts can also play a role in shaping the distribution and accumulation of parasites. This is particularly relevant in larval stages, where intermediate host's behavior could potentially interfere in the molecules secreted by the parasite into the next host during infection. To investigate this hypothesis, we used a proteomic approach to analyze excretion/secretion products (ESP) from *Fasciola hepatica* newly excysted juveniles (NEJ) derived from two intermediate host species, *Lymnaea viatrix* and *Pseudosuccinea columella*. The two analyzed proteomes showed differences in identity, abundance, and functional classification of the proteins. This observation could be due to differences in the biological cycle of the parasite in the host, environmental aspects, and/or host-dependent factors. Categories such as protein modification machinery, protease inhibitors, signal transduction, and cysteine-rich proteins showed different abundance between samples. More specifically, differences in abundance of individual proteins such as peptidyl-prolyl cis-trans isomerase, thioredoxin, cathepsin B, cathepsin L, and Kunitz-type inhibitors were identified. Based on the differences identified between NEJ ESP samples, we can conclude that the intermediate host is a factor influencing the proteomic profile of ESP in *F. hepatica*.

1. Introduction

Fasciola hepatica is the causative agent of fasciolosis, a zoonotic foodborne disease of livestock that causes major economic losses worldwide and, in developing countries, constitutes a threat to human health (Mas-Coma et al., 2005). The adult worm infects a broad range of hosts, including sheep, cattle, wild mammals, and humans. Depending on the definitive host species, fasciolosis has different progressions. The acute phase is present primarily in sheep and might lead to the death of the animal, while chronic fasciolosis is often asymptomatic in cattle (Behm and Sangster, 1999). The snails that serve as intermediate hosts in the parasitic cycle belong to the family Lymnaeidae, and the species vary according to geographic region (Bargues et al., 2001). In America, the most common species are *Pseudosuccinea* (*Lymnaea*) *columella* and *Lymnaea viatrix*, while *Galba truncatula* is predominant in Europe (Relf et al., 2011). However, nowadays these species can also be found in other regions of the globe (Boray, 1978; Caron et al., 2014). In the snail, which inhabits temporary streams bordered by aquatic vegetation and grass or stagnant waters, at the edges of lakes and ponds (Rondelaud et al., 2004), the miracidium completes the parasitic asexual phase and exits the host as a swimming cercaria, to later lose its tail and become a metacercaria. The definitive mammalian host acquires the infection by ingesting vegetation containing metacercariae. Newly excysted juveniles (NEJ) emerge in the duodenum and migrate to the liver and bile ducts, where they undergo maturation, and produce eggs (Mas-Coma et al., 2014). Immature eggs are discharged in the biliary ducts and pass in the stool; each egg releases a miracidium, which invades the snail. The number of ingested

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Anexo 5 Artículo 3

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Data in brief

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Data Article

Dataset supporting the proteomic differences found between excretion/secretion products from two isolates of *Fasciola hepatica* newly excysted juveniles (NEJ) derived from different snail hosts

Lucía Sánchez Di Maggio [a, b], Lucas Tirloni [a, c], Antônio F.M. Pinto [d], Jolene K. Diedrich [d], John R. Yates III [d], Carlos Carmona [b], Patricia Berasain [b, **], Itabajara da Silva Vaz Jr. [a, e, *]

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ABSTRACT

Here we present the proteomic profile datasets of two *Fasciola hepatica* NEJ isolates derived from different snail hosts: *Lymnaea viatrix* and *Pseudosuccinea columella*. The data used in the analysis are related to the article 'A proteomic comparison of excretion/secretion products in *Fasciola hepatica* newly excysted juveniles (NEJ) derived from *Lymnaea viatrix* or *Pseudosuccinea columella*' (Di Maggio et al., 2019)

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Anexo 6 Artículo enviado a la revista International Journal for Parásitology.

Title:

Serpins in *Fasciola hepatica*: insights into host–parasite interactions

Authors:

Lucía Sánchez Di Maggio[1,2], Lucas Tirloni[1,3], Marcelle Uhl[4], Carlos Carmona[2], Carlos Logullo[4], Albert Mulenga[3], Itabajara da Silva Vaz Jr[1,6*] & Patrícia Berasain[2,5*]

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Anexo 7. Trabajos presentados en congresos

2014: XVIII Congresso Brasileiro de Parásitologia Veterinária. Gramado, RS.

Identification and Characterization of Three Serpins in *Fasciola hepatica*.

Sánchez Di Maggio, L.[1,2]; Tirloni, L.[2]; Gambetta, D.[1]; Benavides, U.[4]; Carmona, C.[1]; da Silva Vaz Jr, I.[2,3]; Berasain, P.[1]

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INTRODUCTION: The liver fluke *Fasciola hepatica* is the causative agent of fascioliasis. It leads to significant economic losses in the livestock industries in addition to becoming an emerging pathogen for humans in many countries. Serpins are a superfamily of inhibitors regulating inflammation, complement activation, blood coagulation and fibrinolysis. Here we present sequence and *in silico* analysis of three *F. hepatica* serpins regarding their role in parasite-host relationship. **MATERIAL AND METHODS**: Serpin encoding sequences were retrieved from a public database and specific primers were designed. Total RNA were extracted from eggs, newly excysted juvenile (NEJ) and adults and cDNA were synthetized. Amplicons from PCR were purified and cloned into pGEM-T vector and identity confirmed by DNA sequencing. The deduced amino acid sequences were scanned against Genebank using the BLASTp algorithm. Alignment was performed using ClustalW algorithm in the MEGA6.06 program. *In silico* models for tertiary structure were built with Phyre2 and Swiss-Model softwares. **RESULTS**: It was demonstrated the existence of three serpin encoding genes (FhSPN1, FhSPN2 and FhSPN4) with 1125pb, 1205 pb and 1227pb long ORFs, respectively. FhSPN4 (409pb) has similarity with serpins from *Schistosoma japonicum* while FhSPN1 (375aa) and FhSPN2 (384aa) show similarity with sequences from *Clonorchis sinensis*. The 3D models obtained are consistently with the predicted tertiary structure for serpins. Peptide sequences compatible with FhSPN1 and FhSPN2 have been identified by proteomics assays for excretory/secretory products. **CONCLUSION**: According with the avaliable data, FhSPN1 y FhSPN2 are secreted during the NEJ and adult stage, respectively, suggesting a role of these proteins in parasite-host relationship. Expression of the recombinant proteins of these three serpins are under development for biochemical and functional characterization.

Key-words: Serpin, *Fasciola hepatica*, endopeptidase inhibitor

Acknowledgments: Dicyt-CNPq, CAPES-UDELAR, PEDECIBA, INCT-Entomologia Molecular, FAPERGS

IDENTIFICATION AND *IN SILICO* ANALYSIS OF 3 SERINE-PROTEASES INHIBITORS (SERPINS) IN *FASCIOLA HEPATICA.*

Sánchez Di Maggio, L.[1,2]; Tirloni, L.[2]; Gambetta, D.[1]; Benavides, U.[4]; Carmona, C.[1]; da Silva Vaz Jr, I.[2,3]; Berasain, P.[1]

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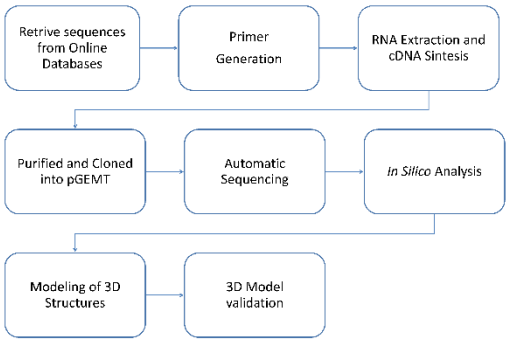
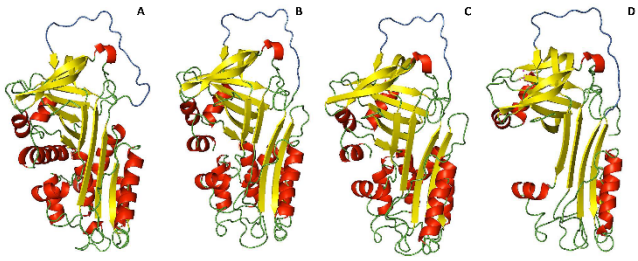
The liver fluke, *Fasciola hepatica*, is the causative agent of Fascioliasis. It leads to significant economic losses in the livestock industries in addition to becoming an emerging pathogen for humans in many countries. Serpins are a superfamily of proteins based on the presence of a single common core domain consisting of three β-sheets and 8-9 α-helices and most of them employ a unique suicide substrate-like inhibitory mechanism. In a previous report our research group discovered two serpins in *F. hepatica*. Now, we clone a third one that shows differences in the amino acid sequences only at the reactive center loop (RCL), and present it in silico analysis.

Materials & Methods



Serpin	Base Pairs	Amino acids	pI	Mw (kDa)	% Identity	Organism
FhSRP1	1125	374	5.97	41.4	36% - leukocyte elastase inhibitor	*Clonorchis sinensis*
FhSRP2.4	1152	383	6.73	42.3	40% - Serpin B	*C. sinensis*
FhSRP2.6	1152	382	6.12	42.2	40% - Serpin B	*C. sinensis*
FhSRP4	1227	408	9.04	46.0	40% - EP45 precursor	*Schistosoma japonicum*

In silico sequence analysis. Theoretical isoelectric point (pI) and molecular weight (Mw) were calculated using the Compute pI/Mw tool in the Expasy portal. The % of identity were obtained using BLATp against the non-redundant protein database in GenBank.

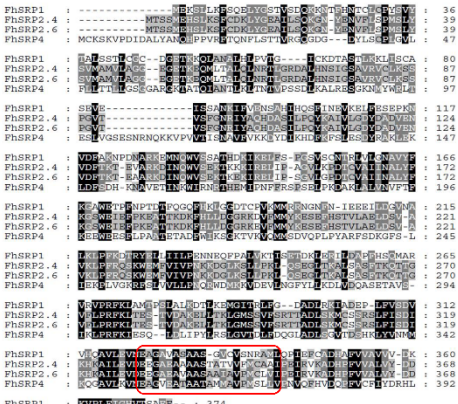


Three-dimentional structures. A: FhSRP1. **B:** FhSRP2.4. **C:** FhSRP2.6. **D:** FhSRP4. In Red: α-helices. In Yellow: β-sheet. In Blue: RCL

Serpin	P17	P16	P15	P14	P13	P12	P11	P10	P9	P8	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'
FhSRP1	E	A	G	A	V	A	S	A	A	S	G	V	C	V	S	N	R	A	M	L
FhSRP2.4	E	E	G	A	E	A	A	A	A	S	T	A	T	V	V	F	M	C	A	A
FhSRP2.6	E	E	G	A	E	A	V	A	A	S	A	A	I	A	V	P	M	C	L	V
FhSRP4	E	A	G	V	E	A	T	A	A	T	A	M	M	A	V	P	M	S	L	L

Comparation of the RCLs sequences and putative cleavage sites for peptidases. The residues are numbered according to the nomenclature developed by Schechter and Berger (1967). In Yellow: Scissile bond. In Bold: predicted P1. In Red: conserved amino acids.

Results



Sequence alignment. The high conserved residues are labeled in black, intermediate and low conserved residues are labeled in dark and light gray, respectively. The RCL is boxed in red.

Serpin	Predicted human proteases
FhSRP1	Trypsin
FhSRP2.4	Chymotrypsin-low specificity
FhSRP2.6	Chymotrypsin-low specificity
FhSRP4	Chymotrypsin-low specificity

Potential proteases that could cleave the RCL of serpins in *Fasciola hepatica* were predicted with the Peptide Cutter tool in the Expasy portal.

Conclusions

- It was demonstrated the existence of three serpin encoding genes
- The 3D models obtained are consistently with the predicted tertiary structure for serpins.
- Peptide sequences compatible with FhSPN1 and FhSPN2 have been identified by proteomics assays for excretory/secretory products.

According with the available data, FhSPN1 y FhSPN2 are secreted during the NEJ and adult stage, suggesting a role of these proteins in parasite-host relationship. Expression of the recombinant proteins of these four serpins are under development for biochemical and functional characterization.

23rd Congress of the International Union for Biochemistry and Molecular Biology
44th Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology
Foz do Iguaçu, PR, Brazil, August 24th to 28th, 2015

THE SECRETOME OF INTRA MAMMAL STAGES OF *Fasciola hepatica*: NEW CLUES UNVEILED IN THE BOVINE HOST-PARASITE INTERACTION

Sánchez Di Maggio, L.[1,2]; Tirloni, L.[2]; Benavides, U.[4]; Pinto, A.F.M[5,6].; Diedrich, J[6].; Yates, J.R[6]; Carmona, C.[1]; Berasain, P.[1]; da Silva Vaz Jr, I.[2,3].

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INTRODUCTION AND OBJECTIVES: The liver fluke *Fasciola hepatica* is the causative agent of fascioliasis. It leads to significant economic losses in the livestock industries in addition to becoming an emerging pathogen for humans. The strategies employed by parasites to establish infections remain poorly understood. Parasite excretory/secretory (ES) products play major roles in the host-parasite interface that protects the parasite from the host defense responses. Identifying proteins secreted/excreted by parasites and their associated functions will improve our understanding of their roles in the host-parasite interaction and parasite biology. **MATERIALS AND METHODS**: *F. hepatica* metacercariae were excysted *in vitro*. Emerging active parasites, newly excysted juveniles (NEJ), were collected, washed and incubated. Adults were collected from the bile ducts of cattle washed and incubated. The supernatant from both samples was removed after culture and concentrated. The proteomic analysis was performed using a shotgun proteomics approach. The resulting MS/MS spectra were searched against *F. hepatica* and *Bos taurus* protein databases. **RESULTS AND CONCLUSIONS**: A total of 416 *F. hepatica* proteins were identified, being 148 in NEJ, 358 proteins in adult, and 108 proteins shared for both stages. In NEJ and adult the most abundant categories are proteinases (60% and 58%, respectively) followed by proteinase inhibitors and heme/iron metabolism-related proteins. Concerning host-derived proteins, we identified 41 proteins in NEJ and 82 in adult, both stages shares 33 proteins. We identified many proteins implicated in parasite protection from the host immune system like proteinase inhibitors, antioxidant enzymes, lipocalins and glycoproteins; migration and tissue penetration proteins as proteinases and heme/iron metabolism proteins. These proteins identified can provide new insights into the biology of the parasite and its relationship with the host.

Keywords: Proteomic analysis, *Fasciola hepatica*, Parasites
Acknowledgments: DICYT-CNPQ, CAPES-UDELAR, PEDECIBA, INCT-ENTOMOLOGIA MOLECULAR, FAPERGS

THE SECRETOME OF INTRA MAMMAL STAGES OF *Fasciola hepatica*: NEW CLUES UNVEILED IN THE BOVINE HOST-PARASITE INTERACTION

Sánchez Di Maggio, L.[1,2]; Tirloni, L.[3]; Benavides, U.[4]; Pinto, A.F.M[5,6].; Driedrich, J[6].; Yates, J.R[6]; Carmona, C.[1]; Berasain, P.[1]; da Silva Vaz Jr, I.[2,3].

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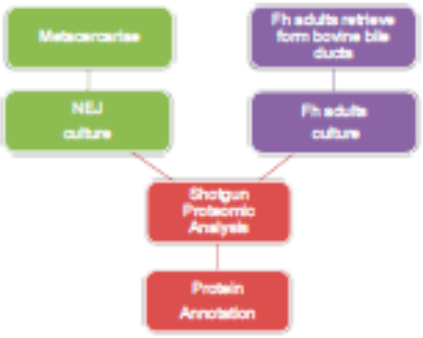
Introduction

The liver fluke *Fasciola hepatica* is the causative agent of fascioliasis. It leads to significant economic losses in the livestock industries in addition to becoming an emerging pathogen for humans. The strategies employed by parasites to establish infections remain poorly understood. Parasite excretory/secretory (ES) products play major roles in the host-parasite interface that protects the parasite from the host defense responses.

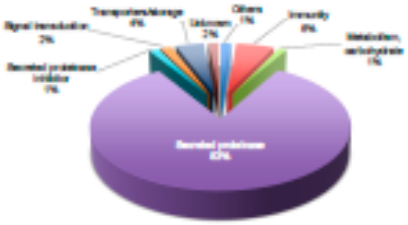
Identifying proteins secreted/excreted by parasites and their associated functions will improve our understanding of their roles in the host-parasite interaction and parasite biology.

Materials and Methods

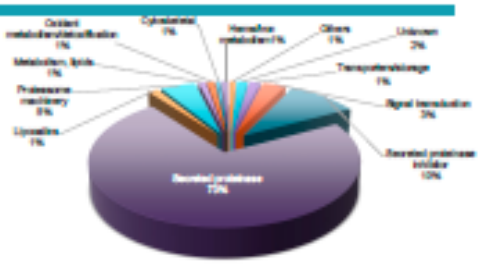


Results and Conclusions

PEI: Protein comparison between juvenile and adult stages. In violet proteins found only in adult proteome. In blue, proteins found only in juvenile proteome. In the middle, proteins present in the proteome for both stages.



Secretory proteins in the juvenile stage (Infection) Percentages corresponding to NSAF (Normalized Spectral Abundance Factor) values for proteins from each class.



Secretory proteins in the adult stage (Feeding/Reproduction). Percentages corresponding to NSAF values for proteins from each class.

In this work we identified the largest number of proteins for the intra-mamma stages of *Fasciola hepatica* until now. A total of 240 *F. hepatica* proteins were identified, being 90 from NEJs, 202 proteins from adult flukes, and 52 proteins shared between both stages. In the NEJ and the adult the most abundant categories are secreted proteases (83% and 73%, respectively) followed by protease inhibitors and proteasome-related proteins in the adult and immunity and transporter/storage-related proteins in the NEJ. In the adult, the predominance of cathepsin L cysteine proteases is clearly evident (65% from all the proteases) whereas in the NEJ the cathepsin B cysteine proteases (38%) are the most abundant product followed by cathepsin L cysteine proteases (21%) as been described before. In relation to shared proteins between the two stages we found that 44% of these proteins are proteases, mostly cathepsin B and L, followed by transport, cytoskeleton and proteinase inhibitors proteins. Concerning host-derived proteins, we identified 82 proteins in the adult, being proteasome machinery and cytoskeletal the most abundant categories. In addition, only a few host proteins were detected in the NEJ preparation.

Secretory host proteins in the adult stage (Feeding/Reproduction). Percentages corresponding to NSAF values for proteins from each class.

We identified many proteins implicated in parasite protection from the host immune system like proteinase inhibitors, antioxidant enzymes, lipocalins and glycoproteins; migration and tissue penetration proteins as proteinases and heme/iron metabolism proteins. These proteins identified can provide new insights into the biology of the parasite and its relationship with the host.

Acknowledgments:

IDENTIFICATION OF HEPARIN-BINDING PROTEINS IN THE TICK *Rhipicephalus* (*Boophilus*) *microplus* LARVAE

Kinappe, L. F. G.[1,2]; Tirloni, L.[1]; Sanchez Di Maggio, L.[1], Xavier, M.[1]; Dourado, T. S. [3];Termignoni, C.[1,2,4]; da Silva Vaz Jr I. [1,2,5]

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Introduction and Objectives: The tick *Rhipicephalus (Boophilus) microplus* is an economically important parasite of cattle. The currently decreased efficiency of acaricides has stimulated the search for new control strategies. Heparin is a sulfated glycosaminoglycan responsible for the modulation of physiological processes by binding to various proteins. Therefore, this work seeks to identify heparin-binding proteins in *R. microplus*. **Materials and methods**: Was selected 10-day larvae for preparation of protein extract and the heparin-binding proteins were purified by heparin affinity chromatography. Proteins were eluted in a linear NaCl gradient (10 mM sodium phosphate, pH 7.4, 0 - 1M NaCl). The elution peaks were determined by optical density in a FPLC. After, the samples were analyzed by SDS-PAGE. The proteomic analysis was performed using a GeLC-MS/MS approach. The resulting MS/MS spectra were searched against a *B. microplus* protein databases. **Results and Conclusions**: The SDS-PAGE revealed that a high number of proteins eluted at low NaCl concentration.This indicates that there are many proteins in the extracts with lower affinity for heparin. The molecular weight of these proteins varies from 14kDa to 66kDa. Protein profiles with ***higher*** affinity were also identified, although in less number. Uma frase dizendo de onde vierma estas proteinas. Among the identified proteins include lysosomal protease is cathepsin-like, bifosfatoaldolase fructose, heme-binding aspartic proteinase, and heat shock proteins (HSP's). Our next step will be choosing some of these proteins based on their biological role and perform expression assays for biochemical characterization and immunogenicity testing.

Keywords: *R. microplus*, heparin-binding proteins, parasite

Acknowledgments: DICYT-CNPQ, INCT-ENTOMOLOGIA MOLECULAR, FAPERGS

HEPARIN-BINDING PROTEINS IN THE TICK *Rhipicephalus (Boophilus) microplus* LARVAE

Kinappe, L. F. G.[1,2]; Tirloni, L.[1]; Sanchez Di Maggio, L.[1], Xavier, M.[1]; Dourado, T. S.[3];Termignoni, C.[1,4,5]; da Silva Vaz Jr I.[1,2,5]

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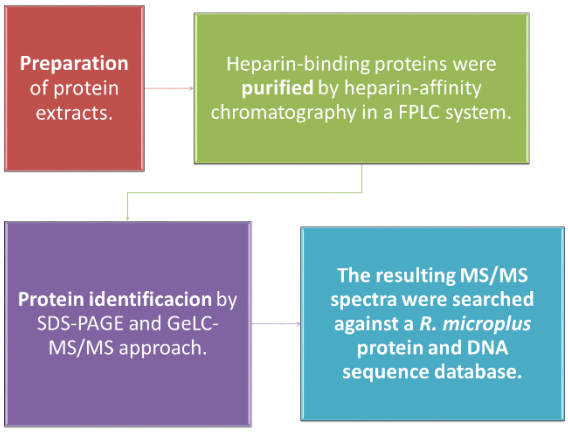
Introduction

The tick *Rhipicephalus (Boophilus) microplus* is an economically important parasite of cattle. Decreased efficiency of acaricides induces the search for new control strategies. Heparin is a sulfated glycosaminoglycan responsible for the modulation of several physiological processes by binding to various proteins.

The aim of this work is to identify heparin-binding proteins in *R. microplus*.

Materials and Methods



Results

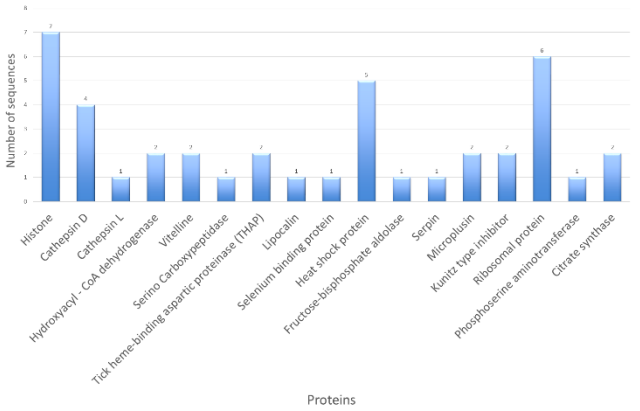


Fig.1: LC-MS/MS results from the identified proteins.

Conclusions

The SDS-PAGE revealed that a high number of proteins, varying from 14 kDa to 66 kDa, eluted at low NaCl concentration, indicating that mainly proteins in the extracts have lower affinity for heparin. Protein profiles with higher affinity were also identified, although in less number. Proteins identified by MS/MS include lysosomal proteases as cathepsin-like and heme-binding aspartic proteinase, fructose-bisphosphate aldolase, heat shock proteins (HSP's), and others. The most abundant categories are binding proteins (42%) and proteins with catalytic activity (34%). The biochemical characterization of some identified proteases are in progress to check heparin influence on protease activity.

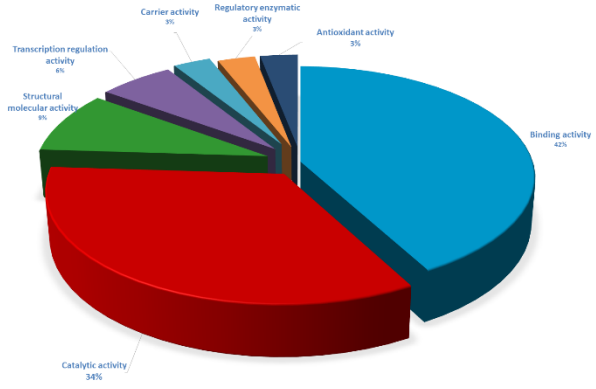


Fig. 2. Predicted molecular functions of the identified proteins against The Gene Ontology® database.

Acknowledgments:

Sociedade Brasileira de Bioquímica e Biologia Molecular — SBBq

45ª Reunião Anual da SBBq
18 a 21 de junho de 2016 - Natal, RN

A Proteomic Study of the Intra-mammalian Stages of *Fasciola hepatica*.

Sánchez Di Maggio, L.[1,4]; Tirloni, L.[1]; Benavides, U.[2]; Pinto, A.F.M[3,5]; Diedrich, J[6].; Yates, J.R[6]; Carmona, C.[4]; Berasain, P.[4]; da Silva Vaz Jr, I.[1,3].

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INTRODUCTION: Fasciolosis is a zoonotic foodborne disease of herbivores and humans caused mostly by the trematode parasite *Fasciola hepatica*. This disease causes economic losses in livestock production and is an emerging pathogen to humans' health. Although control methods are available, re-infection and resistance to drugs demand new control strategies. **OBJECTIVE:** Understanding the molecular mechanisms underlying the host-parasite interaction could provide relevant clues, enhancing our knowledge of this interaction and leading to promising targets for diagnosis and control. **MATERIALS AND METHODS:** *F. hepatica* metacercariae were excysted *in vitro*. Emerging active parasites, newly excysted juveniles (NEJ) were collected, washed and incubated. Adults were collected from the bile ducts of cattle, washed, and incubated in culture media. The supernatant (excretion/secretion products – ESP) from both samples were collected and concentrated. The somatic soluble proteins of NEJ (SSP) were also collected. The proteomic analysis was performed using a shotgun proteomics approach. The resulting MS/MS spectra were searched against *F. hepatica* and *Bos taurus* protein databases. **RESULTS AND DISCUSSION:** More than 700 *F. hepatica* proteins were identified and a core of 45 proteins is shared among the samples. In the ESP 240 proteins were identified, being 90 from NEJ, 202 from adults, and 52 proteins shared between both stages. In the proteomic analysis of the SSP, 575 proteins were identified. Additionally, we were able to identify host-derived proteins in all three samples. Functional classification of the identified proteins revealed the presence of proteinases, proteinase inhibitors and proteins related to oxidation/detoxification, heme/iron, and lipid metabolism. In addition, proteins involved in signal transduction, transport, and metabolism of carbohydrate, energy production, nucleotide, and amino acids were identified. **CONCLUSIONS:** The proteomic analysis of the intra-mammalian stages of *F. hepatica* could lead to a better comprehension of the host-parasite relationship and the infectivity, virulence and development of this helminth.

Keywords: *Fasciola hepatica*, LC-MS/MS, secretome, parasite-host interaction.

Acknowledgments: DICYT-CNPQ, CAPES-UDELAR, PEDECIBA, INCT-ENTOMOLOGIA MOLECULAR, CSIC, FAPERGS

A Proteomic Study of the Intra-mammalian Stages of *Fasciola hepatica*.

Sánchez Di Maggio, L. [1]; Tirloni, L. [2]; Benavides, U. [3]; Pinto, A.F.M.M. [4]; Diedrich, J. [5]; Yates, J.R. [5]; Carmona, C. [3]; Berasain, P. [3] da Silva Vaz Jr, I. [1].

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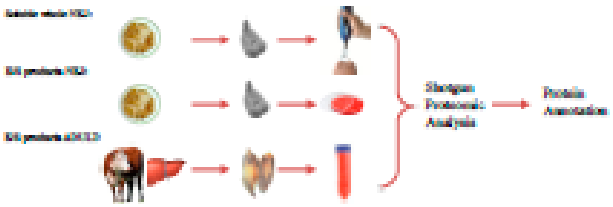
INTRODUCTION

The liver fluke *Fasciola hepatica* is the causative agent of fascioliasis. It leads to significant economic losses in the livestock industries in addition to becoming an emerging pathogen for humans. The strategies employed by parasite to establish infections remain poorly understood.

Understanding the molecular mechanisms underlying the host-parasite interaction could provide relevant clues, enhancing our knowledge of this interaction and leading to promising targets for diagnosis and control.

MATERIALS AND METHODS



RESULTS

Figure 1

Figure 2

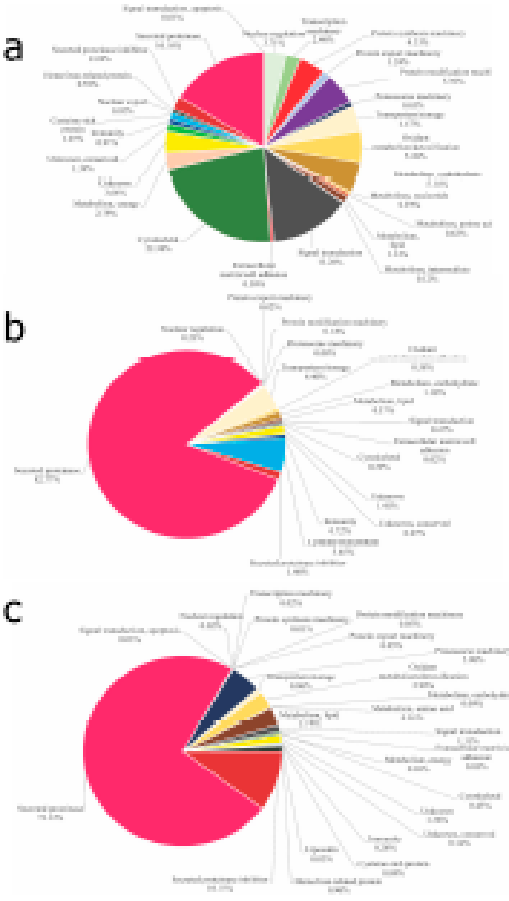
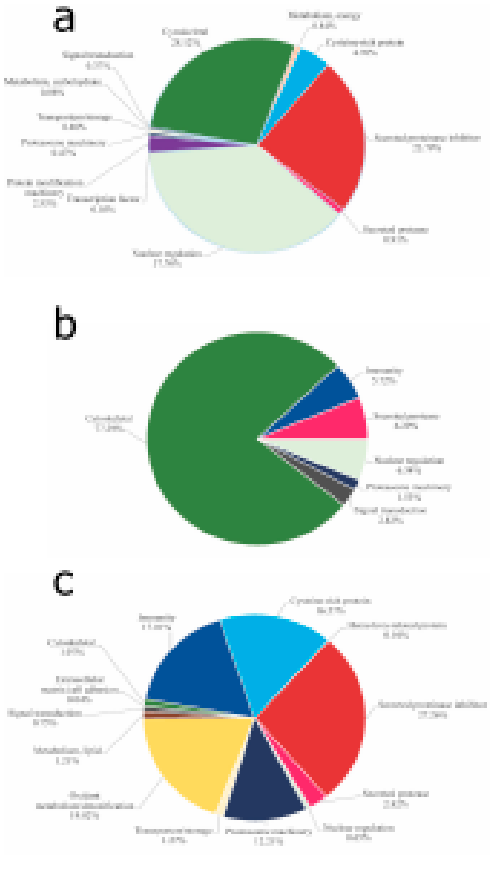


Figure 3



CONCLUSIONS

This work provides in-depth proteomic analysis of *F. hepatica* intra-mammalian stages, and represents the largest number of proteins identified to date for this species.

We identified proteins implicated in parasite protection from the host immune system like proteinase inhibitors, antioxidant enzymes, lipocalins and glycoproteins; migration and tissue penetration proteins as proteinases and hemoglobin metabolism proteins.

Some proteins are differentially expressed during the intra-mammal stages, suggesting a role in that particular developmental stage of the parasite.

CLONING AND CHARACTERIZATION OF SERINE PROTEASE INHIBITORS (SERPINS) IN *Fasciola hepatica*.

Sánchez Di Maggio, L.[1,4]; Tirloni, L.[1,4]; Benavides U.[2]; Mulenga, A[1]; Carmona, C.[4]; Berasain, P.[4] da Silva Vaz Jr, I.[1,3].

[1]Centro de Biotecnologia, UFRGS, RS, Brazil; [2]Unidad de Biologia Parasitaria, Facultad de Ciencias, UdelaR, Montevideo, Uruguay; [3]Faculdade de Veterinária, UFRGS, RS, Brazil; [4]Texas A&M University, Department of Veterinary Pathobiology TX, USA; [5]Departamento de Inmunologia, Facultad de Veterinaria, UdelaR , Montevideo, Uruguay.

INTRODUCTION AND OBJECTIVES: The liver fluke *Fasciola hepatica* is the causative agent of fascioliasis that inflicts significant economic losses in the livestock industries and it is an emerging pathogen for humans in many countries. Serpins are a superfamily of serine protease inhibitors regulating inflammation, complement activation, blood coagulation and fibrinolysis in mammals. The aim of this study was the characterization of four *F. hepatica* serpins. **MATERIALS AND METHODS**: The deduced amino acid sequences of *F. hepatica* serpins (FhS) were scanned against Genbank databases using the BLASTp algorithm. Alignment was performed using ClustalW algorithm in the MEGA 6.06 program. *In silico* models for tertiary structure were built with Modeller v9.14 and Pymol v1.7.4 softwares. The serpin encoding sequences were cloned and expressed in the *Pichia pastoris* system. The recombinant proteins (rFhS2.4 and rFhS4) were used to assay the inhibitory activities against a panel of serine proteases related to host defense systems, heparin-binding and deglycosylation assays. **RESULTS AND CONCLUSIONS**: The 3D models obtained are consistent with the predicted tertiary structure for serpins and putative heparin-binding sites were identified. Peptide sequences compatible with FhS-1 and FhS-2 have been identified by proteomics assays for excretory/secretory products. The two recombinant expressed proteins (rFhS-2.4 and rFhS-4) are glycosylated and, as predicted in silico, rFhS4 bounds onto heparin-Sepharose. These serpins have distinct enzymatic inhibition profiles: rFhS-2.4 has anti-cathepsin G activity and rFhS-4 has anti-chymotrypsin and anti-cathepsin-G activity. Furthermore, at molar excess rFhS-2.4 and rFhS-4 are capable of inhibit rat chymase and human factor XIIa, respectively. Proteases such as chymotrypsin-like and cathepsin G are involved in inflammation and blood coagulation, suggesting that these serpins could interfere in host immune responses during parasitism by *F. hepatica*.

Keywords: *Fasciola hepatica*, functional characterization, serine protease inhibitor

Acknowledgments: DICYT-CNPQ, CAPES-UDELAR, PEDECIBA, INCT-ENTOMOLOGIA MOLECULAR,

CLONING AND CHARACTERIZATION OF SERINE PROTEASE INHIBITORS (SERPINS) IN *Fasciola hepatica*

Sánchez Di Maggio, L.[1,2]; Tirloni, L.[3,4]; Benavides U.[5]; Mulenga, A.[4]; Carmona, C.[2]; Berasain, P.[2], da Silva Vaz Jr, I.[1,3].

[1]Centro de Biotecnología, UFRGS, RS, Brazil; [2]Unidad de Biología Parasitaria, Facultad de Ciencias, UdelaR, Montevideo, Uruguay; [3]Faculdade de Veterinária, UFRGS, RS, Brazil; [4]Texas A&M University, Department of Veterinary Pathobiology TX, USA; [5]Departamento de Inmunología, Facultad de Veterinaria, UdelaR , Montevideo, Uruguay.

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Introduction

The liver fluke *Fasciola hepatica* is the causative agent of fasciolasis. It leads to significant economic losses in the livestock industries and it is an emerging pathogen for humans in many countries. Serpins are a superfamily of serine protease inhibitors regulating inflammation, complement activation, blood coagulation and fibrinolysis.

The aim of this study was the *in silico* study and expression analyses of four *F. hepatica* serpins.

Materials and Methods

Results and Conclusions

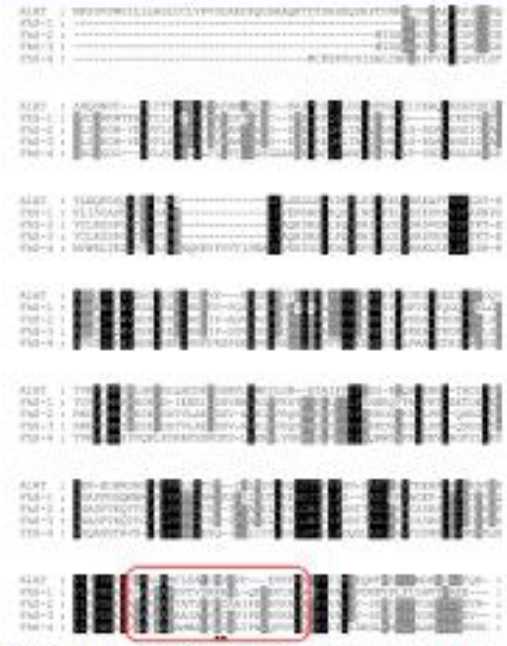

Fig. 1: *F. hepatica* serpin sequence alignment. Sequence alignment was constructed by comparison with human alpha-1-antitrypsin (NIAC Genebank Accession number: AAB59876.1) as template. The high conserved residues are labeled in black and grey. The RCL is boxed in red and the scissile bond is marked with an *.

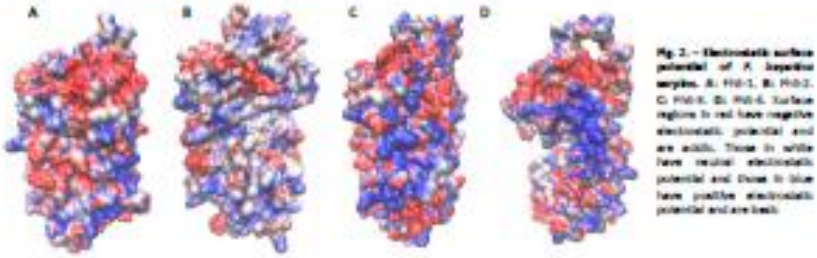

Fig. 2. – Electrostatic surface potential of *F. hepatica* serpins. A: rFh8-1, B: rFh8-2, C: rFh8-3, D: rFh8-4. Surface regions in red have negative electrostatic potential and are acidic. Those in white have neutral electrostatic potential and those in blue have positive electrostatic potential and are basic.

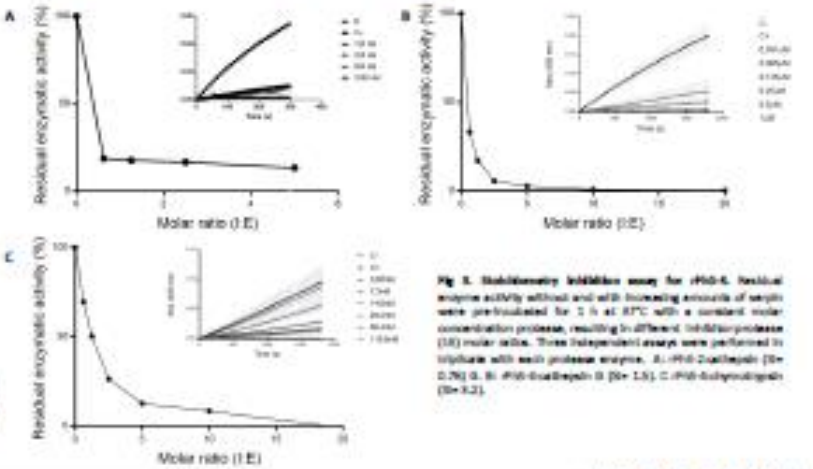

Fig. 3. Stoichiometry inhibition assay for rFh8-4. Residual enzyme activity without and with increasing amounts of serpin were pre-incubated for 1 h at 37°C with a constant molar concentration protease, resulting in different inhibitor:protease (I:E) molar ratios. Three independent assays were performed in triplicate with each protease enzyme. A: rFh8-2:cathepsin (SI= 0.76) B. B: rFh8-4:cathepsin B (SI= 1.5). C: rFh8-4:chymotrypsin (SI= 3.2).

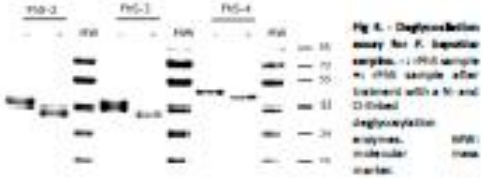

Fig. 4. – Deglycosylation assay for *F. hepatica* serpins. – : rFh8 sample +: rFh8 sample after treatment with a N- and O-linked deglycosylation enzymes. MW: molecular mass marker.

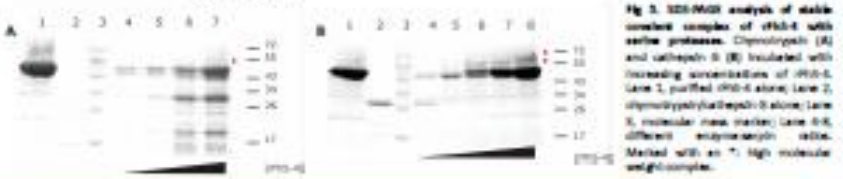

Fig. 5. SDS/PAGE analysis of stable covalent complex of rFh8-4 with serine proteases. Chymotrypsin (A) and cathepsin B (B) incubated with increasing concentrations of rFh8-4. Lane 1, purified rFh8-4 alone; Lane 2, chymotrypsin/cathepsin B alone; Lane 3, molecular mass marker; Lane 4-8, different enzyme:serpin ratios. Marked with an * high molecular weight complex.

Fig. 6. Heparin binding purification. Lane 1, molecular mass marker; Lane 2, purified rFh8-4; Lane 3, pass-through fraction; Lane 4, wash fraction; Lane 5, fraction 10; Lane 6, fraction 11; Lane 7, fraction 12; Lane 8, fraction 14; Lane 9, fraction 16; Lane 10, fraction 18.

The 3 serpins expressed in *Pichia pastoris* are glycosylated. rFh8-2 and rFh8-4 have distinct enzymatic inhibition profiles and both serpins inhibit serine proteases involved in processes enhancing inflammation. rFh8-3 appear to be a non-inhibitory serpin despite of having practicaly identical sequence to Fh8-2 with differences in the RCL region, in amino acids that are critical for the inhibitory function in this family of proteins. rFh8-4 forms high molecular weight covalent complex with both target proteases. As was predicted *in silico*, rFh8-4 bind to heparin with high affinity and this characteristic could enhance the inhibition activity of the serpin.

Sociedade Brasileira de Bioquímica e Biologia Molecular — SBBq

47a. Reunião Anual da SBBq
26 a 29 de maio de 2018 - Joinville, SC

The Host Factor: Comparison Between *F. hepatica* Newly Excisted Juvenile From Different Intermediate Hosts.

Lucia Sánchez Di Maggio[1,4], Lucas Tirloni[1,2], Antonio F.M. Pinto[3], Jolene K. Diedrich[4], John R. Yates III[4], Carlos Carmona[5], Patricia Berasain[6] & Itabajara da Silva Vaz Jr[1,2].

[1]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil; [2]Unidad de Biologia Parasitaria, Facultad de Ciencias, Universidad de la República Oriental del Uruguay, Montevideo, Uruguay; [3]College of Veterinary Medicine, Department of Veterinary Pathobiology, Texas A&M University, College Station, TX, USA. [4]Department of Chemical Physiology, The Scripps Research Institute, CA, Unites States of America. [5]Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

INTRODUCTION: Fasciolosis is a zoonotic foodborne disease of herbivores and humans caused mostly by the trematode parasite *Fasciola hepatica*. This disease causes economic losses in livestock production and is an emerging pathogen to humans' health. Although control methods are available, re-infection and resistance to drugs demand new control strategies. **OBJECTIVE**: the aim of the present study was to perform a comparative proteomic analysis of the NEJ (newly excysted juvenile) stage of *F. hepatica* retrieved from different intermediate hosts: *Lymnaea viatrix* and *Lymnaea columella*. **MATERIALS AND METHODS**: *F. hepatica* metacercariae were excysted *in vitro*. Emerging active parasites, newly excysted juveniles (NEJ) were collected, washed and incubated. The supernatant (excretion/secretion products – ESP) from both samples were collected. The proteomic analysis was performed using a shotgun proteomics approach. The resulting MS/MS spectra were searched against *F. hepatica*, *Bos Taurus* and *Ovis aries* protein databases. **RESULTS AND DISCUSSION**: A total of 93 *F. hepatica* proteins were identified and a core of 54 proteins are shared among the samples. In the *L. viatrix* ESP 57 proteins were identified, being 3 specifically for this host. In the *L. columella* ESP 90 proteins were identified, being 36 exclusive for this sample. Additionally, host-derived proteins were also identified in both samples. Functional classification of the proteins revealed the presence of proteinases as the predominant class, proteinase inhibitors and proteins related to oxidation/detoxification, heme/iron, and lipid metabolism. In addition, proteins involved in signal transduction, transport, and metabolism of carbohydrate, energy production, nucleotide, and amino acids were identified. **CONCLUSIONS**: Both NEJ samples has similar ESP composition but they have differences in the relative amount of key proteins that could be related to parasite survival strategies in the different hosts environment.

Keywords: *Fasciola hepatica*, secretome, parasite-host interaction.

Acknowledgments: DICYT-CNPQ, CAPES-UDELAR, PEDECIBA, INCT-ENTOMOLOGIA MOLECULAR, CSIC, FAPERGS

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INTRODUCTION

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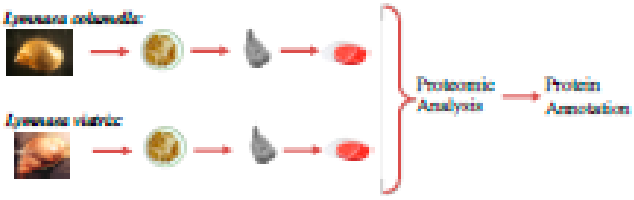
The aim of the present study was to perform a comparative proteomic analysis of the NEJ stage of *F. hepatica* retrieved from different intermediate hosts: *Lymnaea viatrix* and *Lymnaea columella*.

MATERIALS AND METHODS



RESULTS

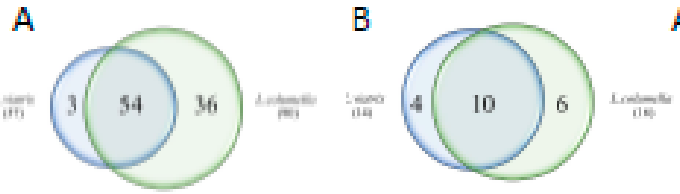


Figure 1. Venn diagram of NEJ ESP proteins identified in *L. columella* and *L. viatrix* samples. The overlap between the circles represent the shared proteins between samples. A: *F. hepatica* database. B: *B. Taurus* database.

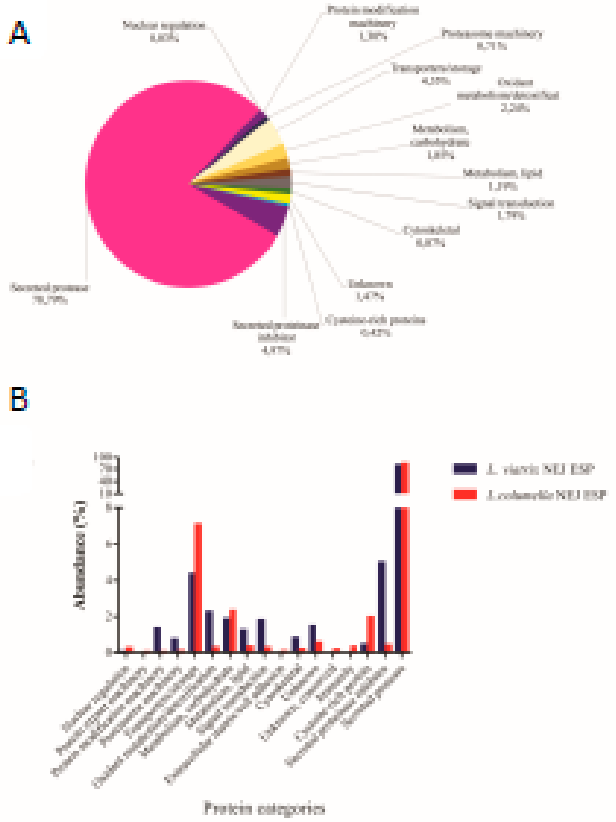


Figure 2. Functional classification of *F. hepatica* NEJ ESP identified *L. viatrix* sample. A: Pie chart represent the percentage of proteins found in each group with respect to their normalized NSAF classified according to their function and/or protein family. B: NSAF comparison between the two NEJ ESP samples.

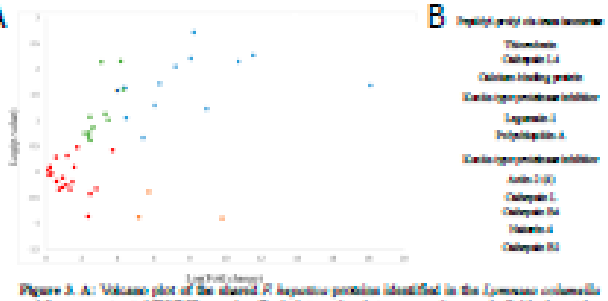


Figure 3. A: Volcano plot of the shared *F. hepatica* proteins identified in the *Lymnaea columella* and *Lymnaea viatrix* NEJ ESP samples. Each dot on the plot, representing one individual protein, represents the difference in expression (log2 fold difference, abscissa axis) between samples plotted against the level of statistical significance (log2 p-value, ordinate axis). Proteins represented by blue dots (16) have an identification that satisfied both fold change and statistical criteria; yellow dots (3) have an identifications that was filtered out by the statistical criteria; green dots (13) have an identification satisfied the fold criteria but, most likely, happened by chance; and red dots (10) when the identification did not meet fold change and p-value criteria. B: Proteins represented by blue dots in the volcano plot.

Table 1. *Bos taurus*-derived proteins identified by LC-MS/MS in *Lymnaea columella* and *Lymnaea viatrix* NEJ ESP samples. The * indicated the presence of the protein in the sample.

Accession number	Description	*L.viatrix*	*L. columella*
sp|A1L3V2|K1C13_BOVIN	Keratin, type I cytoskeletal 13		*
sp|P19035|CBR1_BOVIN	Chloride channel	*	*
sp|P04264|K2C1_BOVIN	Keratin	*	*
sp|P04259|K2C6B_BOVIN	Keratin, type II cytoskeletal 6B		*
sp|P02193|ALBU_BOVIN	Polyubiquitin-C	*	*
sp|P68133|ACTS_BOVIN	Actin, alpha skeletal muscle	*	
sp|Q29RC0|K2C1_BOVIN	Keratin type II cytoskeletal 1	*	*
sp|E1BGS1|E1BGS1_BOVIN	Complement component C1q receptor		*
sp|E1BJS6|E1BJS6_BOVIN	Tubulin		*
sp|Q3SX14|K1C9_BOVIN	Keratin, type I cytoskeletal 9	*	
sp|P13645|K1C10_BOVIN	Keratin		*
sp|P19013|K2C4_BOVIN	Keratin	*	*
sp|P31689|DNJA1_BOVIN	Small GTPase mediated signal transduction		*
sp|P04259|K2C6B_BOVIN	Keratin	*	*
sp|P35527|K1C9_BOVIN	Keratin	*	*
sp|P20848|S10A8_BOVIN	Histone H2A	*	*
sp|Q3SWW5|K2C8_BOVIN	Keratin	*	*
sp|A6QQY9|K2C79_BOVIN	Keratin	*	*
sp|Q3SX09|K1C14_BOVIN	Histone H2A	*	*

CONCLUSIONS

A total of 93 *F. hepatica* proteins were identified and a core of 54 proteins are shared among the samples. In the *L. viatrix* ESP 57 proteins were identified, being 3 specifically for this host. In the *L. columella* ESP 90 proteins were identified, being 36 exclusive for this sample. Additionally, host-derived proteins were also identified in both samples. Functional classification of the proteins revealed the presence of proteinases as the predominant category, proteinase inhibitors and proteins related to oxidation/detoxification, hemefron, and lipid metabolism. In addition, proteins involved in signal transduction, transport, and metabolism of carbohydrate, energy production, nucleotide, and amino acids were identified.

Both NEJ samples has similar ESP composition but they have differences in the relative amount of key proteins that could be related to parasite survival strategies in the different hosts environment.

A Novel Kunitz-type Serino Protease Inhibitor from the tick *Ixodes persulcatus*

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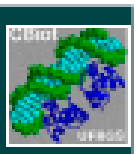
INTRODUCTION: For success in feeding, hematophagous animals developed specialized inhibitors to control host hemostatic system, including thrombin inhibitors belonging to the Kunitz-type family. These molecules can be used as model for new drugs development. OBJECTIVE: The aim of this work was the molecular and structural characterization of persulcatin, a putative thrombin inhibitor of the tick *Ixodes persulcatus*. **MATERIAL AND METHODOS**: Persulcatin coding sequence was amplified from whole adult *I. persulcatus* female cDNA library using primers based on an *Ixodes scapularis* Kunitz-type sequence (XM_002403992.1). The coding sequence was cloned in the pPICZαC expression vector and the *Pichia pastoris* GS115-strain transformed with the construction. Cloning was confirmed by PCR, restriction enzymes and DNA sequencing. Protein expression was tested by SDS-PAGE and trypsin inhibition assay. Persulcatin similar sequences were identified in GeneBank usin BLASTp algorithm. Predicted molecular weight and isoelectric point were determined by Peptide mass tool. Alignments were performed using MUSCLE and phylogenetic analysis done using Neighbor-Joining method. Tertiary structures were constructed by MODELLER, visualized by PyMOL and characterized by molecular docking and dynamics simulations using PatchDock / FireDock and GROMACS softwares. **RESULTS AND CONCLUSIONS**: Persulcatin coding sequence was cloned and trypsin inhibitory activity indicated the expression of the inhibitor in *Pichia pastoris* system. Persulcatin is a protein of 14,5 kDa and pI 4.59, codified by a 430 bp-long sequence. This protein presents two Kunitz-type domains and 12 conserved cysteine residues. *In silico* characterization showed that persulcatin has structure and mechanism of action similar to boophilin, a thrombin inhibitor of *Rhipicephalus microplus* tick (52% identity). In molecular docking and dynamic analysis, persulcatin showed to be able to inhibit thrombin by non-canonical mode. Standardization of heterologous expression and protein purification are underway to enable further functional characterization and evaluation of possible biotechnological applications of the recombinant protein.

Keywords: Anticoagulant, Thrombin inhibitor, Kunitz-type inhibitor, *Ixodes persulcatus*.
Supported by: CNPq, CAPES, FAPERGS, INCT-Entomologia Molecular.

A Novel Kunitz-type Serine Protease Inhibitor from the tick *Ixodes persulcatus*



De Mata, B.[1]; Di Maggio, L.L.S.[2]; Tirloni, L.[3]; Pertal, L.F.[2]; Kist, R.[1]; Caceres, R.A.[4]; Konnai, S.[5]; Ohashi, K.[5]; da Silva Vaz, I.[3]; Seixas, A.[1,6]

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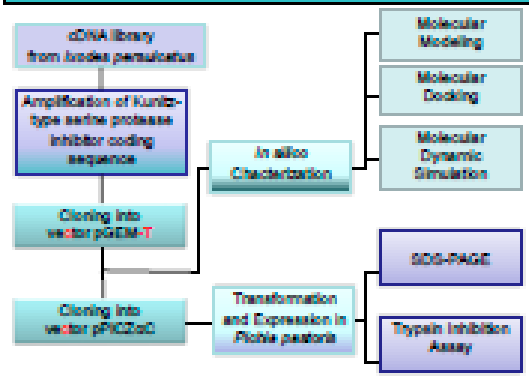
Introduction

For success in feeding, hematophagous animals developed specialized inhibitors to control host hemostatic system, including thrombin inhibitors belonging to the Kunitz-type family. These molecules can be used as model for new drugs development. In this report, the coding sequence of the protein persulcatin from *Ixodes persulcatus* (human ectoparasite) was characterized as a putative thrombin inhibitor, which exhibits typical characteristics of Kunitz-type serine protease inhibitors and high sequential identity with boophilin from the tick *Rhipicephalus microplus*.

Objectives

The aim of this work was the molecular and structural characterization of persulcatin, a putative thrombin inhibitor of the tick *Ixodes persulcatus*.

Materials and Methods



Results

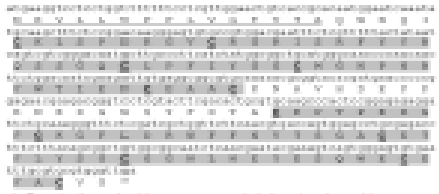


Figure 1. Persulcatin nucleotide sequence and deduced amino acid sequence. The putative signal peptide sequence is underlined. Cysteine residues that form the disulfide bonds are indicated in bold and underlined. Kunitz-type domains are sharkened. The asterisk (*) indicates the stop codon.

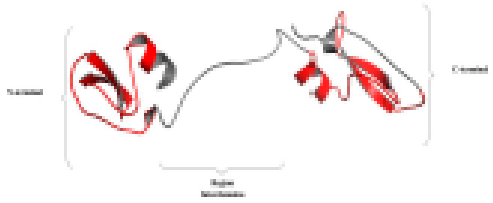


Figure 2. Cartoon representation of 3D structure of persulcatin protein. The domains are colored in red and the interdomain regions in gray.

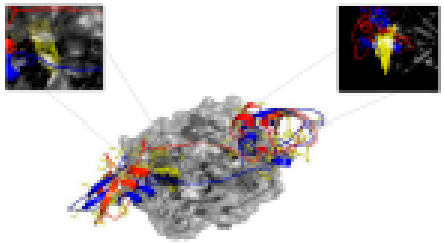


Figure 3. Docking of bovine thrombin in persulcatin - boophilin complex 2OZY revealed similar region of anchorage and interaction with thrombin, however displacements of angulation between N-terminal (left) and C-terminal (right) of persulcatin and boophilin.

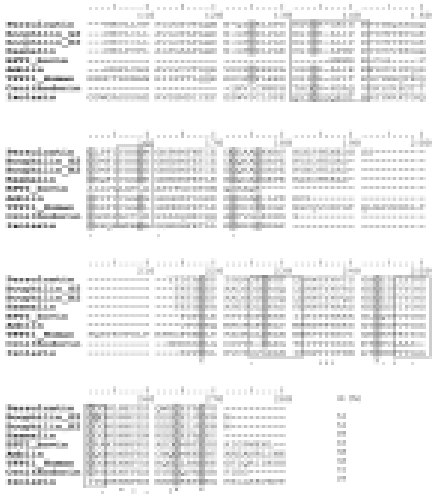


Figure 4. Alignment of deduced amino acid sequences of persulcatin with Kunitz-type inhibitors from other tick species, including boophilin variants 02 (Q8WP03) and H2 (Q8WP02), haemalin (MEDWC), amblin (Q25VJ02), BPTI (P00974), TFPI human (P10646), ixolaris (Q8WQC0), ornithodorin (P56539). Amino acid residues that form protease recognition loops are shown in boxes, and conserved cysteine residues are in bold. The N-terminal extension in TFPI human and ixolaris has been omitted for clarity, as well as C-terminal extension in amblin, TFPI human and ixolaris. Persulcatin showed high identity with thrombin inhibitors from others ticks species.

Table 1. Predicted binding affinity in protein-protein complexes.

	Thrombin-Boophilin	Thrombin-Persulcatin
ΔG (kcal/mol)	14.0	10.9
Kd (M)	1.2×10^{-11}	1.8×10^{-8}

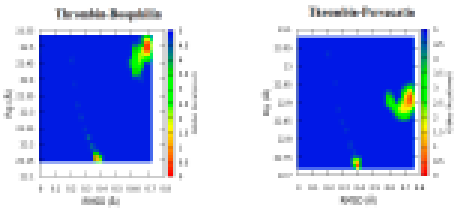


Figure 5. Free energy landscapes from molecular dynamics. The image representing the free energy of the complexes and the projection of the simulated trajectory. The energy trajectories of the T-B and T-P complex are stable throughout the simulation time. Demonstrating structural stability and inhibitory mechanism.

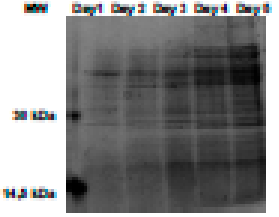


Figure 6. Protein expression analysis by SDS-PAGE. Line 1, molecular mass marker. Line 2, first day of expression. Line 3, second of expression. Line 4, third day of expression. Line 5, fourth day of expression. Line 6, fifth day of expression. Six positive clones were selected from the 11 transformed clones, two of which were active in the trypsin assay.

Conclusion and Perspectives

Our results suggest that persulcatin is a thrombin inhibitor similar to boophilin. Standardization of heterologous expression and protein purification are underway to enable further functional characterization and evaluation of possible biotechnological applications of the recombinant protein.

Supported by: CNPq, CAPES, FAPERGS, INCT-Entomologia Molecular.

Heparin-Binding Proteins from *Rhipicephalus microplus* and *Fasciola hepatica*

da Silveira, L.M.[1,4,A]; Xavier, M.A.[1,A]; Di Maggio, L.L.S.[1]; Vaz Jr., I.S.[1,4]; Termignoni C.[1,3]

[1]Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil; [4]Faculdade de Medicina Veterinária, UFRGS, Porto Alegre, RS, Brazil; [3]Departamento de Bioquímica, UFRGS, Porto Alegre, RS, Brazil.

Introduction: Heparin is a sulfated glycosaminoglycan synthetized by most animal cells with ability to bind proteins due to its multiple active sites, named heparin-binding proteins. Parasites can use heparin as a mechanism of parasite host interaction . The tick *Rhipicephalus microplus* and the helminth *Fasciola hepatica* are hematophagous that parasites cattle, causing damage to animal health and losses in production. **Objective:** (I) characterize the chromatographic profile of heparin binding proteins from *R. microplus* and *F. hepatica* and (II) identify proteins with high affinity by heparin. **Material and Methods:** *R. microplus* larvaeand ovary, gut and salivary glands from partially and fully engorged females had protein extraction. *F. hepatica* gut excretion and body proteins were extracted. The protein extracts were fractionated by affinity chromatography in a resin with immobilized heparin and NaCl gradient elution (0 to 2M). The fractions obtained were analyzed by SDS-PAGE 12%). **Results and Discussion:** It was observed an albundance of proteins with low affinity by heparin in all tick tissues. In guts from partially engorged ticks and larvae it was observed proteins (≤10 kDa) with high affinity (>1.5 M) by heparin. In *R. microplus* partially engorged ovaries and fully engorged salivary glands, proteins (50 kDa) were observed eluting after 1,5 M NaCl elution. In fully engorged R. *microplus* guts and ovaries it was not observed high affinity proteins by heparin. In *F. hepatica* it was observed three protein bands (around 50 kDa) with high affinity (2 M of NaCl) by heparin. **Conclusions:** it was identified the presence of heparin-binding proteins in *R. microplus* and *F. hepatica* tissues. Mass spectrometry analysis are currently being performed in order to identify the observed heparin binding proteins.

Key Words: heparin-binding protein, *Rhipicephalus microplus*, *Fasciola hepatica*.

Acknowledgment: CNPq, CAPES, INCT-EM

Heparin-Binding Proteins from *Rhipicephalus microplus* and *Fasciola hepatica*

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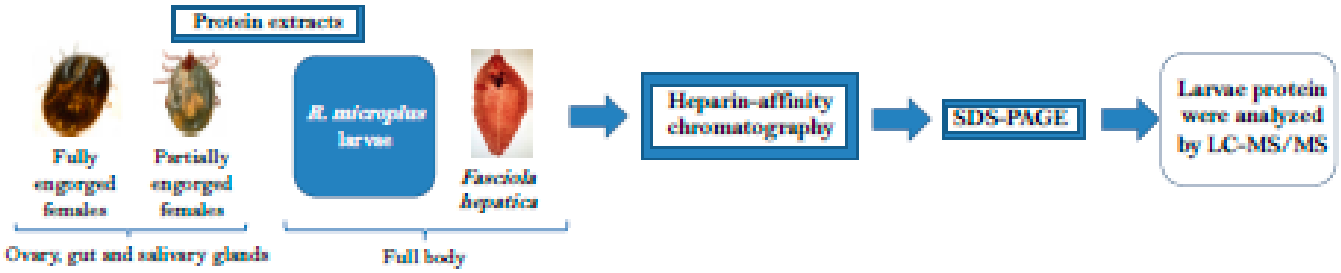
Introduction

Heparin is a sulfated glycosaminoglycan synthetized by most animal cells with ability to bind proteins due to its multiple active sites. Parasites can use heparin as a mechanism of parasite host interaction. The tick *Rhipicephalus microplus* and the helminth *Fasciola hepatica* are hematophagous cattle parasites, causing damage to animal health and losses in production. The aim of this work is to characterize the profile of heparin binding proteins from *R. microplus* tissues and *F. hepatica* and identify proteins with high affinity by heparin.

Materials and Methods



Results and Discussion

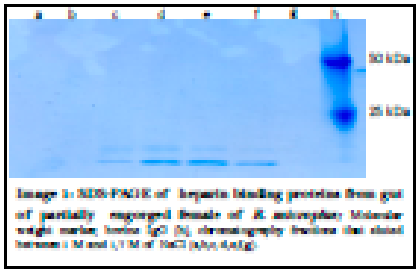


Image 1: SDS-PAGE of heparin binding proteins from gut of partially engorged female of *R. microplus*. Molecular weight marker, bovine IgG (h), chromatography fractions that eluted between 1 M and 1,5 M of NaCl (a,b,c, d,e,f,g).

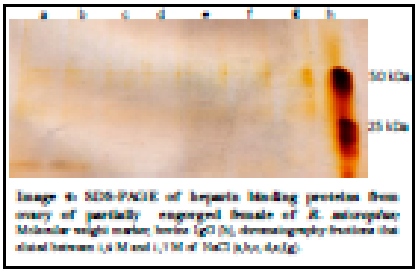


Image 4: SDS-PAGE of heparin binding proteins from ovary of partially engorged female of *R. microplus*. Molecular weight marker, bovine IgG (h), chromatography fractions that eluted between 1,0 M and 1,5 M of NaCl (a,b,c, d,e,f,g).

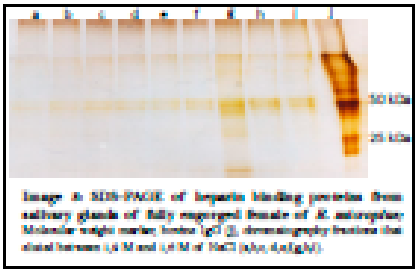


Image 5: SDS-PAGE of heparin binding proteins from salivary glands of fully engorged female of *R. microplus*. Molecular weight marker, bovine IgG (j), chromatography fractions that eluted between 1,0 M and 1,5 M of NaCl (a,b,c, d,e,f,g,h,i).

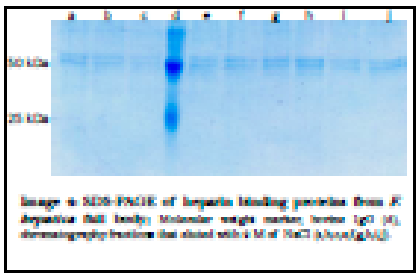


Image 3: SDS-PAGE of heparin binding proteins from *R. hepatica* (full body). Molecular weight marker, bovine IgG (h), chromatography fractions that eluted with 0 M of NaCl (a,b,c,d,e,f,g,h,i).

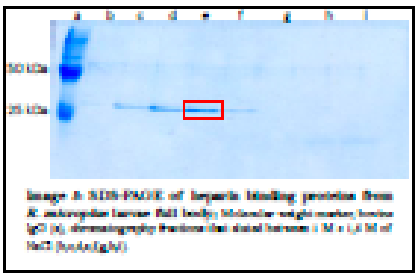


Image 2: SDS-PAGE of heparin binding proteins from *R. microplus* larvae (full body). Molecular weight marker, bovine IgG (h), chromatography fractions that eluted between 1 M e 1,5 M of NaCl (a,b,c,d,e,f,g,h).

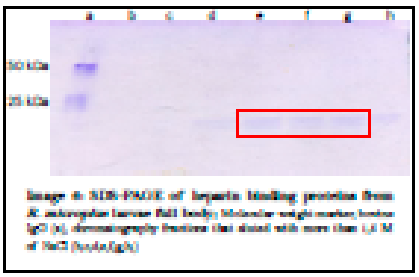


Image 6: SDS-PAGE of heparin binding proteins from *R. microplus* larvae (full body). Molecular weight marker, bovine IgG (h), chromatography fractions that eluted with more than 1,0 M of NaCl (a,b,c,d,e,f,g,h).

SDS-PAGE analysis from fully engorged females (gut and ovary) of *R. microplus* were not shown due chromatography samples presented low affinity to Heparin (<0,8 M of NaCl). Salivary glands from partially engorged females of *R. microplus* did not present considerable amount of heparin binding proteins.

LC-MS/MS analysis, using a database of *R. microplus* tick, identified the protein in line e in the image 3 (boxed in red) as an Histone 1. And as Histone 2A and 2B the mix of samples from lines e to g highlighted in a red box in image 6.

It is known that histones bind heparin and that among histones subtypes, Histone 1 is the one that presents more anticoagulant effects, followed by Histones 2A and 2B. Further enzymatic tests are necessary to identify the profile of this histones found in larvae samples.

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