

del proteosoma y la actividad tripeptidil peptidasa de ~ 10S, son esenciales durante el proceso de enquistación de *G. intestinalis*. Por lo que es muy importante que se inicie la identificación y caracterización de los sustratos blanco y mecanismos de activación y regulación de esta(s) vías enzimáticas.

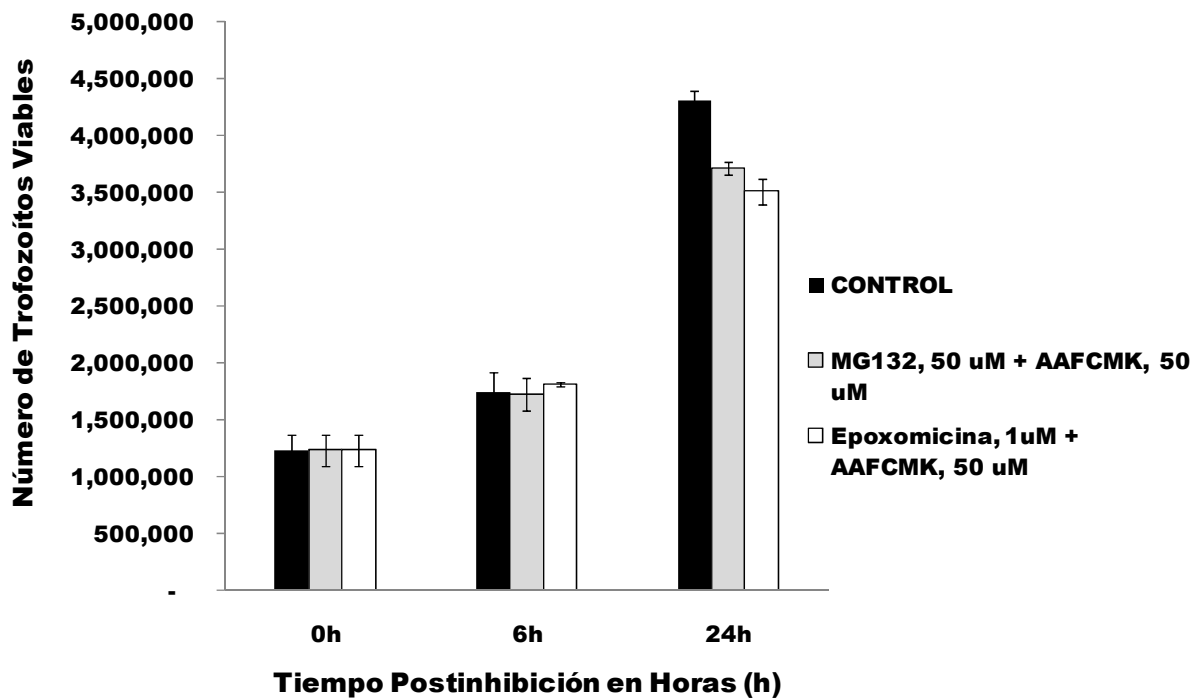


Figura 45. Efecto de la Inhibición Conjunta de TPP II y el Proteosoma en Trofozoítos de *G. intestinalis*. Los parásitos fueron incubados por 24 h con cada uno de los inhibidores señalados, en cada experimento se incluyeron controles negativos adicionados con DMSO. Los datos son el promedio \pm la desviación estándar de un experimento representativo. Cada experimento se repitió al menos dos veces.

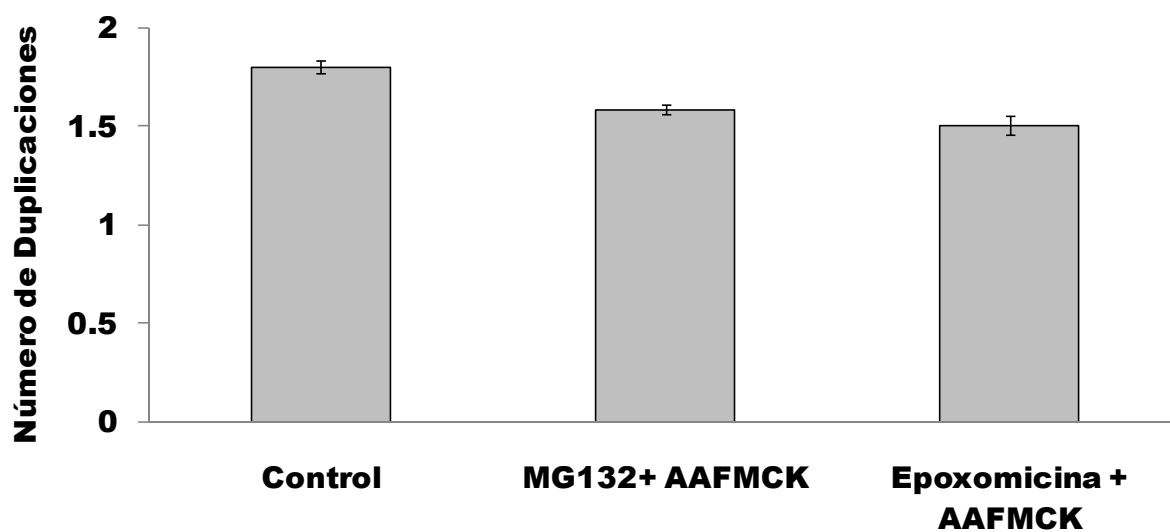


Figura 46. **Efecto de la Inhibición de TPP II y el Proteosoma Sobre el Número de Duplicaciones de los Trofozoítos de *G. intestinalis*.** Los parásitos fueron incubados por 24 h con los inhibidores señalados: H-Ala-Ala-Phe-CH₂Cl (AAFMCK), 50 uM; MG132, 50 uM y Epoxomicina, 1 uM. En cada experimento se incluyeron controles negativos adicionados con DMSO. La gráfica muestra los resultados de Número de Duplicaciones calculadas con la siguiente fórmula: $\text{Log} [\text{Número de células en el tiempo final} / \text{Número de células en el tiempo inicial}] / \text{Log } 2$. Los datos son el promedio \pm la desviación estándar de un experimento representativo. Cada experimento se realizó mínimo dos veces.

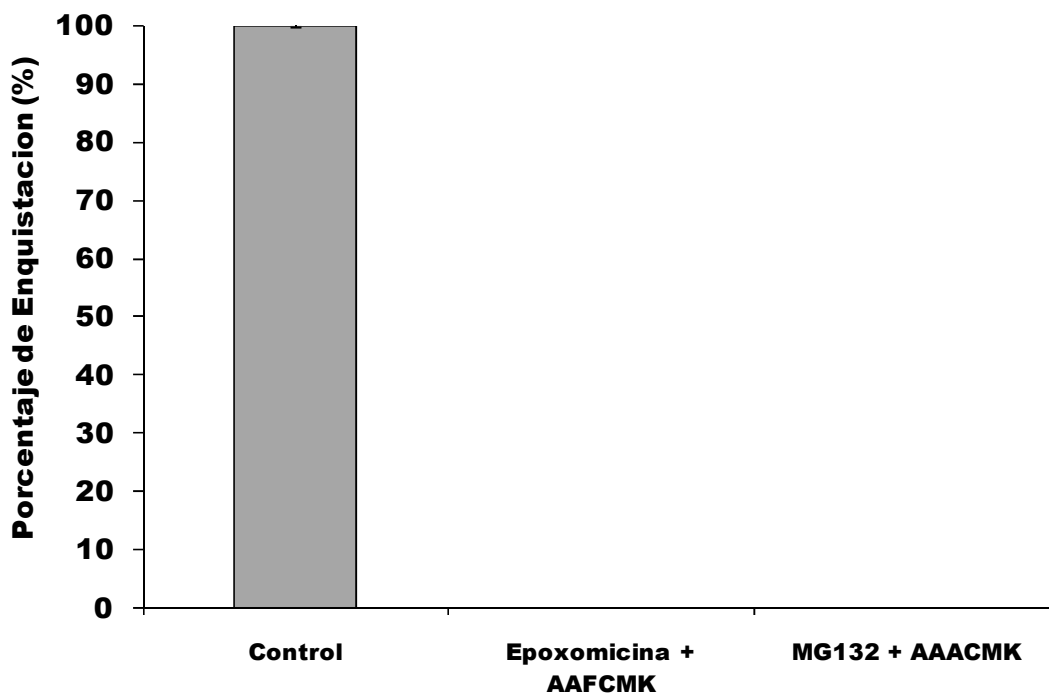


Figura 47. Efecto de la Inhibición de TPP II y el Proteosoma en la Enquistación de *G. intestinalis*. Se indujo el proceso de enquistación *in vitro* (Kane et al, 1991), en presencia de los inhibidores señalados: H-Ala-Ala-Phe-CHCl (AAFCMK), 50 uM, MG132, 50 uM y Epoxomicina 1 uM. Los controles fueron adicionados con DMSO. Los resultados son el promedio \pm la desviación estándar de un experimento representativo. Cada experimento fue realizado mínimo dos veces.

6.14. Purificación Parcial del Proteosoma 20S de *G. intestinalis*

En experimentos preliminares se detectó actividad del proteosoma luego del fraccionamiento de extractos de proteína crudos de *G. intestinalis* (Fig. 36). Con esta información iniciamos la purificación parcial del proteosoma de este protozooario a partir de extractos proteicos obtenidos de cultivos a gran escala de trofozoítos. La precipitación con sulfato de amonio del extracto completo produjo un *pellet* de aproximadamente 60 mg de proteína con actividades peptidasa: similar a quimotripsina (CTL) y similar a caspasa (CL) altamente específicas (Fig. 48). El *pellet* fue fraccionado mediante un protocolo que incluyó separación en gradientes de densidad preparados en glicerol y tres cromatografías en columnas de: 1) DEAE Affi-Gel Blue Gel, 2) Hidroxiapatita (HAP) y 3) DEAE Fractogel. La actividad del proteosoma en las fracciones obtenidas fue seguida mediante ensayos de actividad en placa usando péptidos fluorogénicos para determinar la actividad CTL (Suc-LLVY-AMC) y la actividad CL (Z-LLE-AMC). Como se observa en la figura 49A, después del fraccionamiento en los gradientes de glicerol se evidencian dos picos de actividad enzimática, el segundo pico (fracciones 12—21) fue definido como proteosoma 20S basado en: 1) La ubicación del pico, esta actividad peptidasa coincide con el pico de actividad del 20S bovino empleado como control (datos no mostrados), 2) Los patrones electroforéticos observados en SDS-PAGE, que muestran en esta área un enriquecimiento para proteínas de 22 a 36 kDa (Fig. 49B) y 3) La inhibición específica de este pico por epoxomicina (datos no mostrados). Las fracciones seleccionadas de este paso de purificación fueron mezcladas y fraccionadas en una columna de gel DEAE Affi-Gel Blue. Los ensayos de actividad de las fracciones eluidas de esta columna mostraron algunos picos de actividad similar al proteosoma en las fracciones 29—51 (Fig. 50A), pero solo la actividad de las fracciones 29—40 fue inhibida por epoxomicina y coincidió con las fracciones que presentaban un patrón en SDS-PAGE, rico en proteínas en la región de 22 a 36 kDa (Fig. 50B). Estas fracciones fueron seleccionadas, mezcladas y fraccionadas en una columna de HAP. Luego de esta cromatografía se observó un pico, que eluyó muy temprano, y el cual mostró actividad CTL (datos no mostrados) y CL (Fig. 51A), junto con el patrón electroforético en SDS-PAGE que muestra enriquecimiento de proteínas que migran al mismo nivel de las subunidades del 20S control (Fig. 51B). Además, luego de ser analizadas por PAGE en condiciones nativas, estas fracciones mostraron un bandeo característico de una partícula central 20S parcialmente pura (Fig. 52A). Las fracciones de este pico fueron mezcladas y concentradas mediante su paso a través de una columna de DEAE Fractogel. De la elución de esta columna se encontraron cinco fracciones con actividades peptidasa CTL y CL, específicas del

proteosoma 20S, las cuales mostraron un patrón en SDS-PAGE de al menos 10 proteínas con masas moleculares entre 22--36 kDa (Fig. 52B).

El proteosoma 20S parcialmente puro obtenido en este trabajo muestra las características típicas de actividad peptidasa sobre péptidos fluorogénicos y patrones electroforéticos de las partículas centrales 20S de otros eucariotas (González et al. 1996; Hua et al. 1996; Robertson 1999; Silva-Jardim, Hortab, and Ramalho-Pinto 2004). Este es el primer reporte de purificación parcial del proteosoma 20S de *G. intestinalis* empleando un protocolo reproducible y que permite obtener una partícula central con actividad enzimática detectable. Emmerlich y colaboradores (1999) purificaron el 20S de *Giardia*, pero no lograron identificar actividad peptidasa durante este proceso. La obtención de la partícula central 20S parcialmente pura con estas características, constituye una herramienta muy valiosa que permitirá iniciar análisis enzimáticos sobre proteínas blanco control y determinar la especificidad y características de degradación proteica de esta enzima, empleando aproximaciones experimentales *in vitro*.

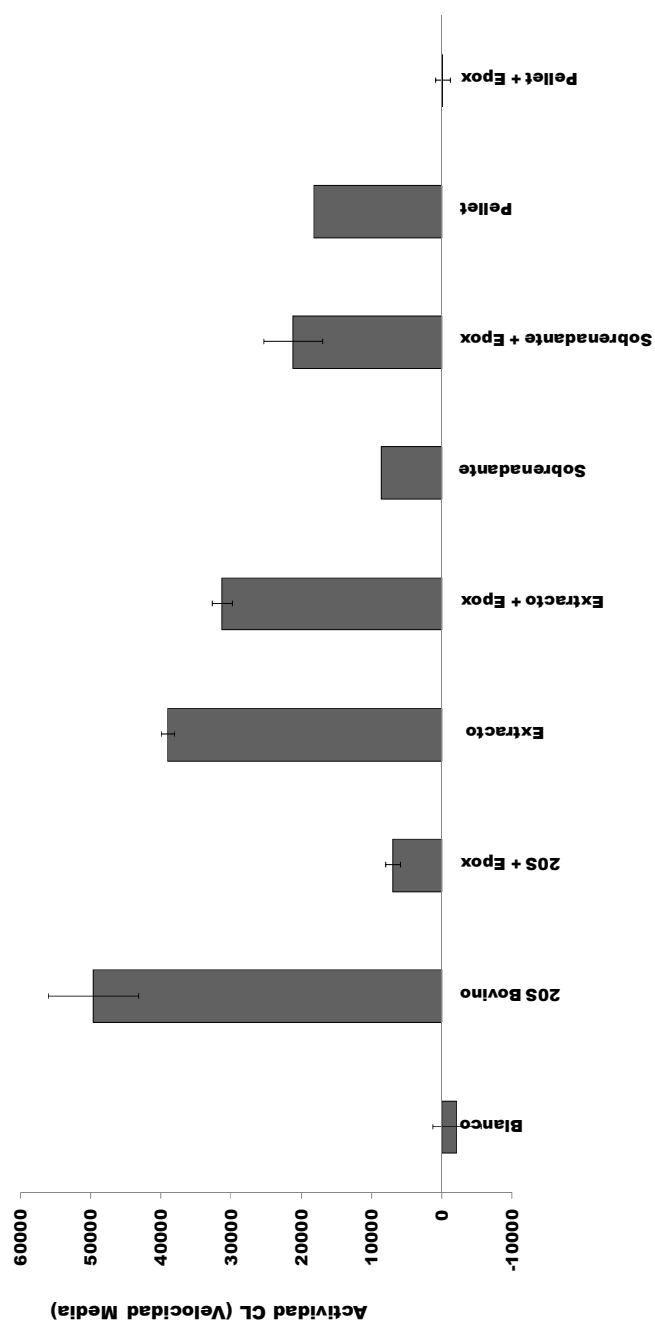


Figura 48. Purificación del Proteosoma de *G. intestinalis*—Fraccionamiento del Extracto Crudo por Precipitación con Sulfato de Amonio. Se produjeron extractos de proteína de cultivos a gran escala de trofozoítos y se fraccionaron por precipitación con sulfato de amonio (40%). Se obtuvo un *pellet* de 60 mg en el cual se conservó la mayor parte de la actividad proteosomal específica, como se observa luego de la inhibición con epoxomicina 1 μ M. Se incluyó 20S puro de bovino como control (m52 20S) y un blanco con *buffer* reacción y sustrato fluorogénico. La concentración de proteína fue normalizada a 20 μ g total, en cada punto analizado. Los resultados son el promedio \pm la desviación estándar de un experimento representativo de actividad peptidasa empleando el péptido fluorogénico Z-LLE-AMC. Cada experimento fue realizado mínimo dos veces.

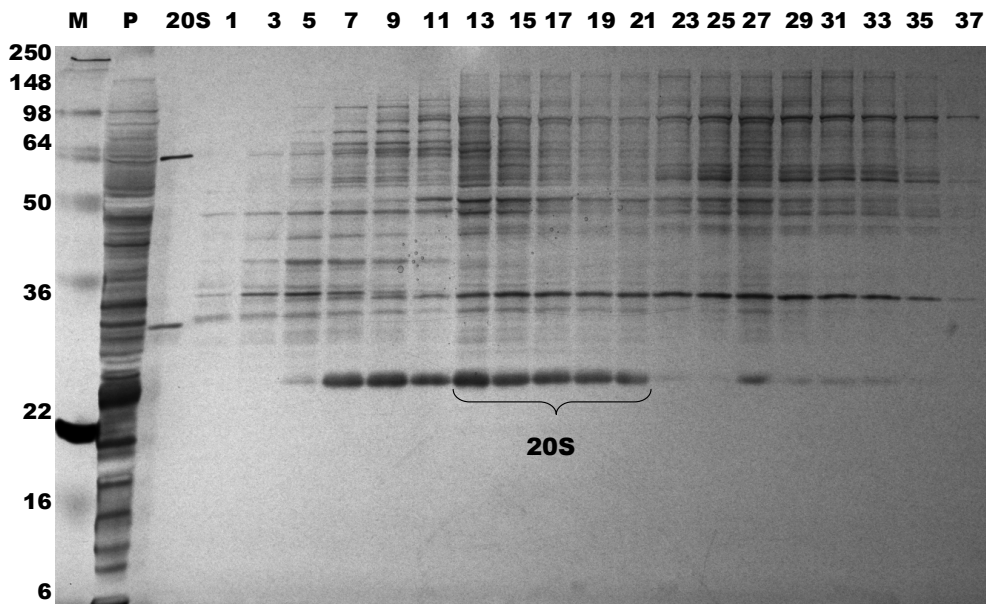
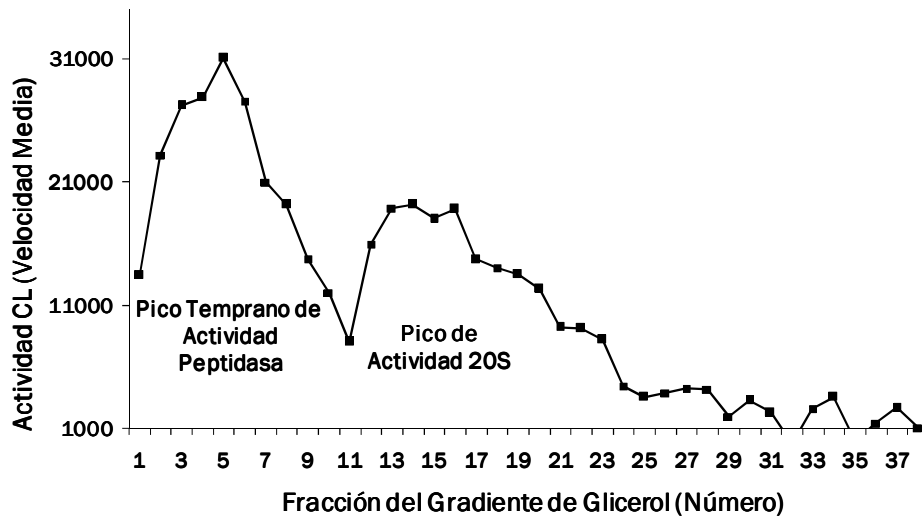


Figura 49. Purificación del Proteosoma de *G. intestinalis* - Fraccionamiento del *pellet* en Gradientes de Glicerol. **A.** Curva de actividad peptidasa similar a caspasa (CL). Se observan dos picos de actividad, el segundo pico (fracciones 12–21) es inhibido específicamente por epoxomicina (datos no mostrados). **B.** SDS-PAGE de las fracciones de la figura A, tinción con plata (Invitrogen, Carlsbad, CA). Se observa que en las fracciones 12–21 hay un enriquecimiento de proteínas de 22 a 36 kDa, este rango de tamaño corresponde a las subunidades de la partícula central 20S del proteosoma. **M**, marcador de peso molecular para proteínas (SeeBlue plus 2); **20S**, proteosoma 20S puro de Bovino (m52 20S); **P**, 5 uL de muestra inicial; **El corchete** señala las fracciones seleccionadas para continuar con el proceso de purificación. La figura representa el resultado de un experimento representativo, de dos realizados.

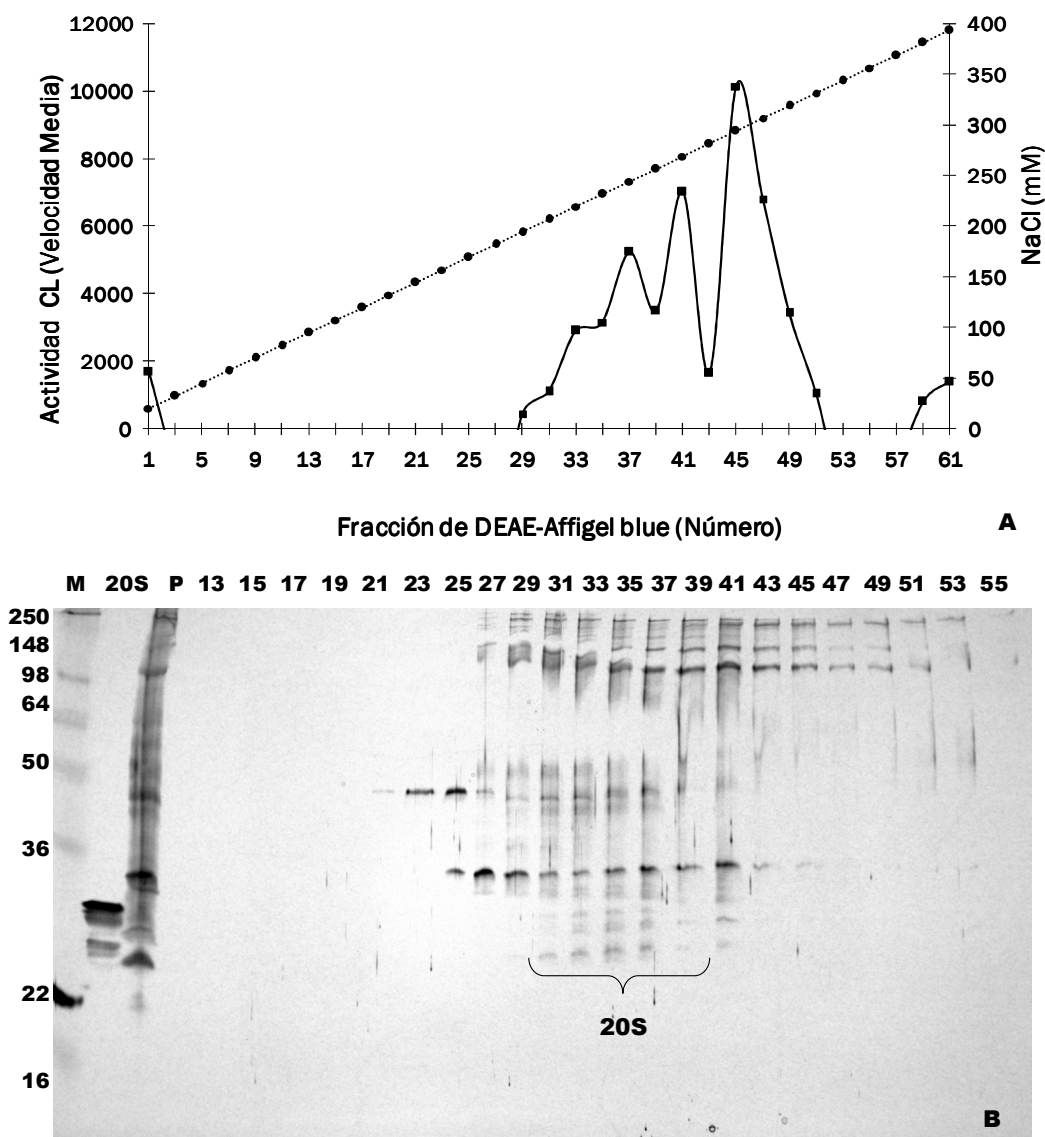


Figura 50. Purificación del Proteosoma de *G. intestinalis* - Fraccionamiento en columna de DEAE Affigel Blue. **A**. Curva de actividad peptidasa similar a caspasa (CL) para las fracciones eluidas, con diferentes concentraciones (mM) de NaCl, de la columna de DEAE Affigel blue. Se observan varios picos de actividad, las fracciones 29 a 40 poseen la actividad específica del proteosoma. **B**. SDS-PAGE de las fracciones de la figura A, tinción con plata (Invitrogen, Carlsbad, CA). Se observa que en las fracciones 20–40 hay un enriquecimiento de proteínas de 22 a 36 kDa, este rango de tamaño corresponde a las subunidades de la partícula central 20S del proteosoma. **M**, marcador de peso molecular para proteínas (SeeBlue plus 2); **20S**, proteosoma 20S puro de Bovino (m52 20S); **P**, 5 uL de muestra inicial; **El corchete** señala las fracciones seleccionadas para continuar con el proceso de purificación. La figura representa el resultado de un experimento representativo, de dos realizados.

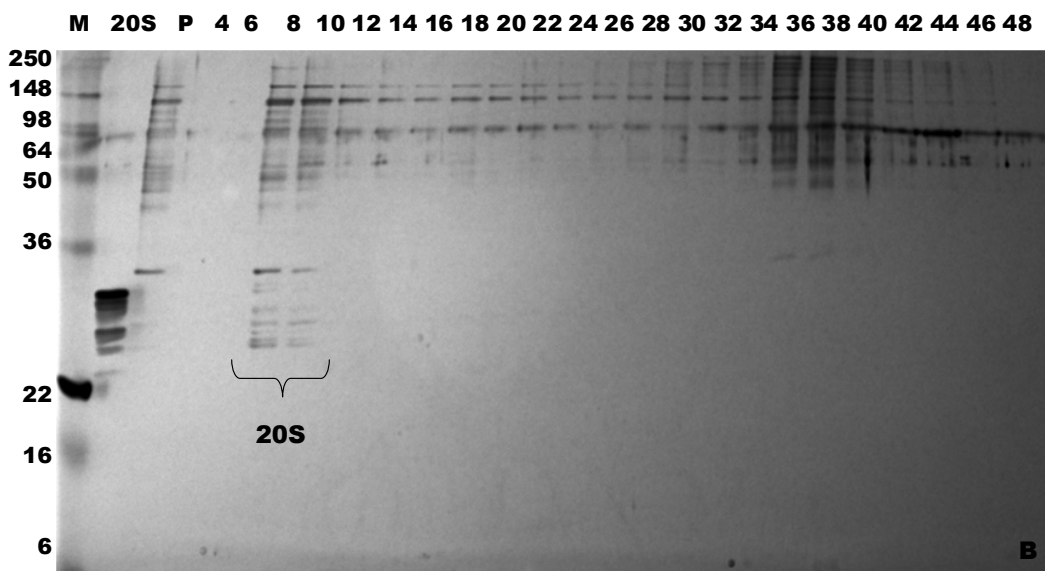
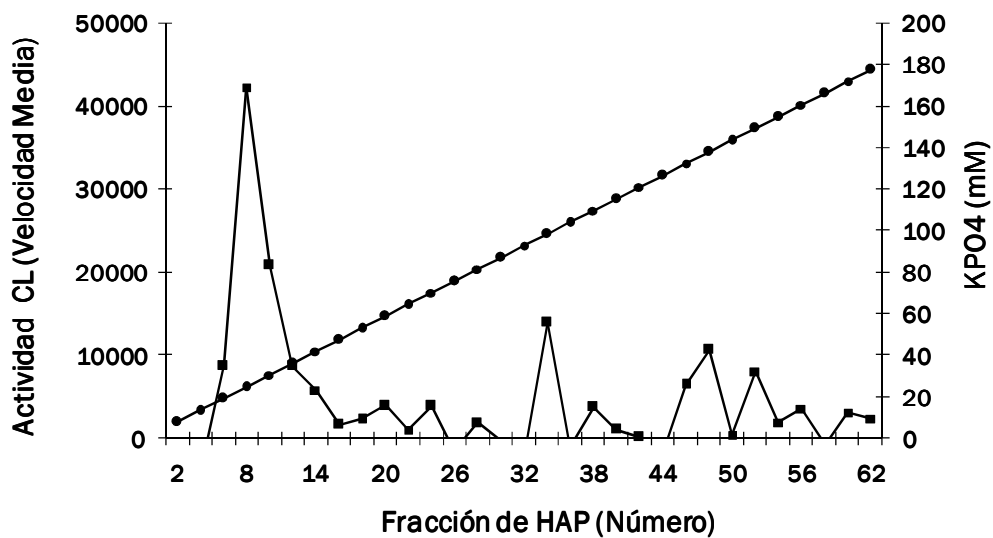


Figura 51. Purificación del Proteosoma de *G. intestinalis* - Fraccionamiento en columna de Hidroxiapatita (HAP). **A.** Curva de actividad peptidasa similar a caspasa (CL) para las fracciones eluidas, con diferentes concentraciones (mM) de KPO₄, de la columna de HAP. Se observan un pico de actividad eluido tempranamente, las fracciones 8 a 10 poseen la actividad específica del proteosoma. **B.** SDS-PAGE de las fracciones de la figura A, tinción con plata (Invitrogen, Carlsbad, CA). Se observa que en las fracciones 8 a 10 hay un enriquecimiento de proteínas de 22 a 36 kDa, este rango de tamaño corresponde a las subunidades de la partícula central 20S del proteosoma. **M**, marcador de peso molecular para proteínas (SeeBlue plus 2); **20S**, proteosoma 20S puro de Bovino (m52 20S); **P**, 5 uL de muestra inicial; **El corchete** señala las fracciones con el proteosoma 20S parcialmente puro de *G. intestinalis*. La figura representa el resultado de un experimento representativo, de dos realizados.

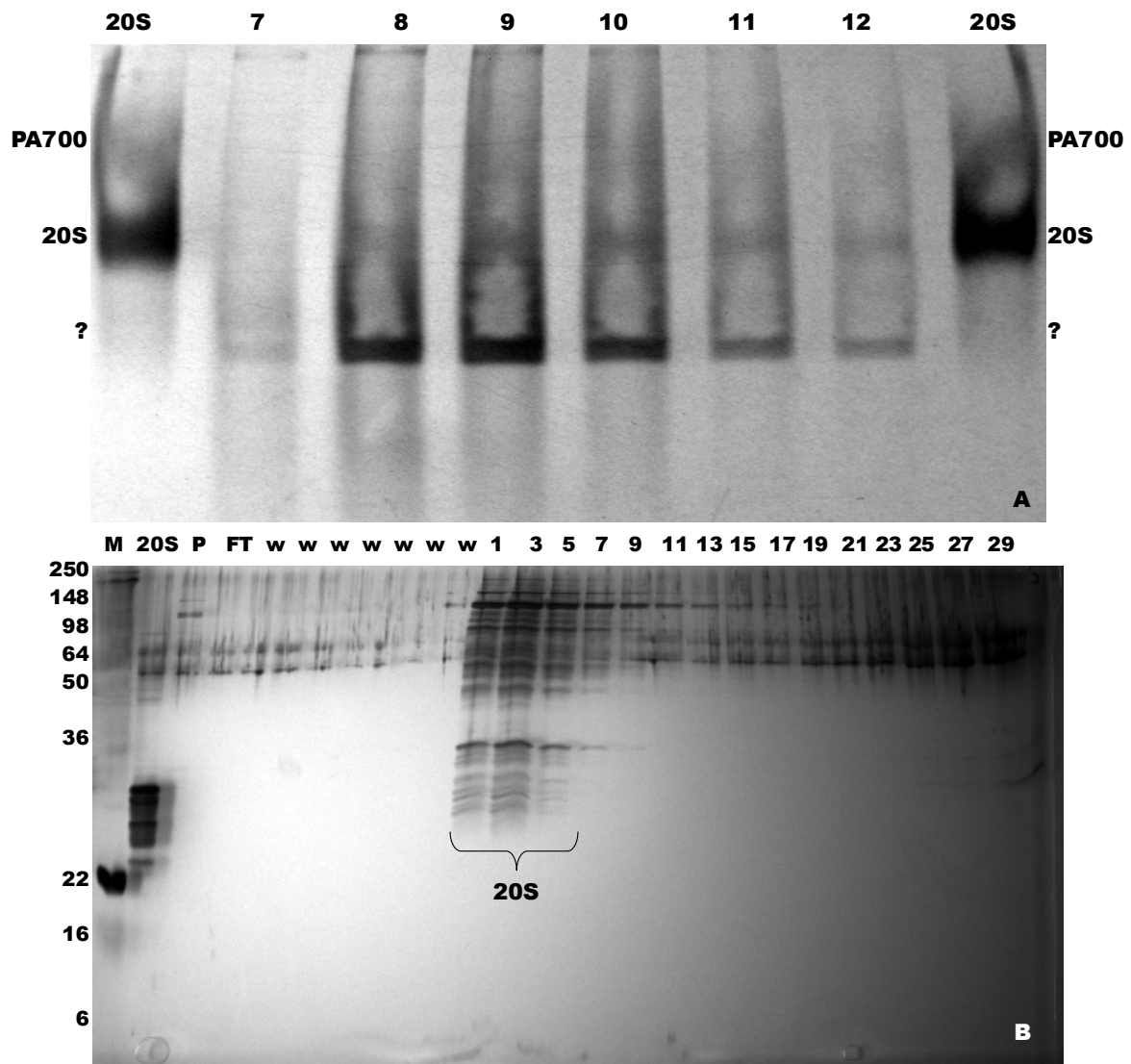


Figura 52. **Purificación del Proteosoma de *G. intestinalis*.** **A.** Evaluación de la pureza del 20S de *G. intestinalis* en PAGE nativo del 4%. Tinción con plata (Invitrogen, Carlsbad, CA) . Se analizaron las fracciones positivas de la cromatografía con HAP y se observó la presencia de la partícula 20S parcialmente pura en las fracciones 8 a 12. **B.** SDS-PAGE, tenido con plata Invitrogen, Carlsbad, CA) , de las fracciones obtenidas luego de la concentración del 20S de *G. intestinalis*, mediante cromatografía en DEAE Fractogel. Se logró la concentración de la partícula central 20S, encontrando un enriquecimiento marcado de proteínas entre 22 y 36 kDa. **M**, marcador de peso molecular para proteínas (SeeBlue plus 2); **20S**, proteosoma 20S puro de Bovino (m52 20S); **P**, 5 uL de muestra inicial; **FT**, 15 uL de muestra no unida a la columna; **w**, 15 uL de los lavados previos a la elución; **El corchete** señala las fracciones con el proteosoma 20S parcialmente puro de *G. intestinalis*.

6.14. Ensayos de Activación del Proteosoma de *G. intestinalis*

Generalmente el proteosoma 20S preparado de extractos frescos y/o cuando es aislado por métodos muy conservadores se encuentra en forma latente. Esta forma es inactiva contra sustratos proteicos y degrada sustratos peptídicos en un nivel muy bajo. Los protocolos de producción de extractos proteicos y purificación del proteosoma de *G. intestinalis* que empleamos en este trabajo incluyeron glicerol al 10%, este compuesto ayuda a mantener la partícula central en este estado de latencia (Tanaka et al, 1986; McGuire et al, 1989) y presumiblemente preserva los complejos 20S y 26S en formas muy cercanas a las fisiológicas (Coux et al, 1996). Se puede lograr una activación dramática de la partícula central 20S con el uso de varios tratamientos: 1) Incubación a 37 °C, 2) Calentamiento a 55 °C, 3) Incubación con polipéptidos básicos, SDS, ácidos grasos, o diálisis contra agua (Tanaka et al, 1986; Rivett et al, 1993; Dahmann et al, 1993). La mayoría de estos tratamientos causan cambios conformacionales en las subunidades catalíticas β , que incrementa su actividad enzimática o generan alteraciones en las subunidades de los anillos α que facilitan la entrada de los sustratos. Se evaluó la capacidad de activación de varios compuestos comúnmente usados en la partícula central 20S de eucariotas. Ninguno de los compuestos ensayados mostró un efecto sobre la actividad peptidasa del proteosoma de *G. intestinalis* evaluada tanto en extractos proteicos como en el 20S parcialmente puro (Fig. 53 y Fig. 54). Los tratamientos con PA28 α y PA700 incrementan entre 10 y 50 veces la actividad peptidasa del proteosoma 20S (Coux et al, 1996). Aparentemente *G. intestinalis* tiene un proteosoma 26 S, con una partícula central 20S y una partícula reguladora 19S (PA700) (Emmerlich et al, 1999; Emmerlich et al, 2001), pero se desconocen los mecanismos regulatorios específicos de la partícula 20S y su asociación con PA700. Es probable que la ausencia de efecto de los experimentos de activación en *G. intestinalis* se deba a la incapacidad de la PA700 pura, obtenida de eucariotas superiores (bovinos), para generar los cambios conformacionales necesarios para la activación de la partícula central 20S de este protozoo. *G. intestinalis* es considerado un eucariota tempranamente divergente y básico en muchos de sus sistemas (Morrison et al, 2007), por lo que es muy probable que las subunidades de su PA700 tengan diferencias importantes, con sus contrapartes bovinas y estas diferencias pueden ser suficientes para evitar el acoplamiento exitoso de estas dos partículas. Además, no se debe descartar que el sistema *in vitro* de activación que nosotros empleamos carezca de algún cofactor esencial en el proceso de activación del proteosoma de *Giardia*.

La ausencia de efecto de activación cuando se empleo PA28 α puede deberse a que esta partícula ha sido identificada solo en organismos con sistema inmune adaptativo (Song et al, 1996) y *G. intestinalis* es considerado un eucariota basal, por lo cual es muy probable que carezca de este tipo de partícula. Esta posibilidad se ve sustentada ya que no fue posible encontrar secuencias homólogas para PA28 o para PA26 en el genoma de *G. intestinalis* (Datos no mostrados). Por otro lado la ausencia de efecto de las bajas concentraciones de SDS puede deberse a un efecto idiosincrático, SDS ha mostrado que tiene un amplio rango de efectos sobre el proteosoma de Archaea (Maupin-Furlow et al, 1998) y existen reportes contradictorios con respecto a su efecto sobre los proteosomas de eucariotas (Arrivas y Castaño 1990; Dalhmann et al, 1993).

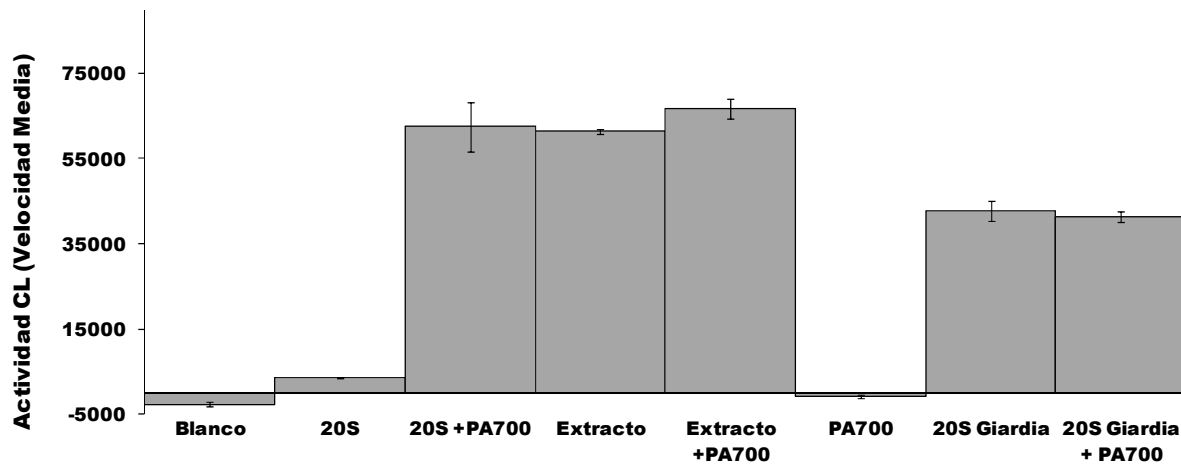


Figura 53. Efecto de PA700 sobre la Actividad Peptidasa de *G. intestinalis*. El extracto de proteínas (20 ug) y la partícula central 20S parcialmente pura (20 uL) de *G. intestinalis* fueron incubados a 37 °C en Buffer reacción por 30 min con 0,1 ug/uL de PA700, para evaluar el efecto sobre la actividad peptidasa similar al proteosoma. Como controles se incluyeron: el blanco con los buffer de reacción, 20S bovino solo (5 ug) y 20S bovino activado con PA700 y PA700 (0,1 ug/ uL) en buffer reacción. La gráfica muestra el resultado promedio \pm desviación estándar de un experimento representativo con el sustrato Z-LLE-AMC. Cada experimento se realizó mínimo dos veces.

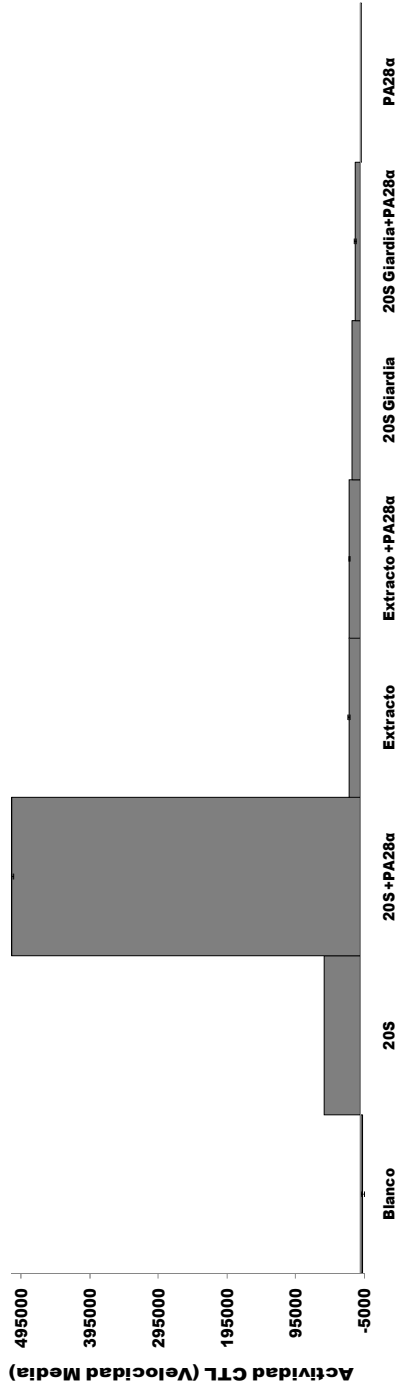


Figura 54. **Efecto de PA28α sobre la Actividad Peptidasa de *G. intestinalis*.** El extracto de proteínas (20 ug) y la partícula central 20S parcialmente pura (20 uL) de *G. intestinalis* fueron incubados a 37 °C en Buffer reacción por 30 min con 0,2 ug/uL de PA28α, para evaluar el efecto sobre la actividad peptidasa similar al proteosoma. Como controles se incluyeron: el blanco con los buffer de reacción, 20S bovino solo (5 ug) y 20S bovino activado con PA28α y PA28α (0,2 ug/ uL) en buffer reacción. La gráfica muestra el resultado promedio ± desviación estándar de un experimento representativo con el sustrato Suc-LLY-AMC. Cada experimento se realizó mínimo dos veces.

7. CONCLUSIONES

7.1. Se estandarizó una metodología para la purificación rápida y eficiente de ubiquitina a partir de extractos de proteína de *G. intestinalis* y se generó un anticuerpo específico contra ubiquitina y los conjugados ubiquitina proteína.

7.2. Se estandarizó una técnica de *western blot* para la detección y posterior análisis de los patrones de ubiquitinación durante el ciclo de vida de *G. intestinalis*.

7.3. Se determinó la presencia de conjugados ubiquitina-proteína en el estadio de trofozoíto y durante el proceso de enquistación de *G. intestinalis*. Esta información junto con trabajos preliminares, que han identificado la presencia de todos los componentes del sistema de ubiquitinación, sugiere que este sistema es activo durante el ciclo de vida de *Giardia*.

7.4. La inhibición específica del proteosoma de *G. intestinalis* no afecta la viabilidad y morfología de los parásitos en estado vegetativo, generando únicamente efectos leves sobre el crecimiento celular. Sin embargo se comprobó que los inhibidores penetran el parásito y causan acumulación de los conjugados ubiquitina-proteína. Por su parte los efectos inhibitorios sobre la enquistación no afectan la eficiencia del proceso pero causan una reducción en la viabilidad de los quistes producidos. Estos datos permiten sugerir la participación del sistema ubiquitina-proteosoma en la regulación de procesos de replicación y diferenciación en *G. intestinalis*.

7.5. Los extractos crudos de proteína de trofozoítos en replicación activa y de trofozoítos inducidos a enquistar poseen actividades peptidasa similares al proteosoma (CTL y CL) y estas actividades provienen de la presencia de dos complejos enzimáticos diferentes: una enzima con actividad tripeptidil peptidasa de aproximadamente 10S y el proteosoma 20S. Las actividades peptidasa similar al proteosoma presentes en la fase vegetativa del ciclo de vida de *G. intestinalis* son basales y se incrementan dramáticamente 24 h después de la inducción del proceso de enquistación y su inhibición causa ausencia de la formación de quistes. Estos resultados sugieren una activación del sistema de degradación específica de proteínas durante la enquistación, activación que parece ser indispensable para el desarrollo adecuado de este proceso de diferenciación en *Giardia*.

7.6. Por primera vez se identificó, en *G. intestinalis*, la presencia de un complejo enzimático de ~ 10S, el cual posee actividades peptidasa CTL, CL y tripeptidil peptidasa y que es inhibido por AAFCMK y MG132. El nivel de actividad de esta enzima es mayor a la actividad del proteosoma tanto en el estadio de trofozoíto como durante el proceso de enquistación de *G. intestinalis*. Mostrando un pico de actividad durante la enquistación. La inhibición de esta actividad enzimática causa un leve aumento en el tiempo de duplicación celular durante la fase vegetativa del parásito y una importante reducción en la eficiencia del proceso de enquistación. Estos resultados permiten sugerir la participación activa y en conjunto con el proteosoma de esta nueva peptidasa en los procesos de degradación proteica en *G. intestinalis*.

7.7. Se estandarizó un protocolo para la purificación parcial del proteosoma 20S de *G. intestinalis* obteniendo una partícula con actividad peptidasa detectable. Esta constituye una herramienta muy valiosa para el estudio y caracterización bioquímica y fisiológica de esta enzima en este protozooario.

7.8. Este trabajo contiene información novedosa y muy interesante que en su conjunto constituye la base orientadora que direccionará los estudios funcionales, moleculares y bioquímicos del sistema de ubiquitinación y degradación proteosomal en *G. intestinalis*.

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