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FACULTAD DE CIENCIAS DEL MAR Y AMBIENTALES
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Caracterización de las enzimas digestivas de la langosta *Panulirus argus* (Latreille, 1804): factores intrínsecos y extrínsecos que intervienen en su regulación

TESIS DOCTORAL

ERICK PERERA BRAVET

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Universidad de Cádiz
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Caracterización de las enzimas digestivas de la langosta *Panulirus argus* (Latreille, 1804): factores intrínsecos y extrínsecos que intervienen en su regulación

Memoria presentada por D. Erick Perera Bravet para optar al Grado de Doctor en Ciencias

Fdo: Erick Perera Bravet

Los directores:

Dr. D. Juan Miguel Mancera Romero
Catedrático de Zoología
Universidad de Cádiz

Dra. Da. Olimpia Carrillo Farnés
Profesora Titular de Bioquímica
Universidad de la Habana

FACULTAD DE CIENCIAS DEL MAR
Y AMBIENTALES
DEPARTAMENTO DE BIOLOGIA
Polígono Río San Pedro
11510 Puerto Real, Cádiz, España
Teléfono: 34-956-016014
Fax: 34-956-016040

JUAN MIGUEL MANCERA ROMERO, Catedrático de Zoología del Departamento de Biología de la Facultad de Ciencias del Mar y ambientales de la Universidad de Cádiz,

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Que la presente memoria titulada **Caracterización de las enzimas digestivas de la langosta *Panulirus argus* (Latreille, 1804): factores intrínsecos y extrínsecos que intervienen en su regulación**, que presenta D. Erick Perera Bravet para optar al Grado de Doctor en Ciencias, ha sido realizada bajo su dirección y autoriza su presentación y defensa.

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Dr. D. Juan Miguel Mancera Romero

UNIVERSIDAD DE LA HABANA
DEPARTAMENTO DE BIOQUÍMICA
FACULTAD DE BIOLOGÍA
CALLE 25, No. 455, e/ J e I, VEDADO
CIUDAD DE LA HABANA, CUBA

OLIMPIA VICTORIA CARRILLO FARNÉS, Profesora Titular del Departamento de Bioquímica de la Facultad de Biología de la Universidad de la Habana,

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Capítulo 1

Introducción general

1.1. LANGOSTAS ESPINOSAS

Las langostas espinosas (Crustacea: Decapoda: Palinuridae) habitan las zonas bajas de las plataformas insulares o continentales, fundamentalmente en ambientes rocosos o de arrecifes, aunque pueden encontrarse también a gran profundidad. Estos crustáceos son uno de los recursos pesqueros más importantes en todo el mundo por su altísimo valor comercial (Lipcius y Eggleston, 2000). De las aproximadamente 47 especies que existen, 33 soportan pesquerías comerciales siendo *Panulirus*, *Palinurus* y *Jasus* los géneros más importantes desde el punto de vista comercial. Las langostas del género *Panulirus* son típicas de regiones tropicales y constituyen el 82,2% de las capturas mundiales de palinúridos (Lipcius y Eggleston, 2000). La especie *Panulirus argus* es la que presenta la distribución geográfica más amplia, localizándose desde Carolina del Norte en EEUU hasta Brasil (Phillips y Melville-Smith, 2006).

1.1.1. Ciclo de vida

La langosta *P. argus* presenta un ciclo de vida largo y complejo (Fig. 1). La puesta tiene lugar en aguas profundas de las plataformas insulares o continentales y tras un estadio larval oceánico (filosoma) de aproximadamente 6 - 12 meses (Lewis, 1951) [6.5 meses en condiciones de laboratorio (Goldstein *et al*., 2008)], ocurre la metamorfosis con la que adquieren una forma similar al adulto (puérulos). Los puérulos de langostas espinosas no se alimentan y nadan activamente a expensas de sus reservas de lípidos, sobre todo lípidos polares, hacia aguas someras para adquirir la forma de vida bentónica característica de la fase adulta (Jeff *et al*., 1999; 2002). Durante esta etapa el tracto digestivo sufre varias transformaciones para adaptarse al cambio sustancial en la dieta que experimentarán en el nuevo hábitat (Wolfe y Felgenhauer 1991; Nishida *et al*., 1990; 1995; Abronhosa y Kittaka, 1997). Durante su ciclo de vida, el crecimiento de las langostas espinosas se sucede a través de las ecdisis o mudas, proceso que en los crustáceos implica cambios significativos en la conducta alimentaria (Lipcius y Herrnkind, 1982), composición bioquímica (Travis, 1955) y fisiología digestiva (Van Wormhoudt, 1974; Fernández *et al*., 1997; Klein *et al*., 1996).

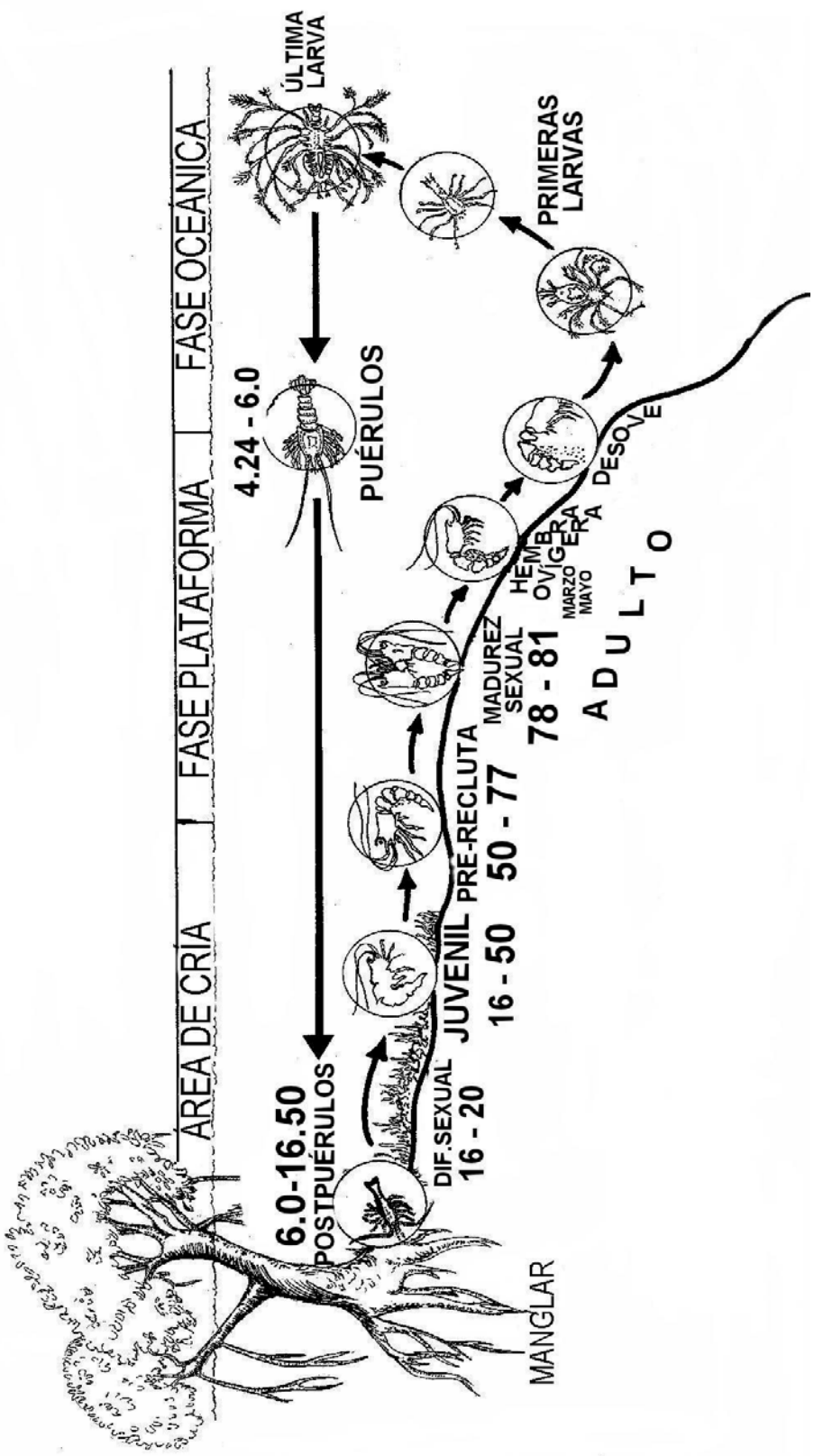

Fig. 1. Representación del ciclo de vida de la langosta espinosa *Panulirus argus* (Tomado de Cruz y de León, 1991).

1.1.2. Hábitos alimentarios

Los palinúridos son considerados carnívoros estrictos y de hábitos nocturnos. Se conoce muy poco sobre la alimentación natural de las larvas filosomas, aunque se asume que se alimenten de organismos plantónicos de cuerpo suave. En cautiverio se han alimentado experimentalmente con *Artemia*, gónadas de mejillón y larvas de peces (Kittaka, 1997). Los alimentos preferidos de postlarvas y juveniles muy tempranos de *P. argus* son los copépodos, anfípodos, isópodos, holoturias, foraminíferos y esponjas (Lalana y Ortiz, 1991). Los estudios de contenido estomacal de juveniles y adultos han podido establecer el carácter de depredación oportunista que caracteriza a *P. argus*, así como su amplio espectro alimenticio. De este modo, en el Caribe mexicano hallaron que en los estómagos de *P. argus* predominan los crustáceos y moluscos (Colinas-Sánchez y Briones-Foorzan, 1990). En especimenes de *P. argus* obtenidos en la plataforma sur-occidental cubana los grupos más frecuentes observados en el análisis estomacal fueron los gasterópodos, bivalvos, anomuros, braquiuros y erizos en el biotopo de seibadal y los gasterópodos, poliplacóforos, erizos y braquiuros en el arrecife (Herrera *et al*., 1991). Resultados similares fueron obtenidos por Cox *et al.* (1997) en especimenes de *P. argus* en la Florida. Los autores postulan que la especie sigue igual estrategia de alimentación en todo su rango biogeográfico, no encontrándose diferencias entre sexos en ninguno de los estudios realizados.

1.2. ACUICULTURA DE LANGOSTAS ESPINOSAS

En los últimos años se ha incrementado la preocupación sobre la sobre-explotación de las poblaciones de langostas espinosas, proceso que ha estado incentivado por la alta demanda de estos crustáceos así como por los elevados precios que mantienen en el mercado internacional. En este escenario, se han intensificado los estudios encaminados a lograr una tecnología para incrementar la producción de langostas espinosas a través de la acuicultura. Sin embargo, el interés en cultivar langostas data del siglo XIX, tanto para langostas espinosas como para las langostas del género *Homarus*.

Los primeros intentos de cultivo de langostas datan de 1885, cuando biólogos estadounidenses realizaron los primeros ensayos para obtener larvas de la langosta americana *Homarus americanus*. En la actualidad la tecnología para la producción de juveniles de langostas del género *Homarus* en hatcheries está disponible (Schmalenbach *et al*., 2009). Aunque los costes de producción aún son elevados, algunos estudios ya han utilizado las langostas producidas en cautiverio para evaluar programas de repoblación y mejora de stocks (Schmalenbach *et al*., 2011). En España se ha cultivado exitosamente el bogavante (*Homarus gammarus*) dentro del programa AquaReg (Galicia-Irlanda-Noruega), en el marco de iniciativa comunitaria INTERREG IIIC (Uglem *et al*., 2006). Sin embargo, la conducta agresiva de estas langostas y el canibalismo, son las causas fundamentales de los elevados costos de producción.

A diferencia de los homáridos, los hábitos gregarios de las langostas espinosas las hacen más apropiadas para el cultivo en altas densidades, aunque las fases larvales son difíciles de cultivar. El primer experimento con larvas de langostas espinosas se realizó en Japón (Hattori y Oishi, 1899, citado por Kittaka, 1997). El ciclo de varias especies de langostas espinosas ha sido cerrado en cautiverio, aunque el cultivo larvario a escala comercial no ha sido factible hasta nuestros días debido a varios factores, tales como una etapa larval muy larga, problemas en la alimentación de las larvas, enfermedades y altas mortalidades obtenidas en el laboratorio (Kittaka, 1997). Sin embargo, recientemente se han obtenido grandes avances en el cultivo larvario de langostas espinosas y se ha abierto nuevamente el debate sobre la posibilidad de obtener semillas de langostas espinosas de hatcheries. La compañía australiana "Lobster Harvest" ha obtenido éxitos en el cultivo larval de *P. ornatus* durante el período 2006-2008, que les ha permitido obtener y cultivar en el 2010 (por primera vez para una langosta espinosa), varias cohortes de segunda generación (F2) en enero del 2010, con supervivencias de 5-10% (de filosoma I a juvenil) (Barnard *et al*., 2011). Aunque mucho más modestos, también se están obteniendo resultados muy alentadores en EEUU con la especie *P. argus* (Goldstein y Nelson, 2011).

Una práctica primitiva es la de mantener las langostas en cautiverio después de la captura por un período de muda para incrementar el peso, permitir la regeneración de apéndices perdidos, o esperar el momento más oportuno para la comercialización. Esta práctica se

realiza por algunos grupos comerciales en el sur de Australia (Van Barneveld, 2001; Bryars y Geddes, 2005) y Nueva Zelanda (Jeffs, 2003), así como en la India (James y Marian, 2003), Brasil y Japón (Booth y Kittaka, 2000).

En la actualidad el mayor interés está centrado en la cría o engorde de postlarvas provenientes de captación natural, debido a la gran disponibilidad de semilla y el crecimiento acelerado de algunas especies tropicales. Se ha estimado que, debido a la alta mortalidad natural de las postlarvas (97-98%), su captura con fines acuícolas presenta un mínimo impacto sobre las pesquerías (Phillips *et al.*, 2003). En los últimos años el engorde en jaulas de langostas espinosas a partir de postlarvas ha tenido un desarrollo acelerado en países asiáticos, sobre todo en Vietnam a partir de 1992 [35,000 jaulas en el 2003; 49,000 jaulas en el 2006 (Nguyen *et al*., 2009)] (Fig. 2). La captura total de semilla de *P. ornatus* en Vietnam en el periodo 2005-2008 fue de 5 196 820, aunque la demanda se ha reducido en los últimos años debido a la ocurrencia de enfermedades (Nguyen *et al*., 2009). Las postlarvas son alimentadas con alimento fresco y generalmente alcanzan 1 Kg. en 18 meses con una supervivencia del 90% (Williams, 2007). La producción de langostas de cultivo en Vietnam varía entre 1400-1900 toneladas y los ingresos ascienden a 90 millones USD (Williams, 2007).

No obstante, existen muchas preocupaciones respecto a la sostenibilidad de esta nueva industria acuícola en relación con el uso de alimento fresco y contaminación ambiental asociada, aparición de enfermedades, variaciones en la disponibilidad de semilla y alimento, así como con la variabilidad en la calidad nutricional del alimento fresco (William, 2007). De todos estos aspectos, la ausencia de dietas artificiales que cubran los requerimientos nutricionales de las diferentes especies y permitan la disminución de los costos de producción y la contaminación, se considera el mayor impedimento tanto para la sostenibilidad de esta industria en la región Asia-Pacífico (William, 2007) como para su desarrollo en otras regiones tropicales como el Caribe (Jeffs y Davis, 2003; Perera, 2008). La cantidad total de alimento fresco (calamares, peces, crustáceos y moluscos) utilizado anualmente en Vietnam para el engorde de langostas espinosas es de aproximadamente 176 420 - 363 440 toneladas (Le, 2003).

Jeffs y Hooker (2000) analizaron por primera vez el engorde de langostas espinosas desde el punto de vista económico. Sin embargo, el estudio estuvo basado en resultados con langostas de aguas frías y el uso de instalaciones en tierra (tanques) para el cultivo. Los aspectos económicos generales de la engorda en jaulas de langostas tropicales fueron analizados por primera vez por Hambrey *et al*. (2001), mientras que Hung *et al*. (2010) analizaron en particular los relacionados con la alimentación (alimento fresco *versus* peletizado). Sin embargo, un análisis económico completo de esta actividad solo fue realizado muy recientemente (Petersen, 2011). El autor demuestra que en el caso de Vietnam, el alimento actualmente representa el 60% del costo de producción, y fundamenta que la introducción de dietas formuladas incrementaría las ganancias anuales de cada cultivador en 15 000 USD, y las de la industria en aproximadamente 24 millones de dólares.

A pesar de que se ha avanzado significativamente en la comprensión de los requerimientos nutricionales de langostas espinosas se continúa careciendo de dietas formuladas apropiadas para estas especies. La mayoría de los estudios encaminados a desarrollar estas dietas (Glencross *et al*., 2001; Ward *et al*., 2003; Smith *et al*., 2003, 2005; Johnston *et al*., 2003, 2007) han seguido la estrategia aplicada décadas atrás para camarones peneidos, mientras que aún existen muchas lagunas en el conocimiento de la bioquímica y fisiología digestiva de las langostas espinosas.



Fig. 2. (A) Jaulas flotantes y (B) encierros para el engorde de langostas espinosas en Vietnam. (C) Jaulas sumergidas para pre-engorde de postlarvas y juveniles tempranos en aguas someras antes de ser transferidos a las instalaciones de engorda. (D) Alimento utilizado en la engorda de langostas espinosas. Fotos tomadas de Hung y Tuan (2008).

1.3. DIGESTIÓN EN CRUSTÁCEOS

1.3.1. Morfología y funciones del tracto digestivo

El tracto digestivo de las langostas espinosas, al igual que en la mayoría de los crustáceos decápodos, es básicamente una estructura tubular (Fig. 3A) que se extiende a lo largo del cuerpo. Este tracto puede dividirse en tres regiones fundamentales: i) intestino proximal o estomodeo, de origen ectodérmico; ii) un intestino medio o mesodeo, de origen endodérmico; y iii) un intestino distal o proctodeo, de origen ectodérmico (Díaz-Iglesias y Mallea, 1971).

El intestino proximal es donde se realiza la digestión mecánica. Está conformado por la cavidad bucal, a la cual se asocian apéndices especializados en la ingestión del alimento. A continuación se encuentra un esófago corto que finaliza con dos cámaras gástricas (cardíaca y pilórica); las paredes internas de estas cámaras forman lámelas de varios tamaños, están revestidas por complejos de quitina y proteínas y presentan estructuras especializadas (Fig. 3B) en la trituración del alimento (Hobbs y Hooper, 2009). Esta capa es remplazada como parte del exoesqueleto, junto con el molino gástrico de la parte posterior de la cámara cardíaca, en cada ecdisis o muda. El alimento de la cámara anterior pasa a través del molino gástrico hacia la cámara pilórica o posterior, para luego volver nuevamente hacia la cámara anterior por las válvulas cardio-pilóricas. Este ciclo se repite varias veces hasta que las partículas sean lo suficientemente pequeñas como para pasar a la glándula digestiva a través de un filtro situado en la cámara pilórica (Barker y Gibson, 1977). El material indigestible se dirige al intestino posterior para su eliminación por las heces.

El intestino medio lo conforma la glándula digestiva o hepatopáncreas, órgano par compuesto por múltiples túbulos, formados a su vez por células diferenciadas (Fig. 3C). El hepatopáncreas es el órgano responsable de la síntesis y secreción de las enzimas digestivas y la asimilación de nutrientes, aunque también está implicado en la excreción, ciclo de la muda, almacenamiento de reservas orgánicas e inorgánicas, el metabolismo de lípidos y carbohidratos, control neuroendocrino y maduración gonádica (Dall y Moriarty, 1983).

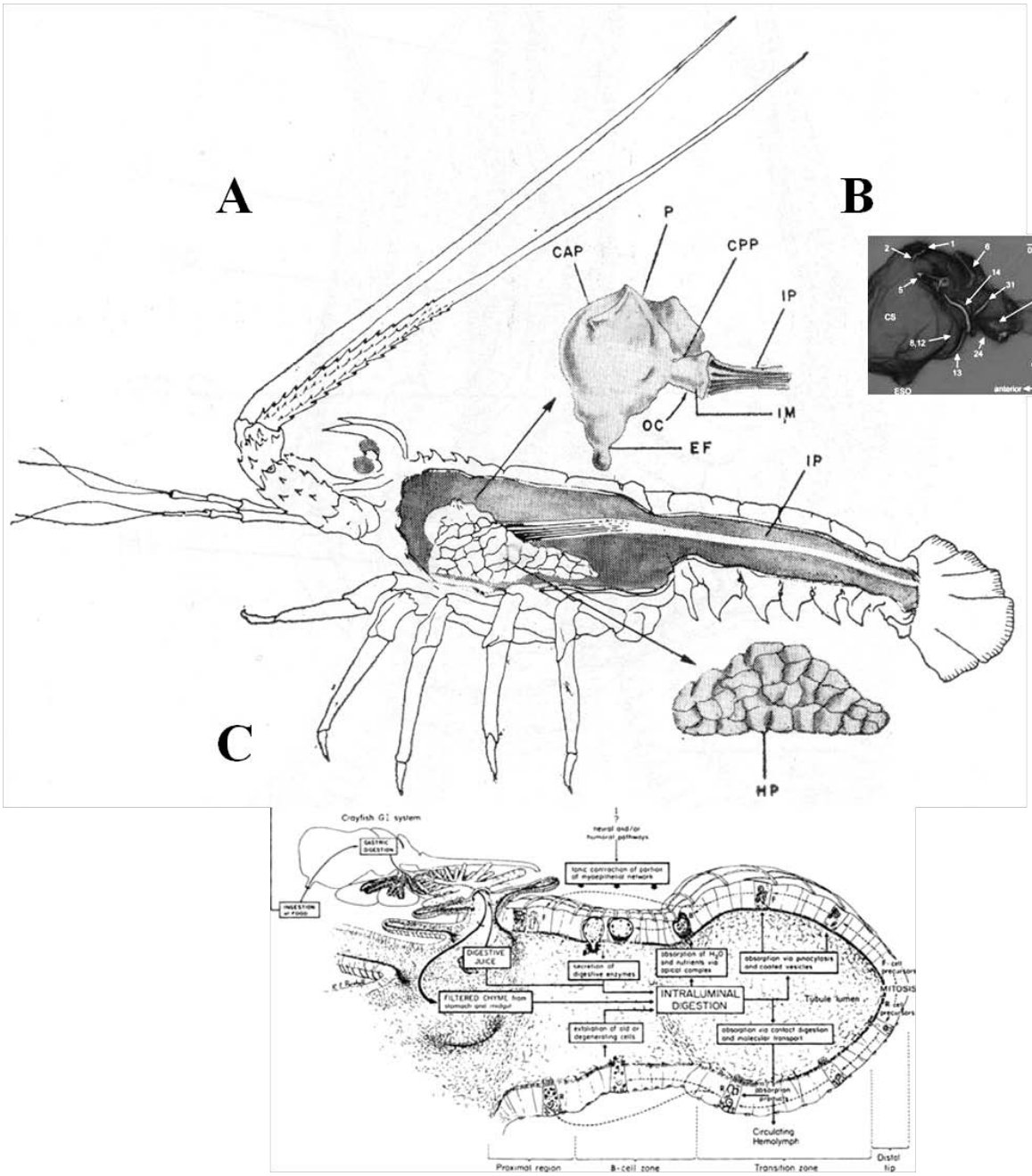

Fig. 3. (A) Sistema digestivo de langostas espinosas (Díaz-Iglesias y Mallea, 1971): EF: Esófago; CAP: Cámara anterior del proventrículo; P: Proventrículo; CP: Cámara posterior del proventrículo; OC: Orificios de comunicación con el hepatopáncreas; HP: Hepatopáncreas; IM: Intestino medio; IP: Intestino posterior. (B) Osículos de la cámara gástrica de langostas espinosas (Hobbs y Hooper, 2009). (C) Túbulo de la glándula digestiva o hepatopáncreas mostrando eje de diferenciación celular y sitio de secreción de enzimas digestivas (Barker y Gibson, 1977).

En el epitelio de los túbulos del hepatopáncreas se han descrito 4 tipos celulares: Embrionalenzellen, Fibrillenzellen, Blasenzellen y Resorptionzellen (Hirsch y Jacobs, 1928). De este modo, y basándose en esta nomenclatura, actualmente los distintos tipos celulares del hepatopáncreas se denominan: E (embryonic), F (fibrillar), B (blister-like) y R (resorptive). Las células E, en el extremo apical de los túbulos (Al-Mohanna *et al*., 1985), se diferencian en las células R y F (Ceccaldi, 1997). Las células F son las encargadas de sintetizar y secretar las enzimas digestivas al lumen del túbulo, para más tarde tomar material para la digestión intracelular y diferenciarse en células B con un sistema vacuolar muy desarrollado (Barker and Gibson, 1977; Al-Mohanna *et al*., 1985; Al-Mohanna y Nott, 1986). Las células R también absorben nutrientes y almacenan lípidos y glucógeno (Al-Mohanna y Nott, 1987, Brunet *et al*., 1994).

El intestino distal, segmento final del tracto digestivo, se localiza entre el intestino medio y la región anal. En esta sección se realiza la reabsorción de agua, se compactan las excretas y se sintetiza la membrana peritrófica. Esta región, igual que el intestino proximal, presenta recubrimiento quitinoso (Díaz-Iglesias y Mallea, 1971).

1.3.2. Fisiología de la digestión

En la actualidad se conocen muy bien las bases fisiológicas de la conducta alimentaria de los crustáceos decápodos, tanto las relacionadas con la búsqueda como con la ingestión del alimento. La mayor cantidad de información en este aspecto ha sido obtenida en *P. argus*, pues ha sido por muchos años el organismo modelo para el estudio de la quimiorrecepción en invertebrados acuáticos (ver revisión de Derby *et al*., 2001). Además, se conocen los mecanismos neuronales involucrados en el control de los movimientos rítmicos de la cámara gástrica de las langostas espinosas (controlado por el sistema nervioso estomatogástrico). De hecho, este sistema es uno de los mejores conocidos en el campo de la neurobiología de invertebrados (Marder *et al*., 1993; Nusbaum y Beenhakker, 2002; Hooper y DiCaprio, 2004; Marder y Bucher, 2007). La mayoría de los demás aspectos de la fisiología digestiva de langostas espinosas se han asumido de otros crustáceos debido a la ausencia de información.

La digestión mecánica en crustáceos comienza con las piezas bucales. Al desarrollar piensos para langostas espinosas se ha tratado de optimizar el tamaño y humedad del pellet, con el objeto de incrementar la cantidad de alimento ingerido y disminuir las pérdidas por manipulación (Sheppard *et al*., 2002). Una vez en la cámara gástrica, el alimento es triturado por el molino gástrico y mezclado con las secreciones enzimáticas de la glándula digestiva, por lo que la digestión química comienza una vez el alimento es ingerido. Esta primera etapa de digestión extracelular en la cámara gástrica presenta una duración variable en los crustáceos estudiados, desde 2 h en camarones peneidos (Nunes y Parsons, 2000), hasta 12 h en crustáceos más grandes como las langostas homáridos (Barker y Gibson, 1977; Sarda y Valladares, 1990) y cangrejos (Hill, 1976). La digestión extracelular continúa en los túbulos de la glándula digestiva, donde tiene lugar la absorción de nutrientes y digestión intracelular. En algunos crustáceos estudiados, esta digestión termina 12-48 h después de la ingestión, con la excreción (holocrina) de los productos de desecho de las células B (Dall y Moriarty, 1983; Brunet *et al*., 1994). La regeneración de las células de la glándula digestiva tiene lugar mediante mitosis de las células E, la cual comienza aproximadamente 24 h después de la ingestión (Al-Mohanna y Nott, 1986).

En general la fisiología digestiva de langostas espinosas ha sido objeto de pocos estudios y sólo recientemente se ha aportado información para la especie *Jasus edwardsii* en aspectos relacionados con la capacidad gástrica, tasa de vaciamiento gástrico e intestinal y variaciones de las principales enzimas digestivas después de la ingestión (Simon, 2009). A pesar de que estos estudios recientes han contribuido a explicar algunas diferencias respecto al aprovechamiento del alimento entre las langostas espinosas y otros crustáceos, todavía existen muchos aspectos de la bioquímica y fisiología digestiva de las langostas espinosas que se desconocen y se continúan asumiendo de estudios en otros crustáceos como los camarones peneidos y langostas del género *Homarus*. Estas lagunas en el conocimiento de la bioquímica y fisiología digestiva de las langostas espinosas han sido ignoradas por los nutricionistas en su intento de desarrollar piensos apropiados para las diferentes especies durante los últimos 20 años.

1.3.3. Enzimas digestivas

Las enzimas presentes en el tracto digestivo de crustáceos decápodos han sido ampliamente estudiadas (ver revisiones de Gibson y Barker, 1979; Carrillo-Farnés *el al*., 2007). La degradación de los carbohidratos de la dieta se realiza por las carbohidrasas, de las cuales la amilasa es la de mayor importancia en los crustáceos (Ceccaldi, 1997). Por otro lado, las lipasas y fosfolipasas son las enzimas encargadas de la digestión de los lípidos de la dieta en crustáceos. La presencia de una verdadera lipasa (glicerol-éster hidrolasa E.C.3.1.1.3) en crustáceos ha sido demostrada en *H. americanus* (Biesiot y Capuzzo, 1990; Brockerhoff *et al*., 1970). Las esterasas, aunque asociadas a muchas otras funciones, también juegan un papel importante en la digestión en crustáceos. Por último, las proteínas de la dieta son digeridas por las proteasas digestivas, las cuales han sido las enzimas digestivas más estudiadas en crustáceos, sobre todo las endopeptidasas (Carrillo *el al*., 2007). Aunque la acción de las endopeptidasas es complementada por la actividad de las exopeptidasas (carboxipeptidadas A y B, y leucin-aminopeptidasa), existe muy poca información sobre estas enzimas en crustáceos.

Las principales endopeptidasas en crustáceos son las serino proteasas colagenolíticas, las quimotripsinas y las tripsinas (Carrillo *el al*., 2007). Las verdaderas colagenasas pertenecen a la familia de las métalo proteinasas, mientras que en los crustáceos (incluyendo las langostas espinosas) la mayoría de las proteinasas con actividad colagenolítica pertenecen a la familia de las serino proteinasas, en ocasiones presentando también actividad tripsina o quimotripsina (Iida *et al*., 1991). Debido a esta variación en la especificidad de las serinopeptidasas de crustáceos, a partir de 1992 el Comité de Nomenclatura (sucesor de la Comisión de Enzimas) de la Unión Internacional de Bioquímica y Biología Molecular (NC-IUBMB) (www.chem.qmul.ac.uk/iubmb/enzyme) recomienda el uso del término “braquiurinas” (EC 3.4.21.32) para las serino endopeptidasas digestivas de crustáceos.

Dentro de las braquiurinas se distinguen dos tipos: i) Tipo I, que pueden hidrolizar el colágeno en su forma nativa, y ii) Tipo II, que tienen solo actividad tripsina (Lu *et al*., 1990). Las braquiurinas Tipo Ia poseen amplia especificidad, presentando actividad

tripsina, quimotripsina y elastasa. Los representantes más estudiados de este grupo son las serino peptidasas colagenolíticas (Eisen *et al.*, 1973; Grant *et al.*, 1983; Tsu y Craik, 1996; Tsu *et al.*, 1997). Sin embargo, las braquiurinas Tipo Ib tienen reducida su actividad tripsina, mientras que mantienen el resto de las especificidades del Tipo Ia, siendo los representantes más estudiados de este grupo las quimotripsinas (Tsai *et al*., 1986,1991; Van Wormhoudt *et al*., 1992).

Aunque las enzimas tipo quimotripsina juegan un papel importante en la digestión de crustáceos (Tsai *et al*., 1986, 1991; Van Wormhoudt *et al*., 1992) incluyendo a las langostas espinosas *Jasus edwardsii* (Johnston, 2003) y *Panulirus interruptus* (Celis-Gerrero *et al*., 2004), las enzimas con actividad tipo tripsina son de las principales enzimas digestivas en la mayoría de las especies.

1.3.4. Enzimas tipo tripsina

La tripsina bovina fue purificada por cristalización a principios de la década del 1930 (Northrup y Kunitz, 1931) y fue una de las primeras peptidasas aisladas con suficiente pureza y cantidad para llevar a cabo análisis bioquímicos (Northrub *et al*., 1948); además, casi toda la secuencia aminoacídica se logró conocer en los años 1960 (Walsh *et al*., 1964; Walsh y Wilcox, 1970). Con el advenimiento de la cristalografía de rayos X, la estructura tridimensional de la tripsina bovina fue resuelta en los años 1970 (Huber *et al*., 1974; Sweet *et al*., 1974; Kossiakoff *et al*., 1977) y en las siguientes décadas la secuencia, estructura y mecanismo de acción fueron estudiados en un gran número de especies, incluyendo invertebrados.

Teniendo en cuenta sus características estructurales, las tripsinas se clasifican en el clan PA, familia S1A, según la clasificación utilizada por la base de datos MEROPS (URL: http://www.merops. co.uk). La estructura tridimensional de las enzimas de la familia S1 está altamente conservada, incluso cuando las estructuras primarias varían en gran medida. En esta familia, los residuos que conforman la triada catalítica se encuentran entre dos dominios barril β, empacados uno contra el otro. En las tripsinas, los residuos involucrados en la catálisis son His57, Asp102 y Ser195 (numeración de la quimotripsina). Las tripsinas

hidrolizan los enlaces peptídicos del lado carboxilo de los residuos arginina y lisina en posición P1. Esta especificidad primaria está determinada fundamentalmente por tres residuos localizados en el bolsillo de la enzima. En las paredes del bolsillo se encuentran dos residuos de glicina, en posiciones 216 y 226 (numeración de la quimotripsina), que permiten el acceso de cadenas laterales grandes como las de la arginina y la lisina. En el fondo, o cerca del fondo del bolsillo, el aspartico en posición 189 (numeración de la quimotripsina) juega un papel importante en la estabilización de las cadenas laterales básicas de arginina o lisina. El puente disulfuro entre las cisteínas en posición 191 y 220 (numeración de la quimotripsina) está altamente conservado en las enzimas tipo tripsina y juega un papel importante en la determinación de la geometría del bolsillo. Estudios de mutagénesis dirigida han mostrado que otras regiones, más alejadas del centro activo, son importantes en determinar la especificidad de estas enzimas (Hedstrom *et al.*, 1992, Hedstrom, 1996; Perona y Craik, 1995). Actualmente sólo se conoce la estructura de la tripsina de un crustáceo (*Astacus leptodactylus*) (Fodor *et al.*, 2005).

Las enzimas con actividad tipo tripsina están ampliamente distribuidas y son de las más importantes en la mayoría los decápodos. La contribución de las tripsinas a la proteólisis total en la digestión de varias especies de crustáceos se ha estimado entre el 40 y 60% (Tsai *et al*., 1986). Las enzimas tipo tripsina estudiadas en crustáceos presentan de 2 a 4 isoformas y masas moleculares alrededor de los 25 kDa (Brockerhoff *et al*., 1970; Galgani y Nagayama, 1987). En el hepatopáncreas de *Litopenaeus vannamei* se han encontrado cinco cDNA para enzimas tipo tripsina (Klein *et al*., 1996), mientras que mediante técnicas de sustrato-SDS-PAGE se han encontrado tres isoenzimas principales de tripsina (Sainz *et al*., 2004, 2005). En otras tres especies de peneidos varias isoformas también han sido descritas (Tsai *et al*., 1991).

La presencia de varias enzimas tipo tripsina en crustáceos y el hecho de presentar el fenómeno de polimorfismo determina que se puedan definir varios fenotipos según la combinación de isoformas presentes (Sainz *et al*., 2004; 2005; Klein *et al*., 1996). En el camarón *L. vannamei* se han descrito mediante la utilización de la técnica sustrato-SDS-PAGE tres fenotipos fundamentales (Sainz *et al*., 2004; 2005). En salmónidos también se ha descrito la presencia de tres zonas de isoenzimas de tripsina (TRP-1, TRP-2 y TRP-3),

pero el uso de técnicas más resolutivas (focalización isoeléctrica) ha permitido la descripción de un mayor número de isoformas y fenotipos (Torrissen, 1987).

En el camarón *L. vannamei* se ha demostrado que no existe variación en los patrones de las isoenzimas (fenotipos) de tripsina durante el transcurso del desarrollo y los estadios de la muda (Sainz *et al*., 2005). La misma ausencia de variaciones en el patrón de estas isoenzimas ha sido descrita durante el desarrollo de salmónidos (Torrissen, 1987; Torrissen *et al.*, 1993; Rungruangsak-Torrissen *et al*., 1998). Sainz *et al*. (2005) estudiaron, además, la segregación de los fenotipos de tripsina en el camarón *L. vannamei* mediante el cruzamiento de parentales con fenotipo conocido y el análisis del fenotipo de la descendencia. Los autores demostraron que las isoenzimas se heredan de manera mendeliana, y sugirieron la presencia de dos genes que se expresan en el hepatopáncreas, uno de ellos heterocigótico con relación de codominancia entre los alelos. Los resultados de estos autores coinciden con los obtenidos a nivel molecular para la misma especie (Klein *et al*., 1996).

Previamente a los resultados que conforman la presente Tesis Doctoral, no existían estudios en crustáceos en cuanto a la ventaja de tener determinado fenotipo de tripsina. Sainz y Córdova-Murueta (2009) plantearon que la comparación de la digestibilidad entre fenotipos en camarones peneidos pudiera tener implicaciones prácticas importantes en la camaronicultura. Las tres isoformas fundamentales de tripsina en *L. vannamei* fueron purificadas y caracterizadas cinéticamente, siendo la isoforma de movilidad relativa media, la de menor eficiencia catalítica (Sainz *et al*., 2004). Por esta razón, estos autores sugieren que los fenotipos compuestos por las otras dos isoformas deben ser más eficientes en la digestión de proteínas. Además plantean que este tema está bajo investigación en su laboratorio para el camarón *L. vannamei*, y sus resultados preliminares (no publicados) sugieren que el fenotipo compuesto por las tres isoformas tiene una capacidad hidrolítica mayor.

Este aspecto solo había sido estudiado con profundidad en salmónidos (Torrissen, 1987, 1991; Bassompierre *et al.*, 1998; Rungruangsak-Torrissen y Male, 2000; Rungruangsak-Torrissen y Sundby, 2000), demostrándose que los grupos que poseen la variante

homocigótica [TRP-2(92/92)] tienen un peso promedio significativamente mayor que los peces con otros fenotipos, sobre todo en invierno (Torrissen, 1987, 1991). Sin embargo, a pesar del rápido crecimiento de los peces con el alelo TRP-2(92), al analizarse el coeficiente de digestibilidad aparente *in vivo* con el marcador óxido crómico, no se encontraron diferencias entre los diferentes fenotipos (Torrissen y Shearer, 1992). La eficiencia digestiva de los diferentes fenotipos de tripsina en salmones fue evaluada posteriormente en estudios de digestibilidad *in vitro* encontrándose las diferencias esperadas (Bassompierre *et al.* 1998). Por esta razón, en la presente Tesis Doctoral se utilizaron métodos *in vitro* de digestibilidad para estudiar las diferencias entre fenotipos tripsina en *P. argus*.

1.3.5. Regulación de la actividad tripsina

La actividad tripsina en crustáceos está influenciada por la proteína de la dieta (Le Moullac *et al*., 1994; 1996; Muhlia-Almazán *et al*., 2003) pero en general el efecto de la dieta sobre estas enzimas ha sido poco estudiado en comparación con vertebrados y otros grupos de invertebrados. De igual manera, se conoce que las enzimas tipo tripsina de crustáceos se secretan luego de la alimentación (Hirsch y Jacobs, 1928; Barker y Gibson, 1977; Al-Mohanna *et al*., 1985; Muhlia-Almazán y García-Carreño, 2002; Simon, 2009) y que están reguladas cuantitativamente a nivel trancripcional (Le Moullac *et al*. 1996; Muhlia-Almazán *et al*., 2003), pero se desconocen los mecanismos de regulación de estos procesos y el efecto del alimento sobre los mismos. En general, existe muy poca información sobre estos mecanismos en invertebrados (Muhlia-Almazán *et al*., 2008) excepto los insectos.

1.4. OBJETIVOS Y PLANTEAMIENTOS

La langosta espinosa *P. argus* es una de las especies de más potencial para el desarrollo de los procesos de engorde en cautiverio con fines comerciales. Sin embargo, un “cuello de botella” en su cultivo es la falta de dietas correctamente formuladas. Por este motivo es necesario contar con información especie-específica en relación con su bioquímica y fisiología digestiva.

De este modo, el ***Objetivo General*** de esta Tesis Doctoral es estudiar las enzimas digestivas de la langosta *P. argus* con énfasis en las enzimas tipo tripsina, así como analizar el valor adaptativo de la presencia de múltiples tripsinas y sus mecanismos de regulación.

El citado ***Objetivo General*** se pretende conseguir mediante los siguientes ***Objetivos Específicos:***

1) Caracterizar parcialmente y estudiar el polimorfismo de las enzimas digestivas de *P. argus*, con énfasis en las enzimas tipo tripsina.
2) Describir los cambios en las enzimas digestivas de *P. argus* durante el desarrollo y los estadios del ciclo de la muda, con énfasis en las enzimas tipo tripsina.
3) Determinar el número de fenotipos en *P. argus* para las tripsinas y analizar las posibles diferencias entre fenotipos en cuanto a la eficiencia en la digestión de proteínas.
4) Clonar y secuenciar los cDNAs de tripsinas y estudiar las enzimas resultantes en cuanto a su estructura, expresión y evolución.
5) Analizar la influencia de la proteína de la dieta en la secreción y la expresión de las enzimas tipo tripsina en *P. argus*.
6) Purificar y caracterizar cinéticamente las principales isoformas de tripsina en *P. argus*.

Dado el desconocimiento total sobre las bases bioquímicas de la digestión en la langosta *P. argus*, en esta Tesis Doctoral se estudia desde la descripción general de las principales enzimas de *P. argus*, hasta el efecto del alimento sobre las principales proteasas digestivas y su regulación. De esta forma, los resultados se presentan de la siguiente manera:

Capítulo 2. Se estudian las principales enzimas digestivas de *P. argus* a partir de extractos crudos de la glándula digestiva y el jugo gástrico, utilizando una combinación de métodos enzimáticos y electroforéticos. Se analiza el polimorfismo en las enzimas digestivas y se describen los diferentes fenotipos presentes en la especie para cada tipo de enzima.

Capítulo 3. Se describen las variaciones de las principales enzimas digestivas de *P. argus* con la muda y el desarrollo, así como las variaciones en la composición de isoformas.

Capítulo 4. Se comparan diferentes fenotipos de tripsina en cuanto a la eficiencia en la digestión *in vitro* de proteínas. Además, se compara la digestibilidad de varias fuentes de proteínas mediante métodos *in vitro*.

Capítulo 5. Se clonan y caracterizan los cDNA de las principales isoformas de tripsina de *P. argus*, estudiándose las diferentes enzimas desde el punto de vista estructural, filogenético y de expresión génica.

Capítulo 6. Se estudia el efecto de la alimentación con diferentes dietas formuladas, en la secreción y la expresión de las diferentes isoformas de tripsina en *P. argus*.

Capítulo 7. Se purifican las principales isoformas de tripsina y se caracterizan en cuanto a sus características cinéticas y operacionales, buscando las bases bioquímicas de las diferencias entre fenotipos en la digestión proteica.

Capítulo 8. Se discuten los principales resultados obtenidos en esta Tesis Doctoral en el contexto de la bioquímica, la fisiología digestiva y la nutrición de langostas espinosas.

Capítulo 9. Se presentan las conclusiones derivadas de la presente Tesis Doctoral.

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Capítulo 2

Polymorphism and partial characterization of digestive enzymes in the spiny lobster *Panulirus argus*

Perera, E., Moyano, F. J., Díaz, M., Perdomo-Morales, R., Montero, V., Alonso, E., Carrillo, O., Galich, G., ***Comp Biochem Physiol B 150: 247–254*** **(2008)**

Resumen

Se caracterizaron las principales enzimas digestivas de la langosta *Panulirus argus* usando una combinación de ensayos enzimáticos y electroforéticos. Las actividades amilasa y proteasa fueron mayores en la cámara gástrica, mientas que las actividades esterasa y lipasa fueron mayores en la glándula digestiva. Estos resultados indican la localización de la digestión extracelular de los principales componentes de la dieta, así como la cronología de la digestión de los mismos: la digestión de carbohidratos y proteínas comienza en la cámara gástrica inmediatamente tras la ingestión, mientras que la digestión de lípidos ocurre posteriormente en la glándula digestiva. La actividad tripsina es mayor que la actividad quimotripsina tanto en el jugo gástrico como en la glándula digestiva. La estabilidad y las condiciones óptimas para las actividades enzimáticas se estudiaron utilizando diferentes condiciones de pH, temperatura y fuerza iónica. Los resultados revelaron características importantes de estas enzimas. Además, mediante el uso de inhibidores específicos se mostró la prevalencia de serino-proteasas y metalo-proteasas. Los resultados para las serino proteasas se corroboraron mediante zimogramas en geles de poliacrilamida, indicando la existencia de varias enzimas tipo tripsina (17-21 kDa) y quimotripsinas (23-38 kDa). También se detectaron varias enzimas amilasas (38-47 kDa) y esterasas. Uno de los mayores aportes fue la descripción del polimorfismo para las principales enzimas digestivas de *P. argus*. Este estudio ha sido el primero en evidenciar con resultados experimentales las bases bioquímicas de la plasticidad en los hábitos alimentarios de *P. argus*.

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Abstract

We characterized major digestive enzymes in *Panulirus argus* using a combination of biochemical assays and substrate-(SDS or native)-PAGE. Protease and amylase activities were found in the gastric juice while esterase and lipase activities were higher in the digestive gland. Trypsin-like activity was higher than chymotrypsin-like activity in the gastric juice and digestive gland. Stability and optimal conditions for digestive enzyme activities were examined under different pHs, temperature and ionic strength. The use of protease inhibitors showed the prevalence of serine proteases and metalloproteases. Results for serine proteases were corroborated by zymograms where several isotrypsins-like (17-21 kDa) and isochymotrypsin-like enzymes (23-38 kDa) were identified. Amylases (38-47 kDa) were detected in zymograms and a complex array of non-specific esterases isoenzymes was found in the digestive gland. Isoenzyme polymorphism was found for trypsin, amylase, and esterase. This study is the first to evidence the biochemical bases of the plasticity in feeding habits of *P. argus*. Distribution and properties of enzymes provided some indication on how the digestion takes place and constitute baseline data for further studies on the digestion physiology of spiny lobsters.

Keywords: Spiny lobster, *Panulirus*, Digestive enzymes, Hepatopancreas, Digestive gland, Characterization, Isoenzyme.

1. INTRODUCTION

Spiny lobsters are predators in tropical and temperate seas feeding on a wide variety of benthic and infaunal species including molluscs, crustaceans, polychaetes and other invertebrates. Ecological studies on the natural diet of *Panulirus argus* have shown the carnivorous and opportunistic nature of their feeding habits (Herrera *et al.*, 1991; Cox *et al.*, 1997). *P. argus* has proven to rely on proteins and lipids for energy when feeding on main items in their natural diet, whereas no sparing of proteins by dietary lipids is possible when lobsters are fed with less important natural preys or unsuitable formulated diets (Díaz-Iglesias *et al.*, 2002; Perera *et al.*, 2005). Yet, digestion in spiny lobsters is poorly understood as evidenced in recent reviews (Williams, 2007a,b) and should be closely related to both feeding ecology and metabolic requirements.

Digestive enzymes of decapod crustaceans have been the subjects of many studies. As a result much information is now available on the digestive biochemistry of different species mainly those cultivated worldwide (e.g. shrimps). In contrast, little information is available on properties of digestive enzymes in spiny lobsters. To date, this knowledge is limited to proteases isolated from the digestive gland or gastric juice of *Panulirus japonicus* (Gagani and Nagayama, 1987) and to the characterization of proteases from crude extract in *Jasus edwardsii* (Johnston, 2003) and *Panulirus interruptus* (Celis-Gerrero *et al.*, 2004). Trypsin and chymotrypsin have been shown to be the major proteinases in spiny lobsters (Johnston, 2003; Celis-Gerrero *et al.*, 2004) although no evidence for chymotrypsin was noted by Gagani and Nagayama (1987). Exopeptidases (Gagani and Nagayama, 1987; Johnston, 2003) and collagenolytic serine proteinases (Iida *et al.*, 1991) have been also found in spiny lobsters. Interestingly, the presence of an acid aspartic proteinase has been recently reported in the gastric juice of *P. interruptus* (Navarrete del Toro *et al.*, 2006). There is little information on features of digestive enzymes other than proteases in palinurids. This paper deals with partial characterization of the main enzymes involved in food digestion in *P. argus.*

2. MATERIALS AND METHODS

2.1. Reagents, sample collection and preparation of extracts

All chemicals were reagent grade and obtained from Sigma-Aldrich, except for casein and DMSO (Merck). Lobsters were collected in the Golf of Batabanó, Cuba. Only intermolt lobsters were used and determination of molt state was done according to Lyle and MacDonald (1983). Animals were placed on ice for 10 min to obtain a chill coma before hepatopancreas extraction. Gastric juice was obtained through the oral cavity into a disposable plastic pipette. Samples were immediately frozen in liquid nitrogen and then lyophylised to be stored at -20°C. Before analysis the powders were homogenised in cold distilled H_2O and centrifuged at 4°C at 8,000 *g* for 15 min. Supernatants were immediately used for enzyme assays or electrophoresis.

Crude extracts for each assay were diluted with reaction buffer to measure enzyme activities at initial rates. Assays were always run in triplicate and activities were expressed as change in absorbance per minute per milligram of protein (ΔAbs min^{-1} mg protein^{-1}) or in percent. The protein content of enzyme extracts was measured according to Bradford (1976) using BSA as standard.

2.2. Enzyme assays for total proteases

Total protease (alkaline) activity was measured by the casein hydrolysis assay (Kunitz, 1947) as modified by Walter (1984). The reaction mixture of 0.3 ml 1% casein in 200 mM Tris-HCl pH 7, 0.5 ml of the same buffer and 0.3 ml crude enzyme extract, was incubated at 25°C for 1 h. Then 0.3 ml of 20% TCA was added to stop the reaction. Tubes were placed for 60 min at 4°C, followed by centrifugation at 8, 000 g for 15 min. The absorbance of supernatant was recorded at 280 nm. The blank used for this assay was prepared by incubating the crude enzyme extract and buffer for 1h at 25°C, followed by the addition of TCA and casein. Additionally, hemoglobin denatured with urea was used as the substrate to assess acid proteolytic activity according to Sarath *et al.* (2001).

2.3. Trypsin and chymotrypsin- like activities

Trypsin-like activity was measured using 1.25 mM *N*-benzoyl-DL-arginine *p*-nitroanilide (BApNA) in 200 mM Tris-HCl, 20 mM $CaCl_2$, and pH 8.4. Chymotrypsin-like activity was measured with 0.1 mM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (SApNA) in the same buffer. Substrate stock solutions of BApNA (125 mM) and SApNA (10 mM) were prepared in DMSO and brought to working concentration by diluting with buffer prior the assays. In a 96-well microplate, 10 µL of enzyme extract were mixed with 200 µL of respective substrate, and liberation of *p*-nitroaniline was kinetically followed at 405 nm in a microplate reader Multiscan EX (Thermolab Systems).

End point assays were performed for estimating optimal temperature for trypsin-like and chymotrypsin-like activities. Crude extract (70µL) was incubated with 500 µL BApNA or SApNA in buffer, for 10 min at different temperature. To stop the reaction 570 µL of 30% acetic acid was added and absorbance was recorded at 405 nm in a spectrophotometer.

2.4. Inhibition assay for proteases

Classes of proteases in the digestive gland were characterized by the effect of protease inhibitors on caseinolytic activity. Inhibitors employed were soybean trypsin inhibitor (SBTI), benzamidine, and aprotinin for serine proteases. Leupeptin was used for serine-cysteine protease inhibition, NEM for cysteine protease inhibition and EDTA for metalloprotease inhibition. Concentrations of inhibitors were as in Table 3.

Additionally, specific inhibitors were used to inhibit trypsin-like and chymotrypsin-like activities on *N*-benzoyl-DL-arginine *p*-nitroanilide (BApNA) and Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (SApNA), respectively. Final concentration of inhibitor for trypsin was 0.5 mM *N*α-p-tosyl-L-lysine chloromethyl ketone (TLCK) while for chymotrypsin were: 0.3 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 0.3 mM carbobenzoxy-Phe chloromethyl ketone (ZPCK) and 0.5 mM chymostatin.

The mixture enzyme: inhibitor was incubated for 60 min at room temperature and then assayed using appropriate substrate as above. Enzyme extracts incubated with buffer instead of inhibitors were used as controls and referred as 100% of enzyme activity.

2.5. Non- specific esterase activity

Esterase activity in extracts was assessed by the hydrolysis of 0.3 mM of *p*-nitrophenyl acetate (*p*-NPA) and 0.3 mM *p*-nitrophenyl butyrate (*p*-NPB) according to Gilham and Lehner (2005) with slight modifications. Stock solutions (100 mM) were prepared for the *p*-nitrophenyl esters in CH_2Cl_2 and diluted immediately prior the assays with 20 mM Tris-HCl, 150 mM NaCl, pH 8.0. Twenty µL of enzyme extract were mixed with 200 µL substrate solution in 96-well microplates and the liberation of *p*-nitrophenol was measured kinetically at 405 nm in a microplate reader.

For optimal temperature and pH, an end-point assay was used. Ten µL extract were incubated with 490 µL substrate solution for 10 min at 37°C. Then, 700 µL of 5:2 (v:v) acetone/ hexane solution were used to stop reaction followed by 300 µL 200 mM Tris-HCl, pH 8. After centrifugation (2 min at 10, 000g) the absorbance of the lower phase was recorded at 405 nm (López-López *et al.*, 2003).

2.6. Lipase activity

Lipase activity was measured using ß–naphthyl caprylate in DMSO. The assay mixture contained: 100 µL of 100 mM sodium taurocholate, 900 µL of 50 mM Tris-HCl pH 7.5, 10 µL enzyme extract, and 10 µL of substrate stock solution (100 mM). The reaction mixture was incubated for 30 min at 37°C for the reaction to proceed and then 10 µL of 100 mM Fast Blue BB in DMSO were added. The reaction was stopped with 100 µL TCA 12%. Finally, 1.35 mL of 1:1 (v:v) ethyl acetate/ethanol solution were added and absorbance was recorded at 510 nm.

2.7. Amylase activity

Amylase activity was determined according to the Somogy-Nelson method using soluble starch (2% w:v) as substrate, as described in Robyt and Whelan (1968). Briefly, 20 µL of enzyme extract and 125 µL of 100 mM phosphate-citrate pH 7.5 were incubated with 125 µL of starch for 30 min. Activity was measured by calculating the reducing sugars released at 600 nm.

2.8. Effects of pH and temperature on the activity and stability of digestive enzymes

The effect of pH on enzyme activity was evaluated using Universal Buffer (Stauffer, 1989). Excluding the reaction pH, enzyme activities were determined as above. Optimal temperature for each enzyme was determined as described earlier by assaying at the optimum pH over the range 20 ^{o}C to 80°C. The effects of different pH and temperature on the stability of digestive enzymes were studied by preincubating the enzyme extract at different pH and temperature for 15, 30 and 60 min prior to the enzyme assays. Results were expressed as residual activity in %.

2.9. Effect ionic strength on digestive enzyme activities

The effect of different NaCl concentrations on the activity of the digestive enzymes was examined including the following NaCl concentration in reaction buffers: 0, 20, 100, 200, 500, 1000, 1500 and 2000 mM. Blanks were prepared using buffer at the appropriate salt concentration.

2.10. Substrate-PAGE of digestive enzymes

Substrate-SDS-PAGE (5% stacking gel, 13% separating gel) was used to determine the composition of proteases in digestive tract (García-Carreño *et al.*, 1993). The enzyme extract was incubated with the various inhibitors for 60 min at 25°C prior to electrophoresis. Distilled water was used instead of inhibitors in the control. Samples were neither boiled nor treated with mercaptoethanol before loading into the gel; they were run at 15 mA and 4^{0}C in a vertical electrophoresis device (Hoeffer SE260, 8 x 10 x 0.75 cm).

The gel was then immersed in 3% casein solution for 45 min at 4^0C and thereafter, the temperature was raised to 25^oC for an additional 90 min. The gel was thoroughly washed with distilled water, fixed in TCA 12% for 30 min, stained with 0.05% Coomassie Brillant Blue in 40% methanol, 7% acetic acid, and finally distained with the same solution without dye. The absence of spots in presence of specific inhibitors indicates a specific type of protease. Molecular mass markers (14-97 kDa, Pharmacia) without reducing agent were used for apparent MW.

Substrate-SDS-PAGE for amylase was performed on 5% stacking gel and 12% resolving gel in the same conditions as for proteases. Gels were immersed in a starch solution (1%) at pH 6 for 60 min and then stained with iodine/KI solution (10%). Molecular weight markers (14-97 kDa, Pharmacia) without reducing agent were used for apparent MW.

Activity and band profile of lobster esterases were strongly affected by SDS; thus zymograms for esterases were performed under native conditions (5% stacking gel and 8% resolving gel). After electrophoresis, the gel was introduced into a mixture of 50 mM Tris-HCl (pH 8), 100 mM α–naphthyl acetate or ß–naphthyl acetate and 100 mM fast blue, and incubated until activity bands were revealed (López-López *et al.*, 2003). For assessing isoenzyme polymorphism, 40 intermolt individuals were analyzed by zymograms as describe above.

3. RESULTS

3.1. Distribution of enzyme activities

Specific activities for the different enzymes are presented in Table 1. Trypsin-like activity was 2.4 times higher than chymotrypsin-like activity in gastric juice whereas tryptic activity was 4.7 times higher than chymotryptic activity in the digestive gland. Activity on *p*-NPB was 2.6 times greater than on *p*-NPA in the digestive gland, thus *p*-NPB was selected for further characterization of esterase activity. Proteases and amylase were several times more active in the gastric juice while esterase and lipase activities were higher in the digestive gland.

Table 1. Specific activities (ΔAbs min^{-1} mg $protein^{-1}$) of major digestive enzymes in *P. argus*. Activities were assayed at optimal pH for each enzyme.

Enzyme	Gastric juice	Digestive gland
Trypsin	20.9 ± 4.462	1.32 ± 0.422
Chymotrypsin	8.56 ± 0.564	0.28 ± 0.078
Amylase	33.38 ± 8.537	10.64 ± 4.330
Esterase (*p*-NPB)	1.76 ± 0.320	20.60 ± 6.305
Esterase (*p*-NPA)	1.81 ± 0.278	7.91 ± 3.265
Lipase	0.07 ± 0.011	4.25 ± 2.732

3.2. Optimal condition for enzyme activities

Optimal pH for total proteolysis, trypsin and chymotrypsin activities was found to be 7 and activity of these proteases remained high over a broad alkaline pH range (Table 2). Acid protease activity on Hb increased 7-8 fold from pH 2.5 to pH 3. Activity continued to increase slowly until pH 3.5-4.5 (0.04 ΔAbs/min/mg prot.). Similar to protease activities, esterase and lipase activities were optimally active over the wide range of pH (Table 2). In contrast, amylase activity had pH optima over the narrow acidic pH range of 4-5 (Table 2). Optimal temperature for the different enzymes varied from 40 to 60°C (Table 2).

Table 2. Optimal temperature and pH for digestive enzymes activities in the spiny lobster *P. argus.*

Enzyme activity	Optimal T°C	Optimal pH	Observations
Proteases	60	7	A minor peak was observed at pH 10-10.5
Trypsin	60	7 and 10-12	Neutral and alkaline peaks are similar in activity. At pH 6 the activity is still high but strongly affected at pH 5.
Chymotrypsin	50	7 and 11	Alkaline peak is slightly higher than at neutral pH. Activity is affected at pH 6.
Esterase	40-50	7, 9 and 11	Three peaks but high activity all over the range pH 7-11
Lipase	40	7-9	High activity all over the range pH 6-9
Amylase	50	4-5	Strong reduction of activity below pH 4. At pH 6 activity is around 50% of maximal activity.

Enzyme activities were observed to increase with ionic strength to maximal activity at 20-100 mM NaCl for amylase, at 100-200 mM NaCl for trypsin, at 200-500 mM NaCl for esterase, and 20 mM NaCl for lipase. Decrease in activity was observed at high salt

concentration for all enzymes studied but chymotrypsin. Severe effect of salt was observed on lipolytic activity above 1 M NaCl (Fig. 1).

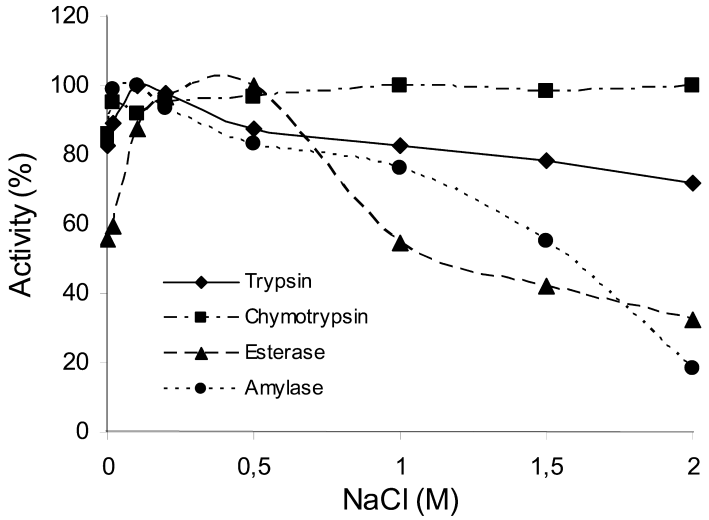

Fig 1. Effect of NaCl on digestive enzyme activities in *P. argus*.

3.3. pH and temperature stability of enzymes

Enzymes responsible for tryptic and chymotryptic activities were stable up to 55°C for at least 1 h but activities were significantly affected at 60°C. Chymotrypsin-like activity was more stable than trypsin-like activity conserving around 40% of activity after 30 min at 60°C whereas trypsin-like activity was almost abrogated after 15 min at this temperature (Fig 2). Chymotrypsin-like activity was also more stable than trypsin-like activity under acid conditions retaining 50-60% of activity after 1h incubation (Fig 2). Amylase activity was stable up to 50°C and had 30-40% activity after 1h at 60°C. Amylase activity was unstable below pH 4 and lost 20% activity at pH 8 (Fig 3).

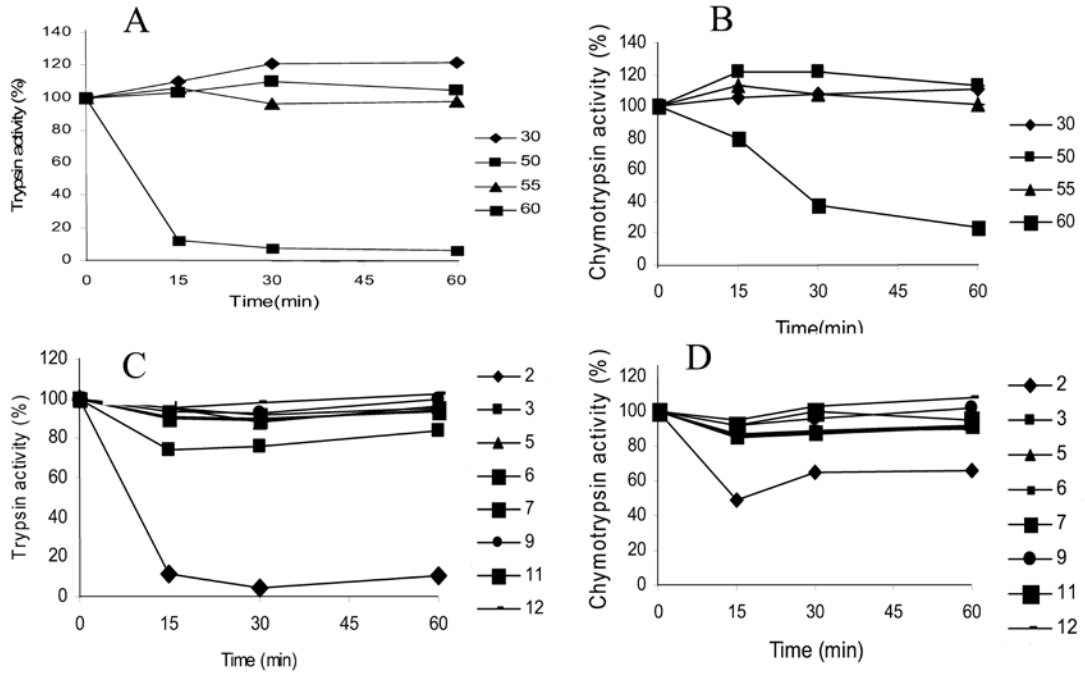

Fig 2. Stability of trypsin and chymotrypsin activities from digestive gland of *P. argus* incubated at different temperatures (°C) (A, B) and pH (C, D).

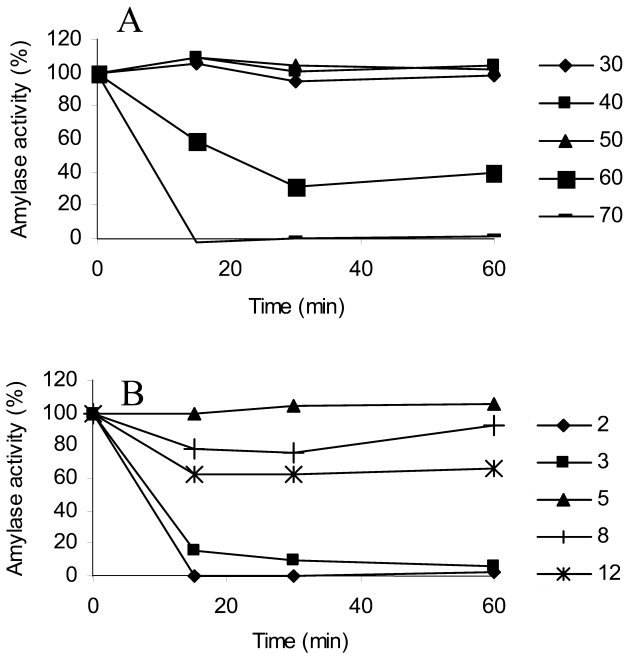

Fig 3. Stability of amylase activity from digestive gland of *P. argus* incubated at different temperatures (°C) (A) and pH (B).

Esterase and lipase activities were found to be less tolerant to high temperature than protease and amylase activities. A significant reduction in esterase activity was observed after 15 min at 50°C while almost 20% lipolytic activity was lost after 1h at 30°C (Fig 4). Non-specific esterase and lipase activities were stable over a wide range of pH from neutral to alkaline (Fig 4). Both activities were more affected by acid media than proteases and amylase (Fig 4).

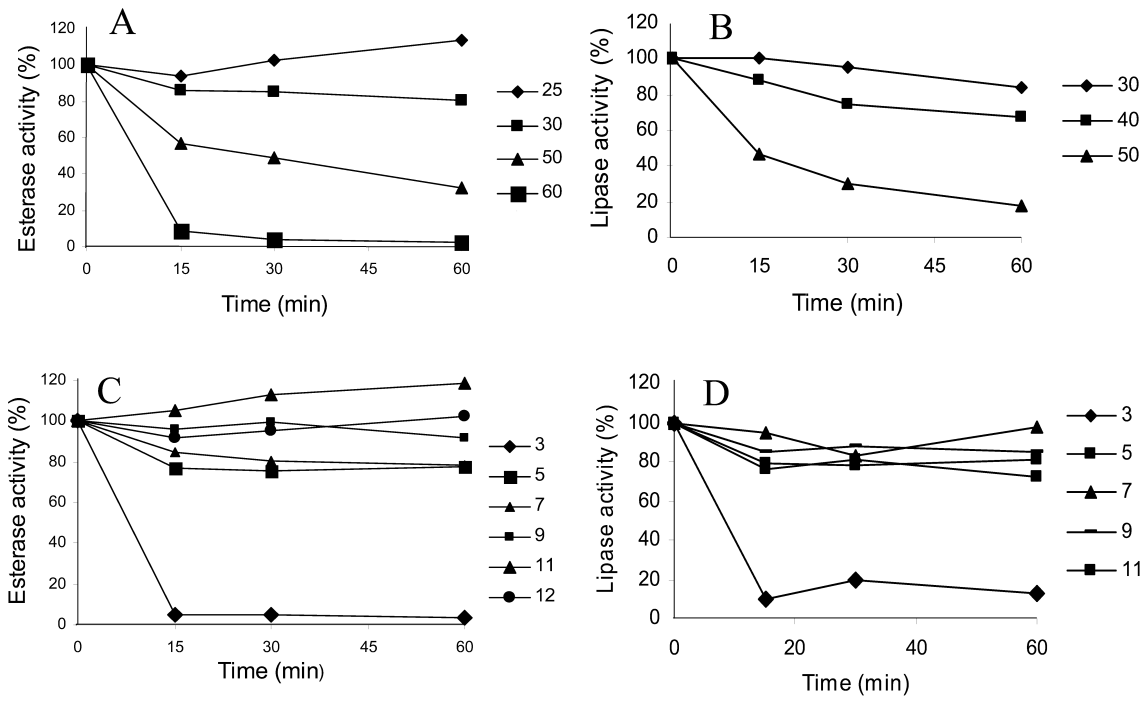

Fig 4. Stability of esterase (C4) and lipase activities from digestive gland of *P. argus* incubated at different temperatures (°C) (A, B) and pH (B, C).

3.4. Effect of specific protease inhibitors

Leupeptin and aprotinin produced 37% and 47% inhibition respectively while SBTI suppressed 54% - 56% of the activity of controls (Table 3). Surprisingly, benzamidine did not inhibit more than 17% of activity (Table 3). An inhibitor for metalloproteases, EDTA, abolished 40%-42% of the activity. Results obtained with SBTI and EDTA suggest that significantly higher percentage of inhibition of serine and metalloproteases cannot be obtained by increasing the amount of inhibitor (Table 3). The SH-enzyme inhibitor NEM produced low percentage of inhibition.

Preincubation with TLCK was always accomplished at pH 6 to ensure stability of inhibitor but activity was evaluated at different pH values. The hydrolysis of BApNA (trypsin-like) at pH 7 was strongly inhibited (80%) by TLCK whereas it only abolished 67% of amidase activity on BApNA above pH 9. Few or no reduction of the hydrolysis of SApNA (chymotrypsin-like) was observed with TPCK or ZPCK at any pH whereas chymostatin suppresses 100 % of activity at pH 7-8.

Table 3. Effect of inhibitors on caseinolytic activity of digestive gland extracts of *P. argus*. Concentration values in the table mean final concentration of inhibitor. The hydrolysis of casein without previous incubation of the crude extract with inhibitor was referred as 100%.

Protease inhibitor	Target	Concentration	% of inhibition
NEM	Binds to SH groups	1 mM	10.1
		10 mM	13.3
EDTA-Na_2	Metallo-proteases	10 mM	40.4
		25 mM	41.8
Leupeptin	Trypsin-like serine proteases	0.1 mM	34.0
	and some cysteine proteases	1 mM	37.0
Aprotinin	Serine-proteases	15 mM	40.7
		30 mM	46.7
Benzamidine	Serine-proteases	1 mM	7.10
		10 mM	17.0
SBTI	Serine-proteases	10 µM	53.6
		20 µM	55.8
		50 µM	54.2

3.5. Substrate-(SDS or native)-PAGE

Digestive gland and gastric juice exhibited the same composition of proteases in preliminary experiments and therefore gastric juice was selected as source of proteases for electrophoresis. Zymograms illustrated 13 active zones with caseinolytic activity (Fig 5, control lane). EDTA and 1, 10-phenanthroline did not affect any activity band (not shown). Several bands were inhibited by SBTI and PMSF corroborating the prevalence of serine proteases in digestive system of lobster. Bands of approximately 43, 32 and 29 kDa were not inhibited by serine protease inhibitors (Fig 5) and remain unclassified. Bands with approximate molecular masses of 17, 18, 19, 20 and 21 kDa were inhibited by serine protease inhibitors and TLCK and thus classified as trypsin-like proteases (Fig 5). A band of 38 kDa was inhibited by serine proteases inhibitors and TPCK, but not by chymostatin.

A 35 kDa enzyme was inhibited by serine proteases inhibitors, TPCK and chymostatin. Other band with high caseinolytic activity of around 23 kDa was inhibited to some extent by TPCK, but strongly inhibited by chymostatin and serine protease inhibitors. The last three bands were classified as chymotrypsin-like proteases (Fig 5). ZPCK did not affect any activity band. Two bands of around 25 and 27 kDa were inhibited by serine protease inhibitors, but do not by TLCK, TPCK, ZPCK or chymostatin (Fig 5) and therefore they could not be assigned to either trypsin-like or chymotrypsin-like protease groups. Proteinase polymorphism could be detected only for trypsin-like enzymes (Fig 5, Panel B).

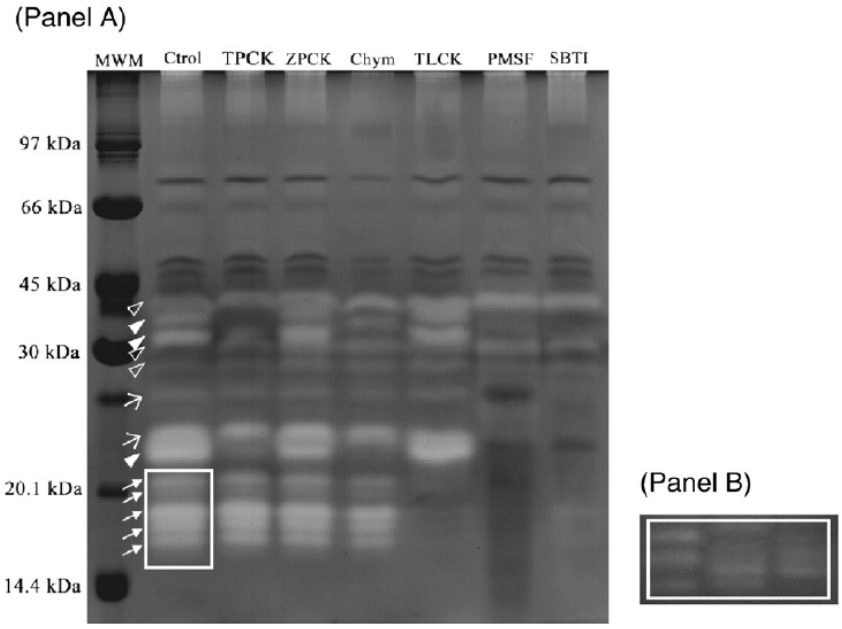

Fig 5. Panel A, 13% substrate SDS-PAGE showing caseinolytic activity bands in gastric juice of *P. argus* (Ctrol) and inhibition by specific inhibitors for chymotrypsin (TPCK, ZPCK and Chymostatin), trypsin (TLCK) and serine proteinases (PMSF and SBTI). Type of proteases is indicated in the control lane as follows: serine proteinases (→), chymotrypsin like proteinases (►), trypsin like proteinases (➞) and unclassified proteinases (▷). Panel B, 14% substrate SDS-PAGE showing polymorphism for trypsin like proteinases within the white rectangle in panel A.

Zymograms showed the existence of up to four starch degrading enzymes in *P. argus* (Fig 6) with apparent molecular masses of 38, 43, 44 and 47 kDa. Several esterases were evidenced in gels with a complex isozyme pattern using α–naphthyl acetate (Fig 7). The same band pattern as in Fig 7 was obtained with ß–naphthyl acetate (not shown).

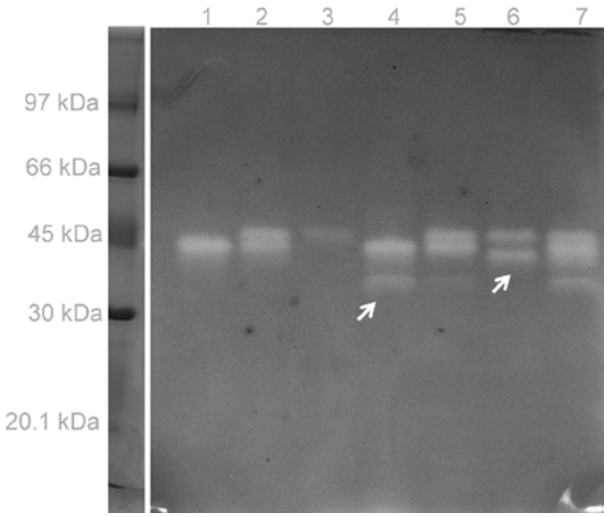

Fig 6. SDS-PAGE in 12% acrylamide of amylolytic enzymes in digestive gland of *P. argus*. Lanes represent the different phenotypes found. One gene appears responsible for the starch degrading enzymes observed in lanes 1, 2 and 3, with two alleles of around 44 and 47 kDa. Band in lane 1 is the more frequent phenotype followed by phenotype in lane 2, whereas lane 3 shown the less common phenotype. Other faster bands of around 38 and 43 kDa (indicated by arrows) were clearly evidenced in just few individuals. From 40 individuals analyzed only one displayed the four enzymes as in lane 7.

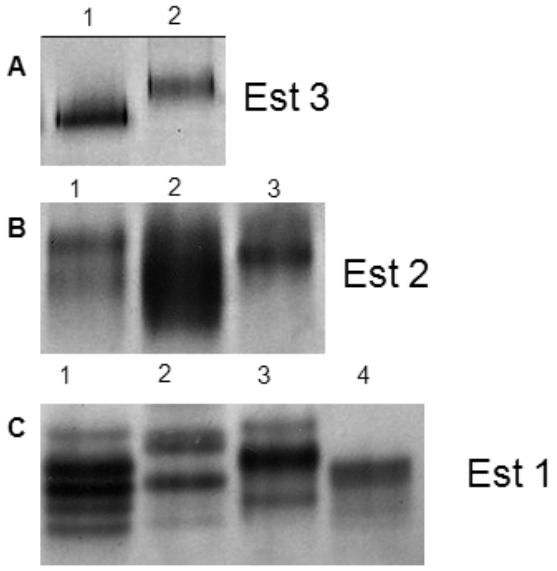

Fig 7. Native-PAGE in 8% acrylamide of α-esterase isozymes in digestive gland of *P. argus*. Phenotypes in lane 1 of panels A, B and C are of the highest frequency (n=40).

4. DISCUSSION

Properties of digestive enzymes from Palinuridae have been reported for just a few species (Gagani and Nagayama, 1987; Iida *et al.*, 1991; Johnston, 2003; Celis-Gerrero *et al.*, 2004; Navarrete del Toro *et al.*, 2006). With few exceptions, such studies have covered mainly biochemical aspects of proteinases. Herein, activities of major digestive enzymes in *P. argus* are presented for the first time and partially characterized.

The lipid content of the digestive gland has been positively correlated with growth in spiny lobster (Johnston *et al.*, 2003). We have previously demonstrated that lipids are an important energy source for *P. argus* (Díaz-Iglesias *et al.*, 2002; Perera *et al.*, 2005). Non-specific esterases in digestive gland were more reactive toward *p*-NPB than on *p*-NPA, both showing normal Michaelis–Menten kinetics in the absence of emulsified agents. Lipase activity was obtained with ß–naphthyl caprylate in the presence of sodium taurocholate, largely in the digestive gland. Lipase activity has been reported in spiny lobsters before (Johnston, 2003) and in other crustaceans like clawed lobsters (Brockeroff *et al.*, 1970), shrimp (González *et al.*, 1994) and crayfish (Figueiredo *et al.*, 2001). Specific activities for esterase and lipase were higher in the digestive gland despite the high content of non-enzyme proteins in this tissue.

In correspondence with predacious behaviour of spiny lobsters, high protease activity was found in the digestive system of *P. argus* especially in the gastric juice. Trypsin-like proteases were more active than chymotrypsin-like enzymes. Results compare well with those presented for *P. interruptus* (Celis-Gerrero *et al.*, 2004) with the same substrates (BApNA and SApNA) but higher activity for chymotrypsin in *J. edwardsii* was found by Johnston (2003) when using benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate.

As observed for proteases, higher amylolytic activity was evidenced in the gastric juice. Our results suggest that efficient lipid digestion starts later in the digestion process whereas a high rate of hydrolysis of dietary proteins and carbohydrates start just after ingestion in the gastric chamber of *P. argus*.

Optimal temperature obtained here for the different enzymes are similar to those reported elsewhere. Enzymes responsible for tryptic, chymotryptic and amylase activities in *P. argus* showed to be thermally robust. Chymotrypsin-like enzymes were more stable than trypsin-like proteases facing high temperature and extreme pH. Similar results were reported for crabs (Díaz-Tenorio *et al.,* 2006).

Enzymes with trypsin and chymotrypsin activities were stable and highly active under neutral and alkaline conditions and interestingly, they were resistant to pH 5 where they did not exert significant activities. Weak bounds that stabilize these proteases could be fairly resistant to extreme pH values or able to renature the disrupted enzymes as environmental pH moves toward the optimum. Whether this behavior only reflects molecular features of enzymes or has any physiological meaning remains to be clarified. As stated before high amount of enzymes with trypsin-like and chymotrypsin-like activities occur in the gastric juice of *P. argus*, where acidic pH occurs (5.9 ± 0.2, mean ± standard deviation, unpublished results) as in other spiny lobsters (Johnston, 2003; Navarrete del Toro *et al.,* 2006). As these proteases are fairly resistant to the pH conditions in the gastric juice, they can aid the proteases in hepatopancreas in later digestion if they migrate to the gland along with food particles which is likely to occur.

Optimal pHs for esterase and lipase activities in *P. argus* were similar to those for proteases. However, esterase and lipase activities were more sensitive to heat and acid pH than proteases and amylase, suggesting a more complex three-dimensional architecture of the active enzymes. Pattern of loss of activity for the different enzymes due to extreme conditions could be affected by the presence of isoforms (Figs 5-7).

The occurrence of acid proteases for early digestion as in the stomach of terrestrial vertebrates and some fishes has been discussed contradictorily in crustaceans. Sometimes the activity at acid pH could not be inhibited by pepstatin A nor separated from the alkaline peak by gel filtration or anion-exchange (Glass and Stark, 1994) suggesting absence of a distinct acidic protease, whereas activity was completed abolished by pepstatin A in other studies (Navarrete del Toro *et al.,* 2006) suggesting the presence of an aspartic proteinase. Also, cysteine proteinases have been found in crustaceans (Laycock *et al.,* 1991; Le Boulay *et al.,* 1995; Le Boulay *et al.,* 1996; Aoki *et al.,* 2003; Hu and Leung, 2007). Weak

acid protease activity in gastric juice of *P. argus* increased 7-8 fold from pH 2.5 to pH 3 and continued to increase slowly until pH 3.5-4.5. The general pattern of activity at acid pH in *P. argus* matched the one for the spiny lobster *P. interruptus*, and the crabs *C. pagurus*, *C. arcuatus* and *C. belicosus* (Navarrete del Toro *et al.*, 2006), but differed from *H. gammarus* (Glass and Stark, 1994; Navarrete del Toro *et al.*, 2006) and *Homarus americanus* (Brockerhoff *et al.*, 1970; Biesiot and Capuzzo, 1990) where high activities and clear peaks are evident. Since palinurids are primitive decapods and brachyurans have long been considered morphologically as a much evolved taxon, similarities between spiny lobsters and crabs have been supposed to be contradictory (Navarrete del Toro *et al.*, 2006), but recent analysis based on molecular data suggest a more basal position of the brachyurans in decapods phylogeny (Porter *et al.*, 2005). Yet, phylogenetic relationships cannot totally explain the different traits since substantial share of proteolytic activity in Caridea is caused by cysteine proteinases (Teschke and Saborowski, 2005).

Amylase has been reported to be the most important carbohydrase in the spiny lobster *J. edwardsii* (Johnston, 2003). *P. argus* amylase activity showed an optimal pH of 4-5 in correspondence to the acidic pH in the gastric juice. Early studies divided crustacean amylases into two groups, one with optimal pH below 6.3 including isopods, amphipods and Astacura, and other group with higher pH optimum comprising shrimps and brachyurans (Robson, 1979). Results obtained here and those from Johnston (2003) in *J. edwardsii* indicate that amylases from spiny lobsters belong to the first group. Other spiny lobster carbohydrases like α ßand -glucosidase, ß-galactosidase and N-acetyl ß-D glucosaminidase are also more active in acidic media (Johnston, 2003). More remarkable is that *P. argus* amylase activity becomes compromised at slight alkaline pH values which might be a limiting factor in carbohydrate digestion in the digestive gland. Low specific activity for amylase in hepatopancreas extracts of spiny lobster in comparison to those exhibited by shrimps and crabs has been reported, although few differences were found between the purified enzymes (Van Wormhoudt *et al.*, 1995). Medium pH appears relevant in regulating carbohydrate digestion in spiny lobsters.

Spiny lobsters are unable to regulate the osmolarity of internal media independently of the environment and thus confined to marine ecosystems with salinities varying in the narrow range of 34-36 $^{o}/_{oo}$. High salt concentration affected most of the digestive enzymes studied.

Electrostatic interactions are relevant for activity of many enzymes and usually decline as salt concentration rise. However, since both crustacean chymotrypsin–like enzymes and SApNA are anionic, increased salinity should reduce the electrostatic repulsion between the enzyme and the substrate, enhancing the activity. This kinetic-salt effect was observed in shrimp chymotrypsins toward SApNA, but not when a neutral substrate was used (Tsai *et al.,* 1991). This salt effect was not observed here, suggesting that lobster chymotrypsin-like proteases could be less anionic than the shrimp enzymes. Another possibility is that the interaction between lobster chymotrypsin-like proteases and the substrate is mainly hydrophobic rather than electrostatic, resembling more the bovine than the shrimp chymotrypsin. Chymostatin could inhibit both shrimp (Tsai *et al.,* 1991) and *P. argus* chymotrypsins (Fig. 5) but results with other inhibitors are inconclusive. Lipase activity in *P. argus* was more affected than any other enzyme by ionic strength. High salt concentration increase hydrophobic interactions, which can lead to the aggregation of lipolytic enzymes and lose of activity.

The inhibition assay showed the prevalence of serine and metalloproteases in the digestive tract of *P. argus.* Results for serine proteases were corroborated by zymograms where several bands with caseinolytic activity were inhibited by SBTI and PMSF. Digestive trypsin in shrimps is polymorphic both by cDNA (Klein *et al.,* 1996) and biochemical (Sainz *et al.,* 2004) studies. Here, five enzymes (17, 18, 19, 20 and 21 kDa) were observed to behave like trypsins since they were inhibited by serine proteases inhibitors and TLCK, with the three slighter bands as the most active enzymes. This pattern is similar to the previously presented for *P. interruptus* (Celis-Guerero *et al.,* 2004). Interestingly, the most reactive forms showed the highest degree of polymorphism. Crustacean trypsins have been estimated from 16 to 25 kDa (Brockerhoff *et al.,* 1970; Brun and Wojtowicz, 1976; Galgani and Nagayama, 1987; Celis-Guerero *et al.,* 2004; Díaz-Tenorio *et al.,* 2006). Apparent molecular masses for trypsin-like enzymes in *P. argus* are consistent with data for other crustaceans, but probably underestimated due to the higher relative mobility of unreduced proteins (incomplete unfolding). The same is true for all enzymes evaluated here under non-reduced or native conditions.

Similar to the spiny lobster *P. interruptus* (Celis-Guerrero *et al.,* 2004), chymotrypsin-like enzymes in *P. argus* have a wide range of molecular mass (23, 35 and 38 kDa) and can be

divided into two groups according to electrophoretic mobilities. Crustacean chymotrypsin-like enzymes have been reported from 21 to 60 kDa (Tsai *et al.,* 1986; Celis-Guerero *et al.,* 2004; Díaz-Tenorio *et al.,* 2006). Activity of shrimp (Tsai *et al.,* 1991) and *P. argus* chymotrypsins toward SApNA are poorly affected by TPCK. However, using casein as the substrate in gels, it was further demonstrated that TPCK inhibits the 38 and 35 kDa chymotrypsin-like enzymes; the smallest (23 kDa) chymotrypsin-like enzyme was poorly inhibited (Fig. 5). In contrast, hydrolysis of SApNA could be almost completely abrogated by chymostatin although this inhibitor totally suppresses only the smallest and most reactive form. All three enzymes were inhibited by serine protease inhibitors like SBTI and PMSF. Results indicate that (i) the two larger chymotrypsin-like enzymes of lobster are not very reactive toward SApNA and (ii) no inhibitor is equally effective on all lobster chymotrypsin-like enzymes. TPCK is known to produce inhibition by alkylation of the active-site histidine by the chloromethyl moiety, whereas the larger chymostatin, as PMSF, reacts with a serine residue in the active site. Altogether, results indicate that these aminoacids are involved in catalysis in all form of the enzyme. Thus, differences in reactivity toward a specific inhibitor are thought to result from differences in the geometry of the active sites. The pocket geometry is important for both the initial docking of the substrate and the formation of the transition state (Wouters *et al.,* 2003). Tsai *et al.,* (1991) suggested that the interaction between the active site of shrimp chymotrypsin and the substrate involve extended subsites, perhaps beyond the enzyme S_4. As in most crustaceans, the small inhibitor ZPCK was a poor affinity-label for *P. argus* chymotrypsin-like enzymes.

Although a collagenolytic metalloprotease has been described in a marine crab (Sivakumar *et al.,* 1999) most crustacean collagenolytic proteases reported so far belong to the serine proteases family (Grant *et al.,* 1983; Iida *et al.,* 1991; Sellos and Van Wormhoudt, 1992; 1999) some times exhibiting tryptic or chymotryptic activity. The hydrolytic activity of identified serine proteases in *P. argus* toward native substrates including collagen should be examined, especially for enzymes for which tryptic or chymotryptic activity were assigned.

Some active bands were not inhibited by serine protease inhibitors. Results from test-tube inhibition assay indicate significant activity of metalloproteases, but this was not evidenced

in gels. It has been stated before that these enzymes do not generate clear zones with this technique (Lemos *et al.,* 2000).

Zymograms showed the existence of four starch degrading enzymes in *P. argus* giving rise to seven different phenotypes. Most individuals had one or two active bands. A high degree of polymorphism has been reported for crustacean amylase with up to 5-6 active bands in some species, but a single molecular form in the spiny lobster *P. interruptus* and other decapods (Van Wormhoudt *et al.,* 1995). One gene appears responsible for the starch degrading enzymes observed in lanes 1, 2 and 3 of Fig 6, with two alleles of around 44 and 47 kDa. Two faster bands (38 and 43 kDa) evidenced in just a few individuals were not specific for either sex or any molt stage (not shown). Doubt remains on whether the two smaller bands come from a second gene expressed in very few individuals or as a result of modification of 44 and 47 kDa enzymes. It has been suggested that glycosylation cannot explain the high degree of polymorphism in crustaceans (Van Wormhoudt *et al.,* 1995), but other post-translational processes like C-terminal processing of amylase by carboxypeptidases (Sogaard *et al.,* 1993) may produce different active forms of a single gene product.

Zymograms resulting from esterase stain showed several isoenzymes with a complex band pattern and different staining intensity as previously reported for shrimps (Lester and Cook, 1987) and spiny lobsters (Menzies and Kerrigan, 1978), but different from the crayfish *Cherax quadricarinatus* where only three bands were reported to hydrolyze ß-naphthyl acetate and butyrate (López-López *et al.,* 2003). Lipase activity could not be evidenced in gels.

The present work highlights for the first time the diversity of enzymes in the digestive system of *P. argus* supporting the plasticity in their feeding habits. Additionally, the distribution and properties of digestive enzymes have provided some indication on how the digestion process takes place. Yet, several issues remain to be studied to fully understand digestion in spiny lobsters.

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Capítulo 3

Changes in digestive enzymes through developmental and molt stages in the spiny lobster, *Panulirus argus*

Perera, E., Moyano, F. J., Díaz, M., Perdomo-Morales, R., Montero, V., Rodríguez-Viera, L., Alonso, E., Carrillo, O., Galich, G. ***Comp Biochem Physiol B*** **151: 250-256 (2008)**

Resumen

Se estudiaron por primera vez para una langosta espinosa (*Panulirus argus*) las variaciones de las principales enzimas digestivas en los diferentes estadios de la muda, así como con el estado de desarrollo. Se describieron relaciones positivas entre las actividades tripsina y amilasa con la talla del animal, mientras que las actividades esterasa y lipasa mostraron una relación inversa con la edad. No se encontró variación significativa entre la relación amilasa/tripsina y la talla de las langostas. Por el contrario, se detectaron tendencias positivas entre las relaciones tripsina/lipasa así como amilasa/lipasa y la talla. Los resultados sugieren que los cambios en la actividad de las principales enzimas digestivas de *P. argus* responden a las necesidades fisiológicas de cada estadio de desarrollo, aunque el análisis multivariante realizado también sugirió que estas variaciones no son totalmente independientes del alimento ingerido en cada momento. Por otra parte, las variaciones de las principales enzimas digestivas respecto a los estadios del ciclo de la muda fueron similares para todas las enzimas. Después de la muda, las actividades tripsina, quimotripsina, amilasa y lipasa se incrementan gradualmente hasta alcanzar los niveles máximos en la intermuda tardía (C4) y la premuda (D). Además, no se encontraron variaciones en el patrón electroforético de las principales enzimas digestivas durante los estadios de la muda o desarrollo, demostrándose que la regulación es cuantitativa y no cualitativa.

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Abstract

Changes in major digestive enzymes through developmental and molt stages were studied for the spiny lobster *Panulirus argus*. There were significant positive relationships between specific activity of trypsin and amylase enzymes and lobster size, whereas esterase and lipase specific activities decreased as lobsters aged. No relationship was found between amylase/trypsin ratio and lobster size. Positive trends were found, however, for trypsin/lipase and amylase/lipase ratios. Results suggest that changes in enzyme activity respond to the lobsters' physiological needs for particular dietary components although multivariate analysis suggested that enzyme activities could be not totally independent of diet. On the other hand, the pattern of changes of major enzyme activities through molt cycle was similar for most enzymes studied. Following molt, trypsin, chymotrypsin, amylase, and lipase activities gradually increased to maximal levels at late intermolt (C_4) and premolt (D). There were no variations in the electrophoretic pattern of digestive enzymes through developmental and molt stages and thus, it is demonstrated that regulation is exerted quantitatively rather than qualitatively. Further studies on the effect of other intrinsic and extrinsic factors on digestive enzyme activities are needed to fully understand digestive abilities and regulation mechanisms in spiny lobsters.

Keywords: Ontogeny, Molt, Digestive enzymes, Spiny lobster, *Panulirus*.

1. INTRODUCTION

Spiny lobsters are ecologically key predators in benthic communities of tropical and temperate seas (Lipcius and Eggleston, 2000) and support important fisheries all around the world. Additionally, tropical species (e.g. *Panulirus argus, Panulirus ornatus*) continue to attract great interest for aquaculture, especially for the growout of post-pueruli collected from the wild. Cage growout of spiny lobsters *P. ornatus* in Vietnam accounted for around US$ 40 million in 2004 (Thuy, 2004) and US$ 100 million in 2006 (Jones and Williams, 2007).

Knowledge of digestive capacities of spiny lobsters through their complex life cycle is limited to a few studies although it is relevant from ecological and aquaculture viewpoints. After hatching, pelagic larvae (phyllosoma) drift in ocean waters for several months feeding on plankton. Information on the natural diet of phyllosoma is scarce but phyllosoma from different palinurids have been successfully reared in laboratory on a diet of *Artemia* and other seafood. Final-stage phyllosoma larvae molt into the colorless lobster-like pueruli and migrate into shallow waters such as mangrove areas for settling to the bottom. There is evidence (Nishida *et al.*, 1990; Wolfe and Felgenhauer, 1991; Lemmens and Knott, 1994; Nishida *et al.*, 1995; Abronhosa and Kittaka, 1997) that pueruli do not feed but rather exhibit secondary lecithotrophy. Shortly after settlement, *P. argus* pueruli molt to become post-pueruli and start to feed on a wide variety of invertebrates such as crustaceans (copepods), holothurians, foraminiferans, and sponges (Lalana and Ortiz, 1991). The natural diet of juvenile and adult *P. argus* comprises mainly gastropods but also bivalves, chitons, anomurans, brachyurans, and sea urchins, depending on availability in the wild (Colinas-Sanchez and Briones-Foorzan, 1990; Herrera *et al.*, 1991; Cox *et al.*, 1997). Ontogenetic variations in digestive enzymes activities in spiny lobsters have been presented before only for *Jasus edwardsii* (Johnston, 2003). However, many other studies have shown that digestive enzymes in Crustacea vary according to the developmental stage. Still, there is little information on the forces that drive these variations and the level at which regulation occur.

Additional information on the physiological meaning of enzyme variations and regulation mechanisms can be obtained from the study of the molt cycle. The molt cycle drives

extensive behavioral, integumentary, physiological, and biochemical changes in crustaceans. Besides its role in digestion, the digestive gland or hepatopancreas actively participates in the molt cycle, being the major site for storage glycogen, fats, and calcium during premolt and thus, in the mobilization of these reserves when needed in subsequent molt stages. Enzyme variations during the molt cycle of crustaceans have been studied for some species (Bauchan and Mengeot, 1965; Van Wormhoudt, 1974; Fernandez *et al.*, 1997; Fernandez-Gimenez *et al.*, 2001; 2002) and results are to some extent contradictory. We are aware of no previous reports for spiny lobsters.

Enzymes present in the digestive tract of spiny lobsters have been studied in *Panulirus japonicus* (Galgani and Nagayama, 1987; Iida *et al.*, 1991), *J. edwardsii* (Johnston, 2003), *Panulirus interruptus* (Celis-Gerrero *et al.*, 2004; Navarrete del Toro *et al.*, 2006), and recently in *P. argus* (Perera *et al.*, 2008). With few exceptions, such studies have covered mainly biochemical aspects of enzymes.

This study was undertaken to examine developmental and molt stage variations in the main digestive enzymes of *P. argus*. Results are correlated with feeding behavior and reveal the capacity of the lobster digestive system to meet the physiological requirement of each developmental or molt stage. Also, results suggest the level at which the regulation of enzyme activity is exerted.

2. MATERIALS AND METHODS

2.1. Staging of lobsters

The distal half of a single pleopod was excised from each lobster upon capture in the wild and used within minutes of removal, to avoid misleading epidermal retraction, for staging according to Lyle and MacDonald (1983).

Differentiation among stage C subdivisions is difficult based only on histology because of variations in onset, rate, and degree of formation of the different cuticle layers, as has been shown in the spiny lobster *Panulirus marginatus* (Lyle and MacDonald, 1983). After molt, clawed and spiny lobsters harden progressively through the different stages (Aiken, 1980;

Quackenbush and Herrnkind, 1983) and although shell hardness is unreliable as a unique indicator of molt stage, it was conveniently used herein to subdivide arbitrarily stage C as in Aiken (1980). In our study, all C substages were considered as "intermolt" and the term "molt" was used in synonymy with ecdysis. Due to limited availability of premolt lobsters, data from premolt were pooled. In summary, for this study lobsters were classified into premolt (D), molt (E), postmolt (AB), and intermolt stages (C_2, C_3, C_4).

2.2. Sample collection and preparation of extracts

Lobsters were collected in the Golf of Batabanó, Cuba. Post-larvae (post-pueruli) were collected by sandwich floating collectors, and juveniles and adults were collected by diving. Small juveniles could not be collected due to difficulties in locating them. Only late intermolt (C_4) lobsters were used to study developmental trends in enzyme activities whereas lobsters at all other stages were analyzed for variations during the molt cycle. Animals were anesthetized by placing them on ice for 10 min and then dissected to collect the hepatopancreas. Samples were immediately frozen in liquid nitrogen, lyophylized, and then stored at -20°C until used. Before analysis, the powders were homogenized in cold distilled H_2O and centrifuged at 4°C at 8,000 *g* for 15 min. Supernatants were immediately used for enzyme assays and electrophoresis.

2.3. Assays for enzyme activity

All chemicals used in this study were reagent grade and were obtained from Sigma except DMSO which was purchased from Merck. The concentration of crude extracts for each assay was adjusted to obtain linearity of enzyme activities with respect to both protein concentration and time. Assays were always run in duplicate and enzyme activities were expressed as change in absorbance per minute per milligram of protein (Δ Abs min^{-1} mg protein^{-1}) or per gram of dry tissue (Δ Abs min^{-1} g dry tissue^{-1}). The protein content of extracts was measured according to Bradford (1976) using BSA as standard.

2.3.1. Trypsin and chymotrypsin- like activities

Trypsin-like activity was measured using 1.25 mM BApNA in 0.2 M Tris-HCl, 20 mM $CaCl_2$, pH 8.4. Chymotrypsin-like activity was measured with 0.1 mM SApNA in the same

buffer. Substrate stock solutions of BApNA (125 mM) and SApNA (10 mM) were prepared in DMSO and brought to working concentration by diluting with buffer prior to assaying. In a 96-well microplate, 10 µL of enzyme extract were mixed with 200 µL of respective substrate, and liberation of *p*-nitroaniline was kinetically followed at 405 nm in a microplate reader Multiscan EX (Thermolab Systems).

2.3.2. Non- specific esterase activity

Esterase activity in extracts was assessed by the hydrolysis of 0.3 mM *p*-nitrophenyl butyrate (*p*-NPB) according to Gilham and Lehner (2005) with slight modifications. Stock solutions (100 mM) were prepared for the *p*-nitrophenyl esters in CH_2Cl_2 and diluted immediately prior to assaying with 20 mM Tris-HCl, 150 mM NaCl, pH 8.0. For assays, 20 µL of enzyme extract were mixed with 200 µL substrate solution in 96-well microplate and the liberation of *p*-nitrophenol was measured kinetically at 405 nm in a microplate reader.

2.3.3. Lipase activity

Lipase activity was measured using ß–naphthyl caprylate in DMSO as the substrate. The assay mixture contained: 100 µL of 100 mM sodium taurocholate, 900 µL of 50 mM Tris-HCl, pH 7.5, 10 µL enzyme extract, and 10 µL of substrate stock solution (100 mM). The reaction mixture was incubated for 30 min at 37°C for the reaction to proceed and then 10 µL of 100 mM Fast Blue BB in DMSO was added. The reaction was blocked with 100 µL TCA 12%. Finally, 1.35 mL of 1:1 (v:v) ethyl acetate/ethanol solution was added and absorbance was recorded at 510 nm.

2.3.4. Amylase activity

Amylase activity was determined according to the Somogy-Nelson method using soluble starch (2% w:v) as substrate, as described in Robyt and Whelan (1968). Briefly, 20 µL of enzyme extract and 125 µL of buffer pH 5 were incubated with 125 µL of substrate for 30 min. Activities were measured by calculating the reducing sugars released at 600 nm.

2.4. Zymograms of digestive enzymes

Substrate-SDS-PAGE (5% stacking gel, 13% separating gel) was used to examine the composition of proteases in digestive tract (García-Carreño *et al.*, 1993) using casein as the substrate. Samples were neither boiled nor treated with mercaptoethanol before loading into the gel and they were run in a vertical electrophoresis device (Hoeffer SE260, 8 x 10 x 0.75 cm). Bands were revealed as described before (Perera *et al.*, 2008).

Substrate-SDS-PAGE for amylase was performed on 5% stacking gel and 12% resolving gel in the same conditions as for proteases. Gels were immersed in a starch solution (1%) at pH 6 for 60 min and then stained with iodine/KI solution (10%) (Perera *et al.*, 2008).

Zymograms for esterases were performed under native conditions (5% stacking gel and 8% resolving gel) as described by Perera *et al.* (2008). High degree of polymorphism for esterases harms us to analyse variation through developmental and molt stages.

2.5. Statistical analysis

Regressions describing the relationship between the enzymes activities and the body size (carapace length, CL) were obtained by the least-squares method. The significance of regression slope *b* was tested by analysis of variance. R^2 values were calculated as a measure of relative goodness of fit of regression curves. Data points were means of duplicate assays for each lobster.

Similarities in digestive capacities among developmental stages were explored by multivariate analysis, taking into account all enzymes studied (multiple dimensions). A hierarchical and agglomerative method was selected and the number of clusters was not pre-established, but randomly generated by the software. Single linkage was selected as amalgamation rule and Euclidean distances were used for computing distances between objects in the multi-dimensional space.

One-way ANOVAs were performed to test for differences among different molt stages. Data were previously checked for normality and homogeneity of variance by Kolmogorov-

Smirnov and Levine's tests, respectively, and $\log_{10}$ transformation of data was necessary to achieve requirement for analysis. SNK tests were used to determine post-hoc differences among means. The software Statistica 6.0 (Statsoft, Inc.) was used for all tests, performed with $\alpha = 0.05$.

The ratio of amylase to protease activity was estimated from the amylase: trypsin ratio as in Lovett and Felder (1990) for shrimp and Johnston (2003) for spiny lobsters. Trypsin accounts for almost 60% of protease activity in *P. argus* (Perera *et al.*, 2008). Additionally, trypsin/lipase and amylase/lipase ratios were calculated.

3. RESULTS

3.1. Postlarvae (post-pueruli) to first juvenile stages: trends in digestive enzyme activity

There were no trends in the relationship between specific enzyme activities (trypsin, amylase, esterase and lipase) and lobster size for animals from 6 to 20 mm CL, i.e. from first post-pueruli to first juvenile stages. Mean specific activities as ΔAbs min^{-1} mg $protein^{-1}$ were as follows: trypsin (6.5 ± 2.36), chymotrypsin (3.1 ± 1.24), amylase (12.2 ± 7.02), esterase C_4 (14.7 ± 12.1), and lipase (1.2 ± 0.63).

3.2. Juvenile to adult: trends in digestive enzyme activity

There was a significant positive relationship between specific activity and lobster size for trypsin and amylase enzymes in juveniles and adults (Fig 1, Table 1). Trypsin and amylase activities expressed as units per gram of dry tissue followed the same trends (not shown). Chymotrypsin activity also increase with age ($F=15.2$, $p \leq 0.001$) but R^2 was extremely low (0.29) thus considered as non-relevant. Esterase and lipase activities exhibited negative linear relationships with lobster size (Fig 1, Table 1). Esterase and lipase activities expressed as units per gram of dry tissue followed the same trends (not shown).

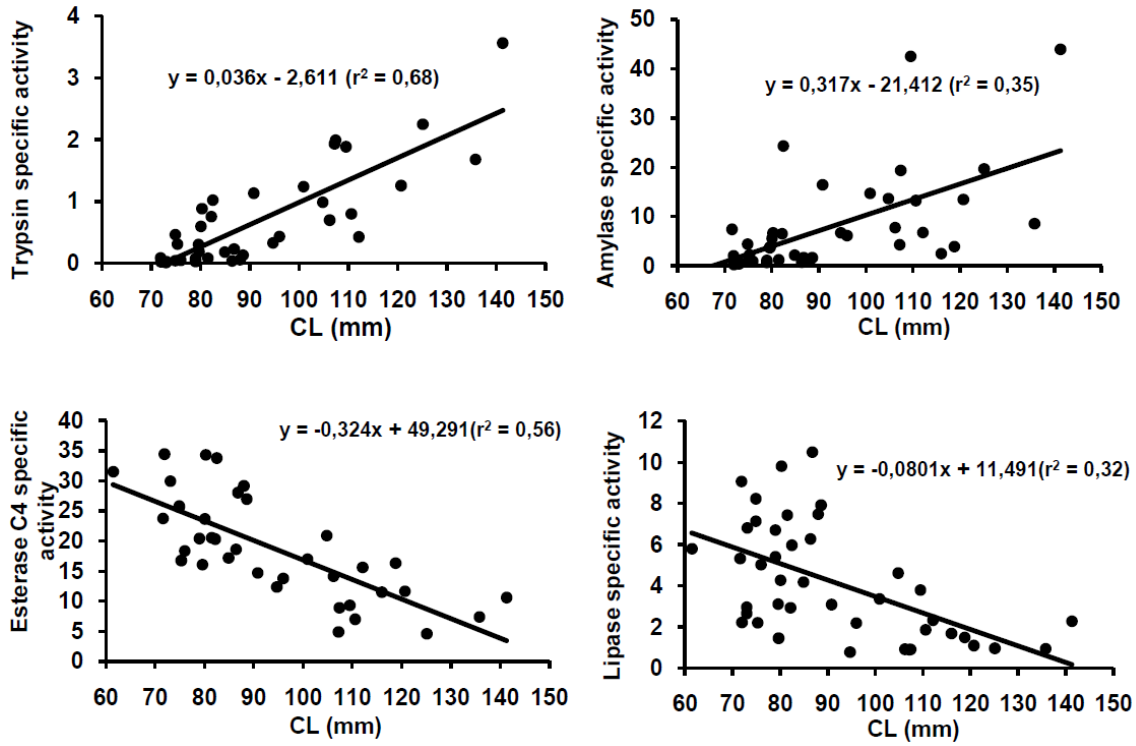

Fig 1. Specific activity (ΔAbs min^{-1} mg $protein^{-1}$) of trypsin, amylase, esterases and lipase in the digestive gland of intermolt *P. argus* from late juvenile to adult. Substrates were BApNA for trypsin, starch for amylase, *p*-nitrophenyl butyrate for esterases and ß–Naphthyl caprylate for lipase. Data points are mean of duplicate measurements for an individual animal.

Table 1. Summary of statistics for the calculated regressions.

Regressions	*b*	*a*	*N*	*r*	*F*
Enzyme activities on size					
Trypsin on CL	0.036	-2.6109	38	0.82	76.19***
Amylase on CL	0.3168	-21.412	41	0.59	20.98***
Esterase C_4 on CL	-0.3244	49.291	37	-0.75	44.47***
Lipase on CL	-0.0801	11.491	42	-0.56	18.40***
Enzymes ratios on size					
A/L on CL	0.2231	-16.668	41	0.74	48.12***
T/L on CL	0.0286	-2.2011	38	0.78	55.46***
A/T on CL	-0.1217	25.652	38	0.36	ns

Enzymes activities as specific activities (ΔAbs min^{-1} mg $protein^{-1}$). CL: cephalothorax length. A/L: amylase to lipase ratio. T/L: trypsin to lipase ratio. A/T: amylase to trypsin ratio. Slope (*b*) and intercept (*a*) are from the regression model $Y=a+bx$. *N*: number of observations, *r*: correlation coefficient. *F*: variance ratio of regression and residual mean squares. The values for *F* correspond to a probability of $p \leq 0.001$ (***). ns: not significant.

Multivariate analysis revealed two main clusters, the one for lobsters of less than 90 mm CL and the one for bigger animals. Postlarvae appear more related to old than to young lobsters (Fig 2).

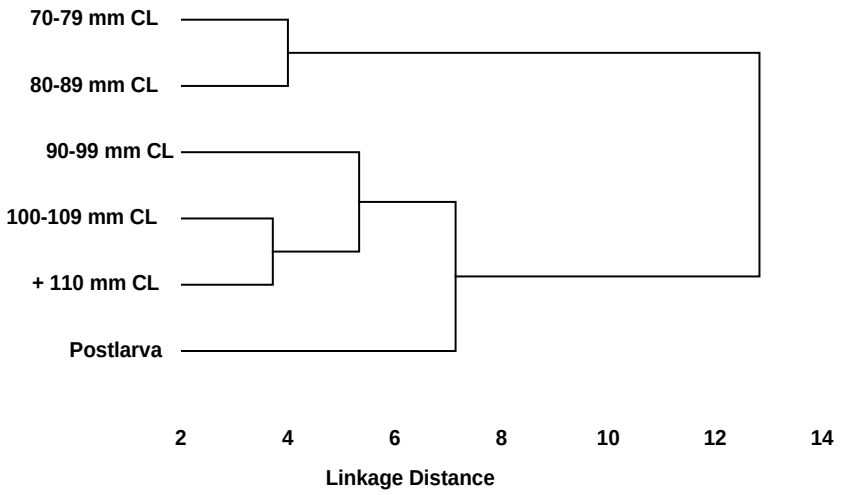

Fig 2. Result of the cluster multivariate analysis carried out using all digestive enzyme activities analysed at different developmental stages.

3.3. Enzyme ratios

There was no significant ontogenetic trend in amylase/trypsin ratio between juvenile and adult lobsters. Positive trends were found for trypsin/lipase and amylase/lipase ratios (Table 1).

3.4. Molt cycle variation in digestive enzyme activities

The pattern of changes in enzyme activity through the molt cycle was similar for all enzymes studied except esterases. After molt, trypsin, chymotrypsin, amylase, and lipase activities gradually increased to maximal levels at late intermolt (C_4) and premolt (D) (Fig 3). Lipase and amylase activities significantly dropped near or at molt while proteases dropped after molt. Most enzyme activities remained relatively low until early intermolt (C_2) (Fig 3). Esterase activity did not significantly vary through the different molt stages except for higher values at late intermolt (C_4) (Fig 3).

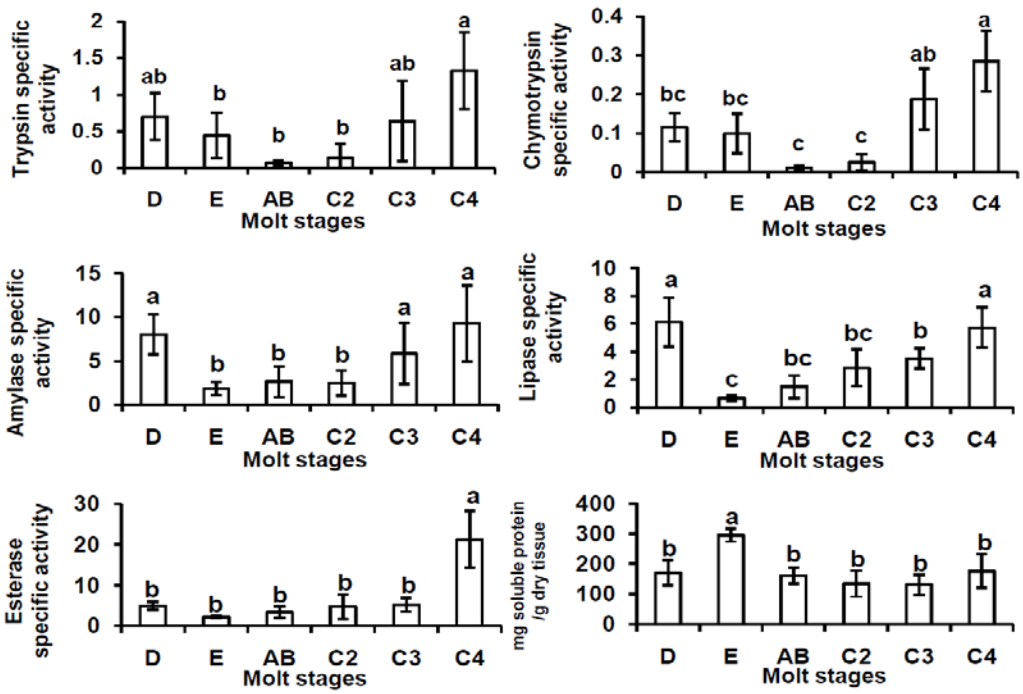

Fig 3. Enzyme activities and total soluble proteins variations in the hepatopancreas throughout the molt cycle of *P. argus*. *F* and *p* values for one-way ANOVAs are shown. Different letters above the bars in each figure represent stages that differ by SNK tests at $p \leq 0.05$. Number of individual analyzed for each molt stage was: 5 individuals in D, 3 individuals at E, 5 individuals in AB, 5 individuals in C_2, 6 individuals in C_3, and 14 individuals in C_4.

Variations described above refer to specific activity of enzymes. No significant variation was found in the protein content of the digestive gland among molt stages, except for higher values at molt (Fig 3). Activities of the different enzymes through the molt cycle are similar than above if expressed as activity units per g of dry tissue (not shown).

3.5. Changes in the isoenzyme pattern of digestive enzymes

There were no variations on the electrophoretic pattern of proteases and amylase through the molt cycle (Fig 4), except those correspond to the polymorphism previously reported for digestive enzymes in *P. argus* (Perera *et al.*, 2008). Also, postlarva and adults present the same electrophoretic pattern of digestive enzymes (not shown).

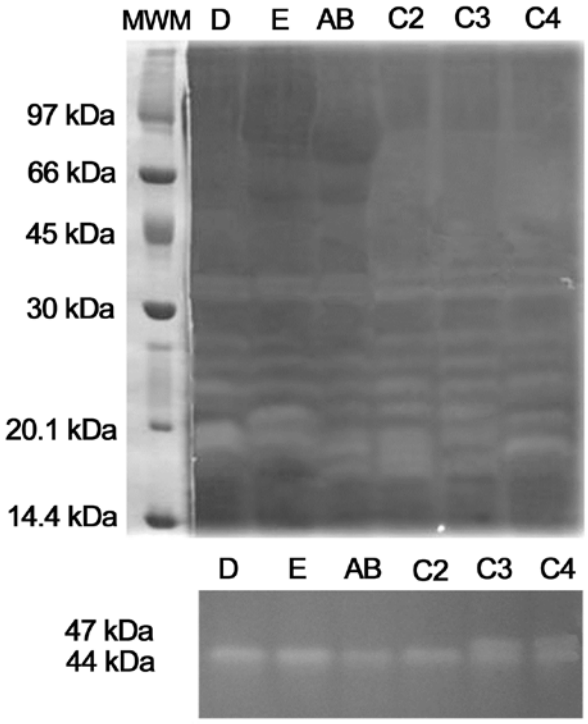

Fig 4. Isoenzyme pattern of proteases (top panel) and amylases (button panel) at different molt stages. Variations correspond to polymorphism for trypsin (proteases of lower MW in the top panel) and amylase (Perera *et al*., 2008) and not to molt stages. For example: many individuals in C_3 and C_4 present only the 44 kDa isoform of amylase whereas individuals from D to C_2 can express also the 47 kDa enzyme (not shown).

4. DISCUSSION

No variations were detected in protein content of hepatopancreas among developmental and molt stages of *P. argus*, except for a significant increase at molt. Thus, variations in specific enzyme activity were assumed to be unaffected by protein content of extracts and reflect actual variation in digestive capacities.

4.1. Variations among developmental stages

After an extended period of feeding on plankton in ocean waters, spiny lobster *P. argus* larvae metamorphose into pueruli, which then migrate into shallow waters to settle. The short-lived pueruli stage do not feed and their digestive system experiences a series of changes adapted for benthic feeding behavior as described for *J. edwardsii* (Nishida *et al.*, 1990), *P. argus* (Wolfe and Felgenhauer, 1991), and *P. cygnus* (Lemmens and Knott,

1994; Abronhosa and Kittaka, 1997). This phase does not feed and although the presence of digestive enzymes has been demonstrated (Johnston, 2003), their meaning is still poorly understood. Our work focuses on feeding stages.

There were no trends in specific enzyme activities (trypsin, amylase, esterase and lipase) in lobsters from 6 to 20 mm CL (from post-pueruli to first juvenile stages). The most frequent items in natural diet of *P. argus* post-pueruli are copepods, holothurians, foraminiferans, and sponges (Lalana and Ortiz, 1991). All *P. argus* post-pueruli stages feed on similar items but brachyurans do not appear in stomachs until post-pueruli of 8 mm CL and increase their importance in diet of larger post-pueruli (11-13 mm CL) (Lalana and Ortiz, 1991). Since this progressive change in diet does not correspond to any trend in enzyme activities, it is proposed that activities of enzymes present since early post-pueruli stages are enough for an efficient digestion of a varied diet. Changes observed in diet composition could reflect just a steady adaptation to preying on larger items. Specific activities for amylase, esterase, and lipase in *P. argus* post-pueruli are similar to those found in adults, which supports the statement above. However, values for proteases in post-pueruli are above the ones for juvenile and adults. Since prey items of post-pueruli are rich in highly digestible proteins, this result could indicate that enzyme activities respond to the physiological requirements of this stage rather than to a compensatory mechanism in the presence of a deficient diet. The requirement for dietary protein has been determined to be very high (45%) in post-pueruli *P. argus* (Fraga, 1996), whereas the estimates are at or below 35% in juveniles (Perera *et al.*, 2005).

Variations in digestive enzyme activities could then reflect changing physiological requirements as lobsters grow. Metabolism of juvenile *P. argus* relies on protein for energy and, when feeding on major natural prey (e.g. gastropods, bivalves, and crustaceans), lipids can somewhat spare proteins from oxidation (Díaz-Iglesias *et al.*, 2002). Higher activity of enzymes involved in lipid digestion in young lobsters could ensure a higher proportion of ingested proteins to be channeled toward growth in correspondence with the higher growth rate of juveniles. The same idea can be drawn from Trypsin/Lipase ratio.

Amylolytic activity was measured here at the optimal pH, which is acidic and thus distant from the physiological pH in the digestive gland. However, high amylolytic activity is

present in the gastric juice of *P. argus* (Perera *et al.*, 2008) where acidic pH occurs (5.9 ± 0.2, mean ± standard deviation, unpublished results) as in other spiny lobsters (Johnston, 2003; Navarrete del Toro *et al.*, 2006). Thus, amylolytic activity measured in the hepatopancreas at acidic pH is likely a good indicator of that occurring in the gastric chamber. The positive trends observed for amylase suggest that the efficiency of carbohydrate digestion increases as lobsters age. Since there is no evidence of a significant role of carbohydrates in energy metabolism of *P. argus* (Díaz-Iglesias *et al.*, 2002; Perera *et al.*, 2005), whether this behavior of amylase activity could energetically pay off for the drop in lipolytic activity in older lobsters remains to be studied, but it is unlikely. No trend in Amylase/Trypsin ratio was observed in this work for *P. argus* and in Johnston (2003) for *J. edwardsii*. An alternative hypothesis is related to the bigger and stronger exoskeleton in larger lobsters and a possible increase in glycogen needs for chitin synthesis after molt as animals grow.

Interestingly, small *J. edwardsii* exhibited higher amylase activity than large lobsters (Johnston, 2003) which is the opposite of our finding for *P. argus*. Feeding habits of *J. edwardsii* are somewhat different from those of *P. argus* (Edmunds, 1995). It is not known to what extent these differences can be explained by a different diet or by a distinct specie-specific pattern of expression of enzymes. Our results indicate that changes in enzyme activities are developmental clued but since our lobsters were not grown in captivity on a single diet but sampled from the wild, further studies are required to determine if activities can be affected by changes in the composition of diet. Despite significant trends, variations in enzyme activities can not be explained to a great extent by the increase in size even for trypsin where lobster size only accounts for 68% of the variation. Our results indicate that factors other than size could influence digestive enzyme activities in lobsters. Multivariate analysis suggested that digestive enzyme activities appear to be not totally independent of diet. Two main clusters were obtained and a size of 90 mm CL, correspond to a size where some prey items appear for the first time in stomachs (e.g. *Strombus gigas*) and other big gastropods become more frequent preys (Herrera *et al.*, 1991).

Cross-reactivity can generate a false association between lipase and esterase activities but the substrates employed in this study discriminate the patterns of lipase and esterase activities through the molt cycle (see below), suggesting that observed connection between

activities correspond to the physiological role of these enzymes rather than to an artifact of methodology. On the other hand, although several proteolytic enzymes exhibit esterase activity, trends for proteases and esterases were reverse. Finally, the method used for esterases does not discriminate digestive esterases from intracellular esterases, but the former were assumed more abundant than the latter.

To our knowledge, there is only one previous study on ontogenetic variations of digestive enzymes in spiny lobsters. Johnston (2003) found a positive relationship between total activity (units per digestive gland) of all enzymes and size in the spiny lobster *J. edwardsii*, coinciding with an increase in size of digestive gland. However, she observed no ontogenetic trend in specific activities except for negative correlations for amylase and laminarinase. In contrast, we report here that trypsin and amylase specific activities tend to increase with lobster size whereas esterase and lipase specific activities fall as lobsters age.

4.2. Molt cycle variations

Variations in the activity of digestive enzymes in *P. argus* resemble the foraging and feeding patterns observed in previous studies. Feeding rate in *P. argus* gradually increases during intermolt to high levels at late C_4 and early D. Then, it gradually decreases to minimal rates at D_3-B_1. Finally, food consumption rises again at late postmolt (B_2) and early intermolt (C_1) (Lipcius and Herrnkind, 1982).

During late stage C, few glycogen granules are evident in the hepatopancreas of *P. argus* but their number increases during stage D both in the hepatopancreas and epidermis. This glycogen disappears some days after molt, indicating glycogen is a necessary precursor for chitin formation (Travis, 1955). In this scenario, the observed increase in amylase activity at late intermolt and during premolt might enhances carbohydrate assimilation and formation of glycogen reserves.

The lipid content of the digestive gland is positively correlated with growth in spiny lobsters (Johnston *et al.*, 2003). Lipids are continuously stored in the hepatopancreas of *P. argus* during intermolt and early premolt (Travis, 1955) and in correspondence high

lipolytic activity occurs in these stages. Also, during late premolt some glycogen is thought to come from the conversion of fat stored in the hepatopancreas (Travis, 1955).

Protease activity increases gradually following molt to maximal activity at late intermolt in correspondence to the carnivorous behavior of lobsters. Protease activity remains high during premolt and thus allowing the animal an efficient use of dietary protein during the feeding phase of premolt. Results in other crustaceans indicate that active synthesis of digestive enzymes remains during early premolt. Chymotrypsin mRNA (Van Wormhoudt *et al.*, 1995) and trypsin mRNA (Klein *et al.*, 1996) increase during premolt in the shrimp *L. vannamei*. Also, the high protease activity in premolt lobsters could be associated to the storage of enzymes produced. Both active synthesis and storage in premolt could promote the high activities observed.

Esterase enzymes are involved in different functions besides digestion and perhaps that is why there is not a tight connection between enzyme activity and feeding behavior.

In general, the activities of the main digestive enzyme in *P. argus* were highest at late intermolt and premolt. Our results totally or partially agree with those observed in *Palaemon serratus* (Van Wormhoudt, 1974) and *Penaeus notialis* (Fernandez *et al.*, 1997) but differ from other studies. Bauchan and Mengeot (1965) reported for *Carcinus maenas* high protease activity in postmolt and intermolt and low activity during premolt. In *Pleoticus muelleri*, the activities of trypsin and chymotrypsin were highest in postmolt (Fernández-Gimenez *et al.*, 2001) whereas in *Artemesia longinaris* they were highest during intermolt and dropped at premolt (Fernández-Gimenez *et al.*, 2002). Many factors can contribute to these contradictory results in different crustacean species, such as criteria for staging, duration of each stage, and species-specific patterns of expression and synthesis of digestive enzymes. Klein *et al.* (1996) studied trypsin expression through the molt cycle of *L. vannamei* and found highest mRNA levels at premolt and lowest levels near molt. Trypsin expression gradually increased, especially during the intermolt. These results compare better to those obtained in this work.

There were no variations on the electrophoretic pattern of digestive enzymes through the molt stages, except those correspond to the polymorphism previously reported for digestive

enzymes of *P. argus* (Perera *et al.*, 2008). Also, postlarva and adults present the same electrophoretic pattern of digestive enzymes (not shown). Individual enzymes in some fishes develop independently during ontogenesis with variations related to species, temperature and feeding habits (Kolkovski, 2001; Rathore *et al.*, 2005). Our results shown that the lobsters *P. argus* express all proteases and amylase enzymes (Fig. 4) at first benthic feeding and all molt stages. Several studies have reported the influence of different environmental factors on trypsin mRNA levels, but to our knowledge, never the absence of isoforms. Sánchez-Paz *et al.* (2003) and Sainz *et al.* (2004) have concluded that trypsin activity in *L. vannamei* is regulated quantitatively at the transcription level, but not qualitatively. The lobster *P. argus* appears to regulate trypsin (and other digestive enzymes) activity in a similar fashion. Additionally, the high degree of polymorphism for trypsin enzymes in *P. argus* (Perera *et al.*, 2008) provides several points for this regulation to occur. Yet, electrophoresis has been the method of choice in most studies and thus only isoforms with different electrophoretic mobilities could be detected. Five trypsin-like enzymes were found in gels by Perera *et al.* (2008) but current studies in our laboratory have shown that after gel filtration and anion-exchange chromatography, up to seven trypsin-like enzymes occur in *P. argus* digestive gland (unpublished results). Sainz *et al.* (2004) showed that the three isotrypsins of *P. vannamei* have different kinetic properties. The high amount of trypsin isoforms makes spiny lobsters good models for studying regulation mechanisms in decapod crustaceans.

Our results suggest that changes in the activities of digestive enzymes of *P. argus* across developmental and molt stages respond to the lobsters' physiological needs for particular dietary components and thus, are thought to be closely regulated by internal factors. It has been reported that endocrine cells in the midgut of the cockroach are stimulated to synthesize and secrete crustacean cardioactive peptide (CCAP) by nutrients, and CCAP then up-regulates the activity of digestive enzymes like α-amylase and proteases (Sakai *et al.*, 2006). However, Chung *et al.* (2006) found that the expression patterns of CCAP mRNA in crustaceans thoracic ganglia throughout the moult cycle shown few changes. Yet, other internal signal like cholecystokinine-like, secretin-like, gastrin-like substances and ecdysteroids could be related with digestive enzyme regulation during development and the molt cycle. Also here it is demonstrated that this regulation is exerted quantitatively rather than qualitatively. Further studies are needed on the effects of other

intrinsic and extrinsic factors on digestive enzyme to fully understand digestive abilities of spiny lobsters and regulation mechanisms.

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Capítulo 4

In vitro digestion of protein sources by crude enzyme extracts of the spiny lobster *Panulirus argus* (Latreille, 1804) hepatopancreas with different trypsin isoenzyme patterns

Perera, E., Moyano, F. J., Rodríguez-Viera, L., Cervantes, A., Martínez-Rodríguez, G., Mancera, J. M. ***Aquaculture 310, 178–185*** **(2010)**

Resumen

Se estudió la digestibilidad *in vitro* de proteínas en la langosta espinosa *Panulirus argus* mediante dos técnicas diferentes: i) la digestión en celda, en la cual los productos de la digestión son removidos continuamente mediante diálisis, y ii) la electroforesis en geles de poliacrilamida, la cual permite la visualización de la digestión de las diferentes fracciones proteicas del alimento. Las tripsinas, principales proteasas digestivas, presentan tres fenotipos o patrones electroforéticos. Por este motivo se estudiaron las diferencias en la eficiencia en la digestión de los principales fenotipos. Los resultados de este estudio mostraron por primera vez para un crustáceo que los fenotipos de tripsina difieren en la eficiencia con que digieren diferentes fuentes de proteína.

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Abstract

The development of cost-effective and nutritionally adequate formulated diets is a key step in the sustainable expansion of spiny lobster aquaculture. Despite proteins are the major and most expensive component of diets, few studies are available on protein digestibility in spiny lobsters and such assessments never have been performed by *in vitro* methods. Two techniques were used for studying *in vitro* protein digestion of some common aquafeeds ingredients by the spiny lobster *Panulirus argus*: i) the digestion cell in which the digestion products are removed by dialysis, and ii) electrophoresis which allows the visualization of the different protein fractions in the tested meals. Since three main trypsin isozyme patterns or phenotypes have been recently described in *P. argus*, the potential differences in protein digestion between individuals with different trypsin isozyme patterns were assessed. Results herein presented demonstrate for the first time in a crustacean species that the different trypsin phenotypes differ in protein digestion efficiency. Also, the digestion cell method was applied for the first time to a crustacea, proving to be sensitive to small changes in digestion efficiency. This method could be used in further *in vitro* studies for examining other aspects of spiny lobsters digestive process.

Keywords: Spiny lobster, *Panulirus argus*, Trypsin isoenzymes, Protein digestion, *In vitro*, Digestibility, Squid

1. INTRODUCTION

There is a great interest in the development of commercial aquaculture of spiny lobsters based on the growout of wild-caught postlarva, especially for tropical species (Jeffs and Davis, 2003). Seed availability and natural mortality rates of this stage are high (Phillips *et al.*, 2003; Cruz *et al.*, 2006) and a minimum impact of this activity on fisheries has been predicted (Phillips *et al.*, 2003). There is a flourishing industry in Vietnam accounting for around US$ 100 M per year (Thuy and Ngoc, 2004) but feeding practices based on fishery bycatch have proven to produce a deleterious effect on environment. Also, many other disadvantages of fresh feeding make the development of cost-effective and nutritionally adequate formulated diets a key step for progressing to large-scale industry worldwide.

Nutritional requirements of some spiny lobsters are known (see Williams, 2007 for a review) but growth rates on formulated diets are still suboptimal for most species. However, Smith *et al.* (2005) and Barclay *et al.* (2006) developed a high-protein pelleted diet for *Panulirus ornatus* which produced better growth results than fresh food (i.e. mussels). This was the first artificial diet achieving a superior growth than fresh food and results were attributed to high feeding frequency as well as to the high amounts of krill meal included in the diet. This expensive component was also present at a high percentage in a recently reported diet which produced good growth rates in *Panulirus argus* (Cox and Davis, 2009).

The use of highly digestible ingredients in formulated diets enables a better use of nutrients for growth on a least-cost basis. However, only two studies have tested *in vivo* digestibility of artificial diets ingredients for spiny lobsters. These studies have shown that spiny lobsters (*Jasus edwardsii*: Ward *et al.*, 2003; *P. ornatus*: Irvin and Williams, 2007) are able to efficiently digest proteins from several sources, but no result is available for *P. argus*. Moreover, the mentioned studies yielded surprisingly low digestibilities for squid meal. The low nutritional value of squid for the temperate lobster *J. edwardsii* was further corroborated by Radford *et al.* (2007) who reported poor growth and reduced survival through successive molts when animals were fed exclusively on squid, but this opposed to our preliminary results on good growing *P. argus* with frozen squid (unpublished).

Digestive enzymes are key factors determining digestibility. Some studies are available on the digestive enzymes of spiny lobsters, mostly on their biochemical characterization (Galgani and Nagayama, 1987; Iida *et al.*, 1991; Celis-Gerrero *et al.*, 2004; Navarrete del Toro *et al.*, 2006; Perera *et al.*, 2008a), variations of activities throughout development and molt stages (Johnston, 2003; Perera *et al.*, 2008b) and time course of activities after ingestion (Simon, 2009). As occurred in most crustacea, trypsins are the main proteases in the digestive tract of spiny lobsters, accounting for up to 60% of digestive proteolysis (Celis-Gerrero *et al.*, 2004; Perera *et al.*, 2008a). We have recently described the existence of at least three different trypsin isozyme patterns or phenotypes in *P. argus* (Perera *et al.*, 2008a).

The aim of this study was to study two novel aspects related to lobster digestive physiology: *i)* the evaluation of protein *in vitro* digestion and *ii)* the potential differences in protein digestion between individuals of *P. argus* with different trypsin isozyme patterns. To achieve the second goal, *in vitro* digestion trials were performed using crude extracts of the digestive gland with three different trypsin phenotypes facing several meals of suspected different grade, following a similar strategy to Bassompierre *et al.* (1998) for testing the same hypothesis in Atlantic salmon.

2. MATERIALS AND METHODS

2.1. Animals and biological samples

Spiny lobsters (80-100 g) were collected by diving in the Gulf of Batabanó, Cuba. Intermolt lobsters according to Lyle and MacDonald (1983) were anesthetized by immersing them into ice-cold water before digestive gland extraction. Samples were immediately frozen in liquid nitrogen and then lyophylized and stored at −80 °C. Before analysis, the powders were homogenized in 200 mM Tris-HCl buffer pH 7.5 and centrifuged (8000 g) at 4 °C for 15 min. Supernatants were immediately used for trypsin activity determination, protease zymogram or *in vitro* digestion.

2.2. Trypsin activity

Other studies have demonstrated that trypsin activity is appropriated for normalizing activity in *in vitro* digestion assays using crude extracts (Rungruangsak-Torrissen *et al.*, 2002) and this strategy well fit to our objective of comparing trypsin phenotypes. Crude extracts were diluted with reaction buffer to measure enzyme activities at initial rates. Trypsin activity was measured using 1.25 mM N-benzoyl-DL-arginine *p*-nitroanilide (BApNA) in 200 mM Tris–HCl pH 7.5. Substrate stock solution (125 mM) was prepared in DMSO and brought to working concentration by diluting with buffer prior the assay. Ten μL of enzyme extract were mixed with 200 μL of substrate and the liberation of *p*-nitroaniline was kinetically followed at 405 nm in a microplate reader ELx808 IU, BioTek. Assays were run in triplicate. The protein content of enzyme extracts was measured according to Bradford (1976) using BSA as standard. Trypsin activity was expressed as arbitrary units (Abs/min) per mL or per mg protein as needed.

2.3. Classification of individuals by trypsin isoenzyme pattern (phenotypes)

Substrate (casein)-SDS-PAGE (5 % stacking gel, 13 % separating gel) was used to determine the composition of proteases in digestive tract as recommended by García-Carreño *et al.,* (1993) and successfully used before in lobsters (Celis-Gerrero *et al.*, 2004; Navarrete del Toro *et al.*, 2006; Perera *et al.*, 2008a). Samples were neither boiled nor treated with mercaptoethanol before loading into the gel. Running conditions and staining procedure were as described in our previous work (Perera *et al.*, 2008a). Clear bands indicated the presence of protease enzymes. Since the electrophoretic pattern (three main isoenzyme zones) of trypsin enzymes is known for *P. argus* (Perera *et al.*, 2008a), this technique allowed the classification of 102 of the 118 individuals analyzed, according to three phenotypes (Fig 1). Lobsters with the three isoenzyme (or isoenzyme zones) are named phenotype A. Individuals lacking the isoenzyme of higher electrophoretic mobility are named phenotype B, while lobsters lacking the isoenzyme of middle electrophoretic mobility are named phenotype C.

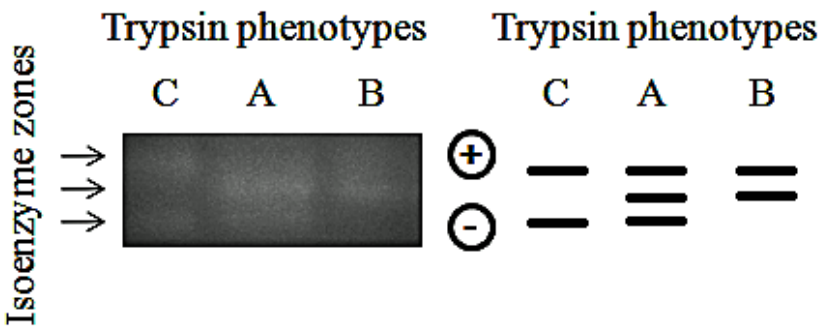

Fig 1. Trypsin isoenzyme patterns or phenotypes in *P. argus* revealed by casein zymography. Lobsters with the three isoenzyme (or isoenzyme zones) are named phenotype A. Individuals lacking the isoenzyme of higher electrophoretic mobility are named phenotype B, while lobsters lacking the isoenzyme of middle electrophoretic mobility are named phenotype C.

2.4. Preparation of meals

Soybean meals were obtained from local suppliers, while meals from animal origin were prepared at the laboratory, thus not totally equivalent to those used in aquafeeds. Frozen squid (*Loligo gahi*), jack mackerel (*Trachurus murphyi*) and Atlantic thread herring (*Opisthonema oglinum*) were purchased at fish market. Squid and herring were used intact whereas jack mackerel was deboned. Raw materials were boiled for 3-5 min, ground and dried at low temperature (60-65 °C). Next, feedstuffs were ground again, now using a 0.5 mm screen and stored at -80 °C until used. Proximate analysis of the different meals indicated the following crude protein and crude lipid contents: soybean meal (SBM) 57.1 % proteins, 1.4 % lipids, 8.2 % moisture; soybean isolate (SBI) 89.4 % proteins, 0.5 % lipids, 10% moisture; herring meal (HM) 79.4 % proteins, 16.2 % lipids, 5.2 % moisture; jack mackerel meal (JM) 79.1 % proteins, 16.8 % lipids, 5.5 % moisture; squid meal (SQM) 76.6 % proteins, 10.8 % lipids, 8.7 % moisture. Additionally, a protein extract of fresh squid muscle was prepared to be used as control.

2.5. In vitro ***digestion by the digestion cell method***

Digestions were performed using digestion cells modified from that described by Gauthier *et al.*, (1982) and Savoie and Gauthier (1986). Briefly, each digestion cell is composed by an inner reaction chamber formed by a cellulose dialysis membrane with molecular cut off of 1000 Da (Spectra/Por 6, Spectrum Medical Industries, Inc., Los Angeles, CA) fixed

within an inverted 50 mL Corning tube that forms an outer chamber. The molecular weight cut-off of 1000 Da allows that both free amino acids and small peptides (up to ten amino acids) are separated for quantification. The inner chamber is continuously agitated by a multiple magnetic stirrer (Variomag). Through the outer chamber a continuous flow of buffer is maintained by a high precision multichannel peristaltic pump (Ismatec, Idex Corp.), which allows the constant removal of digestion products. Nine of these digestion units were used simultaneously and several runs were required to complete ten digestions per phenotype per protein source (150 digestions). Three randomly selected individuals per phenotype were analyzed in each run.

The procedure for digestions was as follows: Protein samples were poured into the reaction chambers and stirred for 30 min in boric acid-borax buffer pH 7.5 for the solubilization of proteins. The amount of each meal to be added into the dialysis bags was previously determined in order to obtain 2 mg of soluble protein after 30 min of stirring, ensuring the same amount of starting soluble proteins. Then, the outer chambers were filled with boric acid-borax buffer pH 7.5 at 26 °C and individual enzyme extracts were added to each reaction chamber (zero time). Also, at this point a continuous flow of buffer (26 °C) at a rate of 0.5 mL/min was activated through the outer chambers. The ten individuals of a higher trypsin activity from each phenotype were selected for assays. The amount of extract added was adjusted in order to place the same units of trypsin activity (50 U) in all digestions (E:S ratio at time zero of 25 U/mg proteins). Dialysates were collected at different digestion time (30 min and 1 to 6 h) for determination of total amino acids using *o*-phthaldialdehyde (Church *et al.*, 1983) using L-leucine as standard. Results were expressed as total amount of amino acids (L-Leu equivalents) released taken into account the dialysates volumes at each sampling time. Blank assays without addition of enzyme extract were carried out for each protein source.

Additionally, a control digestion was performed with the JM meal and with a phenotype A digestive extract (containing the three isoforms) that was previously incubated for 60 min at room temperature with the trypsin inhibitor Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) at a final concentration of 0.5 mM to assess the role of trypsin enzymes on *in vitro* digestion. Preincubation was done at pH 6 to ensure stability of inhibitor and then rose before assay.

2.6. In vitro ***digestion followed by SDS-PAGE***

The time course of digestion of the protein sources was also followed by SDS-PAGE, as reported before for shrimps (Lemos *et al.*, 2004) and fishes (Klomklao *et al.*, 2006). Preliminary trials indicated that delipidization of animal meals with acetone (Coligan, 2007) is necessary for improving solubility and for obtaining a clean protein sample for electrophoresis. The different meals were stirred for 30 min in 200 mM Tris–HCl pH 7.5 for solubilization of proteins and additional vortex was applied to animal meals. Samples were then centrifuged to eliminate insoluble materials. One mL of each meal supernatant (1 mg/mL) was placed in each of three 1.5 mL Eppendorf tubes (zero time) and enzyme extracts belonging to the three different phenotypes were added to each of the three tubes. The enzyme extracts used for each phenotype was prepared by pooling the extracts of the ten individuals used in 2.5. The amount of digestive gland extract added was adjusted in order to face all meals to the same units of trypsin activity. Tubes were shaken through all the digestion time and samples were taken at zero time and at 15 min, 30 min, 1 h, 3 h and 6 h.

Immediately after collection of samples, the electrophoresis sample buffer containing β-mercaptoethanol and SDS was added, the samples were heated at 100 °C for 5 min and centrifugated at 8000 g for 5 min. Samples were analyzed by SDS-PAGE as described by Laemmli (1970) in 10% polyacrylamide gels. High and low molecular weight markers (Amersham Bioscience) were used as standards. Disappearance or clearing up of protein bands were interpreted as degradation. After scanning of gels, the decrease in optical density of main protein fractions was also analyzed using SigmaGel v1.0.5.0. (Jandel Scientific).

2.7. Statistical analysis

Data of total amino acid released after 6 h of *in vitro* digestion were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests, respectively, with $p \leq 0.05$. Total amino acids released were subjected to two-way ANOVA ($p \leq 0.05$) according to a randomized-block design with true replication. Lobsters were divided by trypsin phenotypes (3 blocks). This way the source of variation “phenotype” was fixed in

each block but varied from block to block. All members of a block were considered to be at identical condition except for the protein source tested (5 treatments). Ten individual lobsters (replicates) were analyzed for each combination of phenotype and protein source. After two-way ANOVA, the Tukey test was used to determine differences among means ($p \leq 0.05$).

The progress of *in vitro* digestion was modeled by linear regression analysis. Regressions describing the relationships between cumulative amino acid released and time of digestion for the different meals and phenotypes were obtained by the least-squares method. Although there should be not amino acids in the dialysate at time zero, lines were not forced to pass through origin for statistical analysis, since this involves the extrapolation of regression outside our data. The significance of regression slopes were tested by ANOVA for $p \leq 0.05$ and R^2 were calculated as a measure of relative goodness of fit of regression curves. Differences among initial rate of digestion (slopes) were assessed with ANCOVA by making all pairwise contrasts with a Bonferroni adjustment of significance levels to correct for multiple testing (Quinn and Keough, 2002). The $\log_{10}(X+1)$ transformation was applied for data to meet requirements of analysis. The Software packages Statistica 6.0 Soft, Inc. and StatGraphics Plus 5.1 were used for statistical analyses.

3. RESULTS

3.1. Frequencies of trypsin isozyme patterns

From the 118 lobsters analyzed, 102 individuals could be classified by their trypsin phenotype (Fig 1). Most of the classified lobsters exhibited the phenotype A (41.2%), followed by the phenotype B (35.3%) and finally the phenotype C (23.5%).

3.2. In vitro ***digestion by the digestion cell method***

The trypsin inhibitor TLCK was able to suppress all BApNA activity of extracts. A control digestion using one of these inhibited extracts (phenotype A) showed that efflux of free amino acids was negligible under this condition (data not shown). Although it is known that non-trypsin enzymes in crude extracts still produce protein hydrolysis under this

condition, this control experiment showed that trypsins are the key enzymes driving the responses observed.

Total amino acid released after 6h of digestion were compared by two-way ANOVA. There were significant differences among meals ($F=35.6$, $p\leq0.001$) and among phenotypes ($F=16.75$, $p\leq0.001$). Although the interaction meal x phenotype resulted also statistically significant ($F=4.64$, $p\leq0.001$), because the many means involved, comparisons were made only within meals for simplicity (Fig 2). Higher values of total amino acid release from SQM and JM were obtained with phenotype A enzymes, being those obtained with phenotype B significantly lower (Fig 2). Phenotypes C digestions of these meals produced intermediate levels of total amino acids (Fig 2). HM was similarly digested by both phenotypes A and B and to a less extent by phenotype C (Fig 2). There were no differences among phenotypes in the digestion of SBM but it was observed a non-significant trend to be phenotypes A and C most efficient than phenotype B. This trend became significant when analyzing SBI digestions (Fig 2).

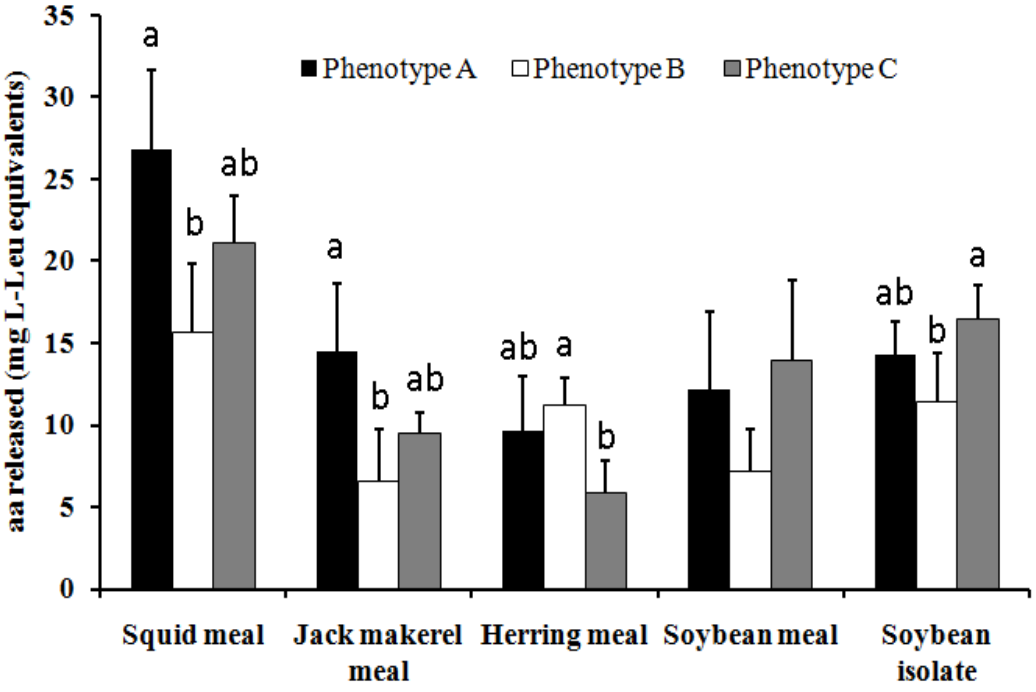

Fig 2. Total amino acids (mean ± SD, n=10) released from different protein sources after 6 h of digestion by digestive extracts of *P. argus* with three different trypsin isoenzyme patterns or phenotypes (A, B and C). Different letters above bars within the same protein source indicate statistical differences according to the Tuckey test ($p\leq0.05$).

The kinetics of amino acid release was assessed by analyzing the cumulative production of amino acid through time of digestion. These relationships were best described by linear regressions, all of them with high determination coefficients (R^2=0.73-0.96). The initial rate of digestion measured by means of the slopes were compared by ANCOVA and showed significant differences. The most evident result was the higher rate of amino acid release from SQM, irrespective to the phenotype of *P. argus* (Figs 3, 4 and 5).

Phenotype A was unable to discriminate among meals other than SQM (Fig 3) while phenotype B was the most sensitive phenotype. Digestive enzymes from phenotype B individuals were particularly efficient hydrolyzing HM and SBI, while JM and SBM were hydrolyzed at a lower rate (Fig 4). Phenotype C was also sensitive to meal quality but showing a gradation in responses (Fig 5). Interestingly, this phenotype degraded SBM and SBI at a similar initial rate (Fig 5).

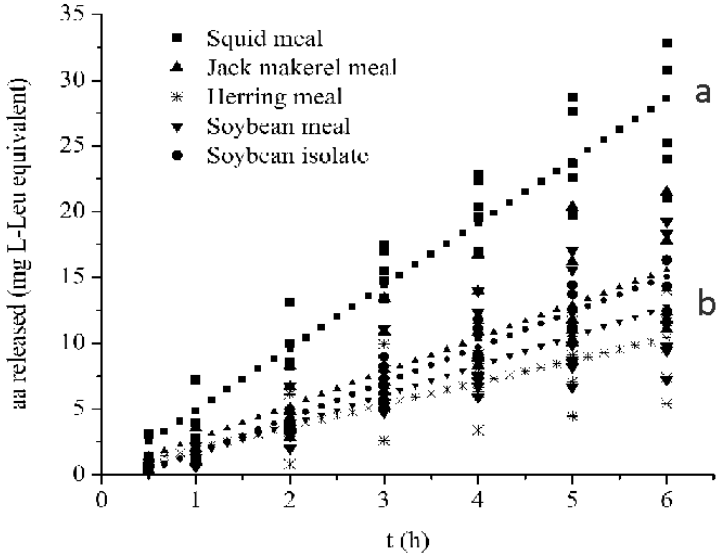

Fig 3. Kinetic of free amino acid released from different protein sources during *in vitro* digestion by crude digestive extracts of *P. argus* (n=10) with trypsin phenotype A. Data points and regression lines of cumulative values against time for each meal are represented with the same symbol. Letters to the right of regression lines indicate differences ($p \leq 0.05$) among slopes.

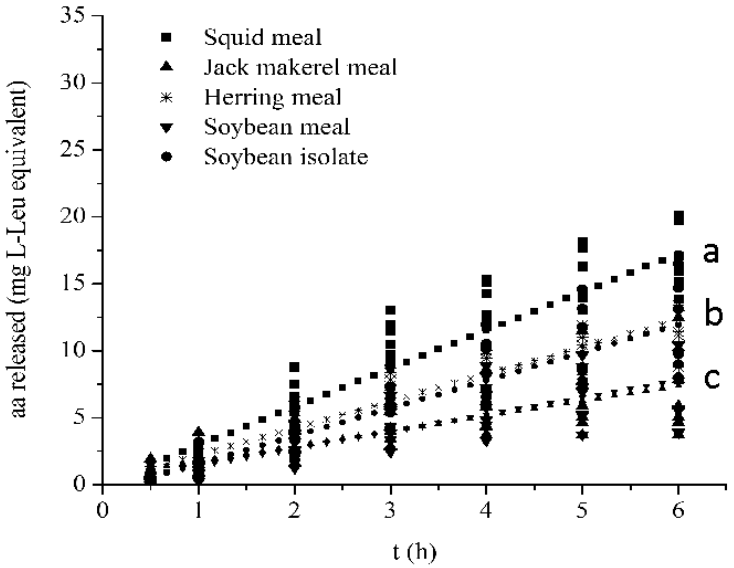

Fig 4. Kinetic of free amino acid released from different protein sources during *in vitro* digestion by crude digestive extracts of *P. argus* (n=10) with trypsin phenotype B. Data points and regression lines of cumulative values against time for each meal are represented with the same symbol. Letters to the right of regression lines indicate differences ($p \leq 0.05$) among slopes.

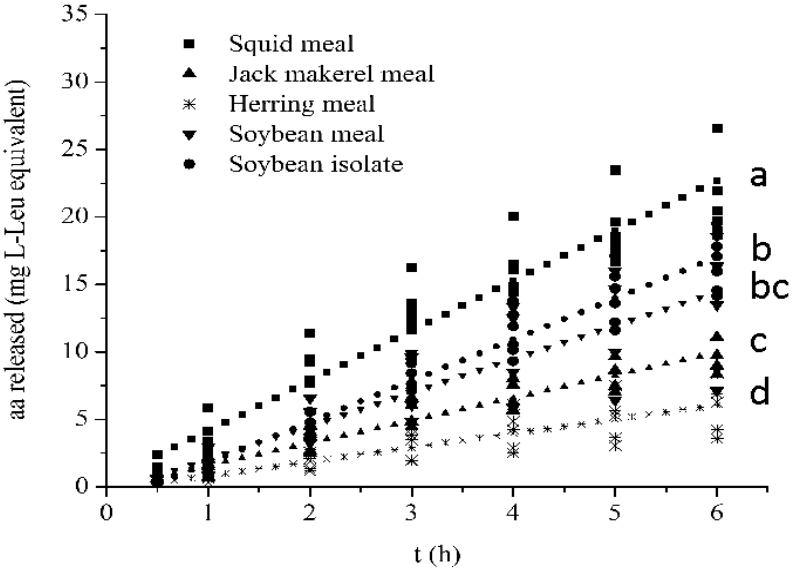

Fig 5. Kinetic of free amino acid released from different protein sources during *in vitro* digestion by crude digestive extracts of *P. argus* (n=10) with trypsin phenotype C. Data points and regression lines of cumulative values against time for each meal are represented with the same symbol. Letters to the right of regression lines indicate differences ($p \leq 0.05$) among slopes.

3.3. In vitro ***digestion followed by SDS-PAGE***

SBM and SBI presented a high solubility and a complex array of protein fractions whereas fish meals and SQM were difficult to dissolve. In animal meals, few fractions contribute to the total protein content.

Main protein fractions of SBM were of 76 kDa, 71 kDa, 50 kDa, 37 kDa and 20 kDa (Fig 6). SBI presented a similar protein pattern to those of SBM but enriched in the 32 kDa fraction (Fig 6). In both meals, the most digestible fractions were those higher than 30 kDa, although in general poor digestion for most fractions was observed (Fig 6). HM was shown to be highly digestible, being evident the clearance of fractions below 40 kDa after 15 minutes of digestion (Fig 7). Fractions of more than 40 kDa were also digested but at a lower rate. Susceptibility of JM to digestion differed from those of HM. The more evident digestion in JM occurred for the 45 kDa fraction (Fig 7). However, two of the most abundant fractions in JM (33 kDa and 38 kDa) were poorly digested (Fig 7).

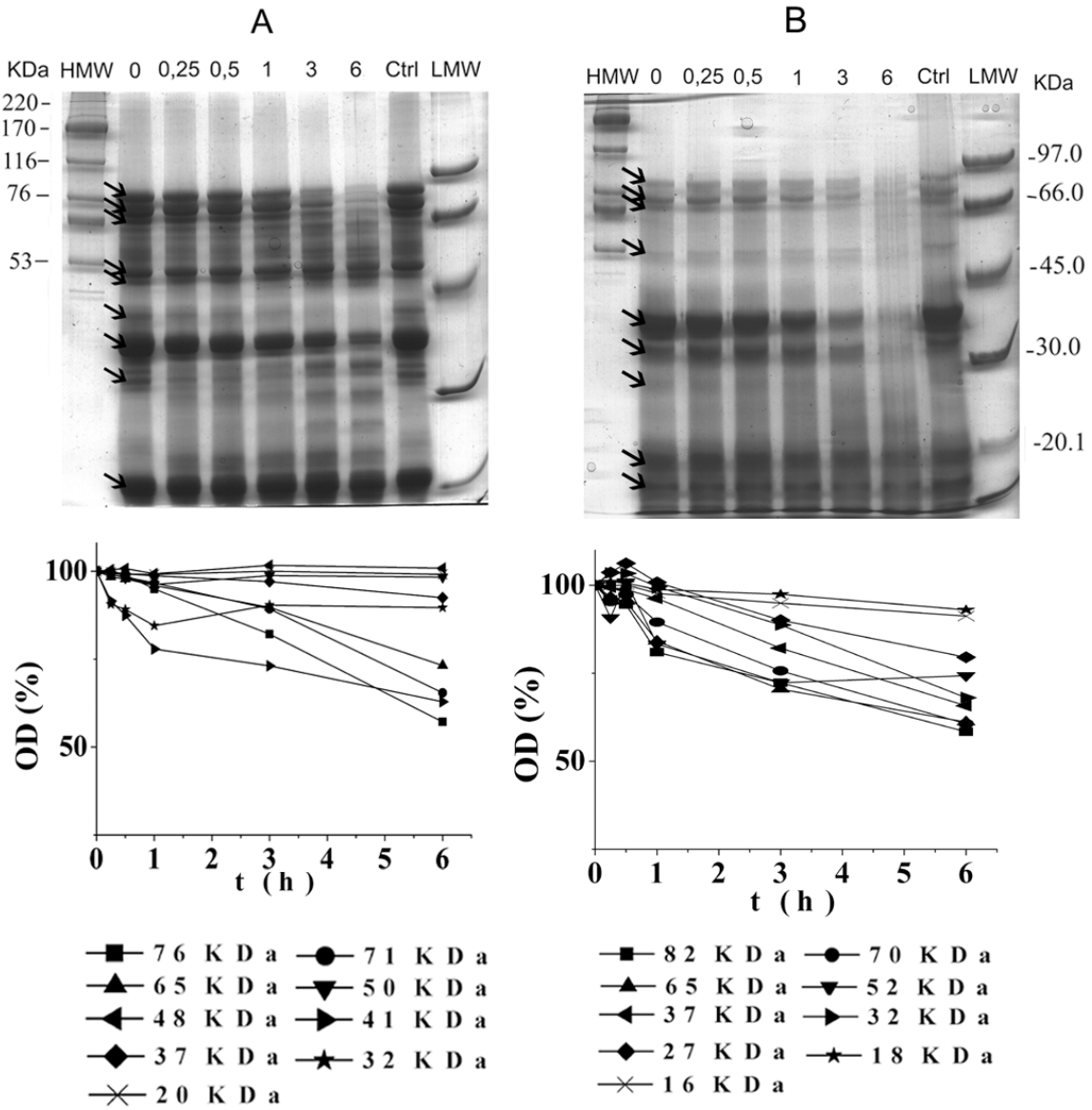

Fig 6. Digestion of main protein fractions (black arrows) of soybean meal (A) and soybean isolate (B) by crude digestive extracts of *P. argus* with trypsin phenotype A, followed by SDS-PAGE. Digestions using extracts with phenotypes B and C were similar (not shown). Numbers above each lane mean time of digestion in hours. Ctrl: control of 6h without the addition of enzyme extract. HMW: high molecular weight markers, LMW: low molecular weight markers. Graphs below each panel represent the decrease in optical density for main protein fractions.

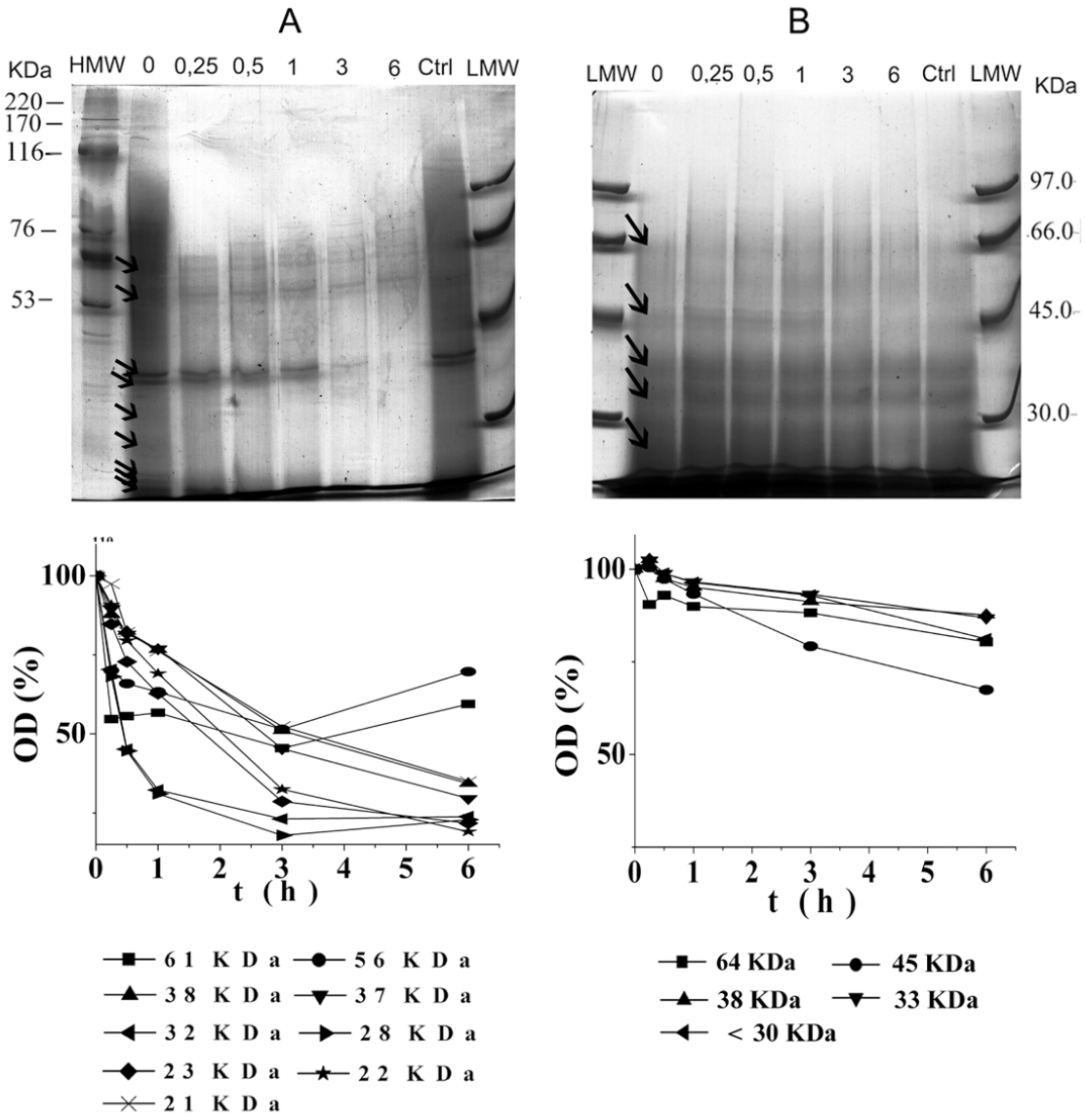

Fig 7. Digestion of main protein fractions (black arrows) of herring meal (A) and jack mackerel meal (B) by crude digestive extracts of *P. argus* with trypsin phenotype A, followed by SDS-PAGE. Digestions using extracts with phenotypes B and C were similar (not shown). Numbers above each lane mean time of digestion in hours. Ctrl: control of 6h without the addition of enzyme extract. HMW: high molecular weight markers, LMW: low molecular weight markers. Graphs below each panel represent the decrease in optical density for main protein fractions.

Main protein fractions observed in SQM were of 44 kDa, 42 kDa, 30 kDa and 27 kDa (Fig 8). The fraction of 30 kDa presented a modest digestion but all other fractions were not digested (Fig 8). The protein rich fraction below 23 kDa showed no defined bands, being difficult to analyze. Proteins from fresh squid muscle were very soluble and contained several protein fractions. Some of these fractions (>100 kDa) showed evident digestion, as well as that of 38 kDa (Fig 8).

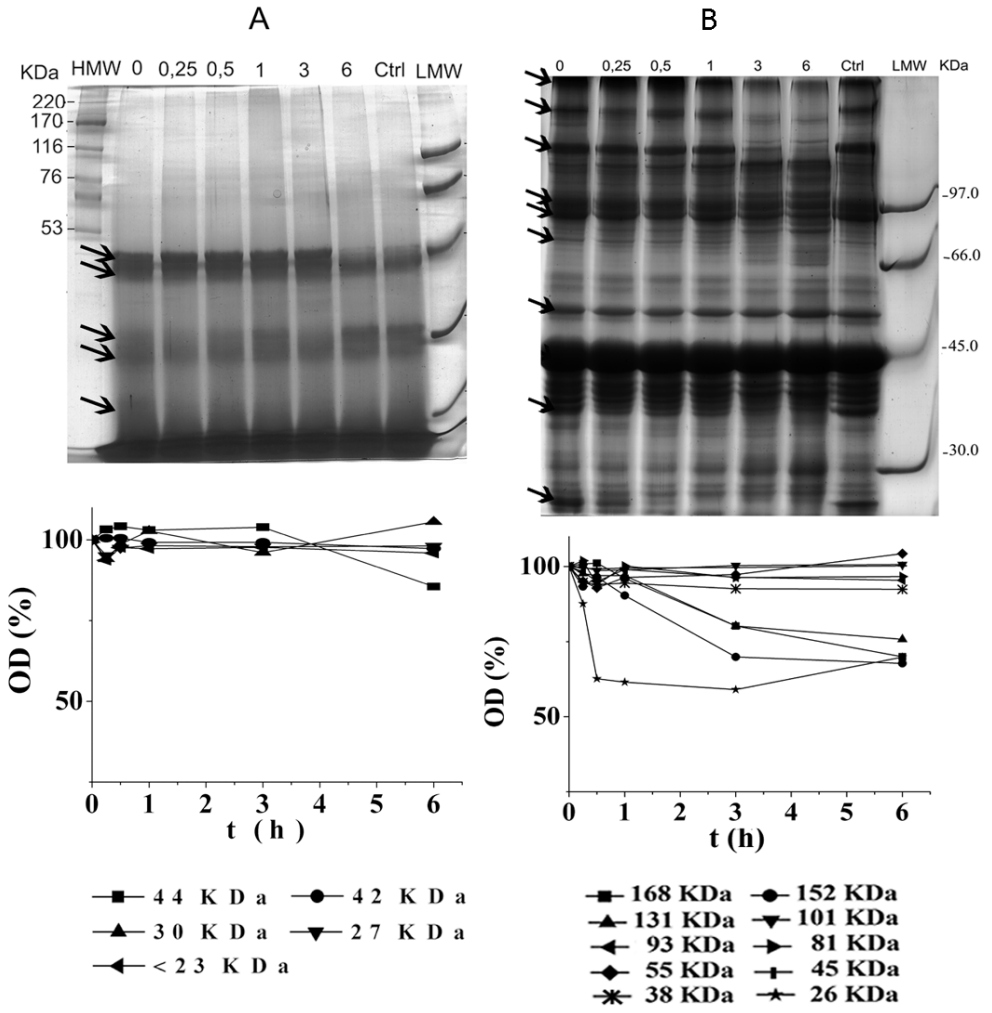

Fig 8. Digestion of main protein fractions (black arrows) of squid meal (A) and fresh squid extract (B) by crude digestive extracts of *P. argus* with trypsin phenotype A, followed by SDS-PAGE. Digestions using extracts with phenotypes B and C were similar (not shown). Numbers above each lane mean time of digestion in hours. Ctrl: control of 6h without the addition of enzyme extract. HMW: high molecular weight markers, LMW: low molecular weight markers. Graphs below each panel represent the decrease in optical density for main protein fractions

No differences were found among the three phenotypes analyzed with this method, regarding their efficiency in digesting the meals studied.

4. DISCUSSION

4.1. In vitro ***digestibility of protein sources***

Several in vitro methods have been developed as alternatives to expensive and time consuming in vivo digestibility trials. Digestibility has been assessed in vitro by single enzymatic systems (pepsin, trypsin or papain), two-step systems (pepsin and trypsin/pancreatine) or three-step systems (pepsin, trypsin/pancreatine and microbial enzymes), being the two-step hydrolysis (acid and alkaline) the most used. However, since pH of the gastric fluid of spiny lobster is around 6.0 (Johnston, 2003; Navarrete del Toro *et al.*, 2006; Perera *et al.*, 2008a), the commonly used acidic phase was not included in this study.

Generally, *in vitro* methods mentioned above have been applied in closed systems. The digestion cell method (open system) used in this study better reproduces the *in vivo* process with no chance for inhibition by end-products. In addition, a well known advantage of using an open system in digestibility studies is its ability to discriminate meals during the early stages of digestion (Bassompierre *et al.*, 1997) detecting small differences in protein bioavailability (Gauthier *et al.*, 1982; Moyano and Savoie, 2001). These points made this method well suited to the aims of the present study.

In vivo digestibility of SBM in spiny lobsters has been reported to be only slightly small (81%) than the one of fish meal (84%) (Irvin and Williams, 2007). Results in the present study agree with previous *in vivo* studies since the amount of amino acids released from SBM and fish meals were similar at least for two of the three phenotypes evaluated (Figs 3, 4 and 5). Also, results suggest that high digestibility of SBM previously reported for spiny lobsters may be related to the high solubility and number of protein fractions available, since most of these are separately poorly digested by the lobster digestive enzymes (Fig 6). Similar results were obtained for the shrimp *Farfantepenaeus paulensis*: SBM showed a higher number of protein bands not digested by shrimp proteinases compared to other ingredients and feeds (Lemos *et al.*, 2004). The hydrolysis of some protein fractions in the SBI was very similar, but overall seems to be slightly faster than in the SBM. Soybean

trypsin inhibitor was checked to be inactive in the soybean meals tested by means of reverse zymography (data not shown) thus it was considered not determinant in this study.

Both methods used demonstrated that there were differences in the protein digestibility between the two fish meals evaluated, being higher for HM than for JM. This was more evident in SDS-PAGE assays and for phenotype B in the digestion cell method. Most fractions in HM were readily digested, especially those below 40 kDa.

In vivo digestibility of SQM proteins by spiny lobsters has been reported to be only 59% (Irvin and Williams, 2007) and even smaller values were obtained by Ward *et al.*, (2003). SQM has proven to be more digestible for other crustaceans (Akiyama *et al.*, 1989; Catacutan *et al.*, 2003; Lemos *et al.*, 2009). Irving and Williams (2007) suggested that their poor results obtained with SQM, as well as those in Ward *et al.* (2003), were influenced by the poor quality of meals examined. It is worthy to remark that we were very careful in preparing all animal meal, especially SQM. Temperature never exceeded 65 °C and meals were stored at -80 °C until used. Córdova-Murueta *et al.* (2007) have suggested the drying at 65 °C as a safe dehydration process.

Digestion of SQM in the cell resulted in the greatest release of amino acids of all the assayed protein sources (Figs 3, 4 and 5). Control assays ensured that no spontaneous hydrolysis occurred. Results from the digestion cell method in this study must be taken only as a measure of initial rate of digestion (Bassompierre *et al.*, 1997). However, SDS-PAGE revealed that major fractions in SQM remained undigested, this being in agreement with *in vivo* studies in spiny lobsters (Ward *et al.*, 2003; Irving and Williams, 2007). Also, when assessing *in vitro* digestibility by measuring total undigested nitrogen, SQM showed lower digestibility than several fish meals, krill meal and soybean meal assayed for tuna (Carter *et al.*, 1999). This is not the first time that contradictory results between digestibility methods have been obtained for SQM. Studying SQM digestion in shrimp, a negative correlation has been observed on degree of hydrolysis (pH-stat) vs. apparent digestibility coefficient (chromic oxide), and these results has been explained by the presence of hydrolyzed protein in feeds (Córdova-Murueta and García-Carreño, 2002). Taking together our results, it can be concluded that in spite of the poor *in vitro* digestion of its main protein fractions (Fig 8), SQM digestion by *P. argus* rapidly release a high

amount of free amino acids (Figs 3, 4 and 5), which should had been hydrolysed from low molecular weight proteins or peptides not revealed as single bands in SDS-PAGE gels.

Since major protein fractions in SQM are poorly digested by lobster and diet formulations are based on total protein measured in raw ingredients, it is suggested that high inclusions of SQM could led to an insufficient amino acid supply for lobsters. Growth enhancement properties of SQM on the shrimp *P. vannamei* has been demonstrated only at low rates of feed supplementation and related to the presence of small peptides and free amino acids, similar to that produced by fish hydrolysates (Córdova-Murueta and García-Carreño, 2002). The increase in free amino acids during early digestion of SQM probably has an anabolic effect on *P. argus*, as suggested in a previous study (Perera *et al.*, 2005) and already demonstrated on shrimps (Cruz-Ricque *et al.*, 1989). Our results suggest that the same growth promoting effect of squid on shrimps could occur in lobsters.

Since preliminary trials in our laboratory showed that tropical *P. argus* grows quite well when fed exclusively on fresh squid, digestibility differences may exist between fresh squid and SQM proteins. To test this hypothesis, an aqueous extract was obtained from squid muscle and subjected to *in vitro* digestion followed by SDS-PAGE. Differing to the SQM, SDS-PAGE analysis revealed digestion of several high molecular weight fractions (<100 kDa) and the one of 38 kDa (Fig 8). Results suggest that most digestible protein fractions in squid muscle are susceptible to gently meal processing conditions.

4.2. Differences among trypsin isoenzyme patterns

Using substrate-SDS-PAGE, three similar isoenzyme zones for trypsins have been observed in the shrimp *L. vannamei* (Sainz *et al.*, 2004 and 2005; Muhlia-Almazán *et al.*, 2008; Sainz and Cordova-Murueta, 2009) and the lobster *P. argus* (Perera *et al.*, 2008a), giving rise to at least three phenotypes as shown in Fig 1. Also, three trypsin isoenzyme zones have been reported for Atlantic salmon named TRP-1, TRP-2 and TRP-3 (Torrissen, 1987) although the author used a more resolutive technique (isoelectric focusing on agarose gel) and therefore described a higher number of trypsin phenotypes. The isoenzyme pattern in shrimps is not affected by dietary treatment, molt cycle, or time (Sainz *et al.*, 2005). Also, there is no variation in *P. argus* trypsin isoenzyme pattern

throughout developmental and molt stages (Perera *et al.*, 2008b). The developmental stage do not affect isoenzyme pattern either in Atlantic salmon (Torrissen, 1987; Torrissen *et al.*, 1993; Rungruangsak-Torrissen *et al.*, 1998). Knowing this variation to occur for the key enzymes in protein digestion, the question of whether these phenotypes differ in digestion efficiency arose early for fish and more recently for crustaceans.

The effects of different trypsin isoenzyme patterns in salmon have been extensively studied (Torrissen, 1987; Torrissen, 1991; Torrissen and Shearer, 1992; Torrissen *et al.*, 1994; Bassompierre *et al.*, 1998; Rungruangsak-Torrissen *et al.*, 1999). In Atlantic salmon, groups possessing the trypsin variant TRP-2(92) presented an average weight significantly higher than that of fish without this variant (Torrissen, 1987). The *in vivo* apparent digestibility coefficient of Atlantic salmon and Arctic charr were reported to be similar for groups with and without this variant (Torrissen and Shearer, 1992). However, in a further study Bassompierre *et al.* (1998) demonstrated in *in vitro* trials that salmons with the variant TRP-2(92) were relatively insensitive to changes in protein quality, while fish without this variant are able to distinguish among proteins of varying quality.

Our results confirm that trypsin enzymes are responsible for the responses observed because the use of trypsin-inhibited extracts did not released a significant amount of amino acids from the digestion cell. Individuals of *P. argus* with the phenotype A were insensitive to protein quality (Fig 3), except for SQM which resulted in a higher release of free amino acids irrespective of the lobster phenotype (Fig 3, 4 and 5). This insensitivity of phenotype A may be related to the presence of the three isoenzymes (or isoenzymes zones) working in conjunction to hydrolyze the peptide bounds of substrates in a wider range of circumstances (e.g. residues in positions other than P1, association of dietary proteins with carbohydrates during the preparation of meals, etc.). This seems to be true, since differences in digestion efficiency were found for phenotypes B and C, each lacking a particular trypsin isoenzyme. The phenotype B was the more sensitive to protein quality, and discriminated between the two fish meals (HM and JM) and between the two soybean meals (SBM and SBI) (Fig 4). Lobsters with phenotype C could differentiate the two fish meals but not to the same extent that phenotype B. Crude extracts with phenotype C hydrolyzed the SBM at a similar rate that the SBI (Fig 5).

Results demonstrate that there are differences in the digestion efficiency of the three phenotypes being the more frequent phenotype A the most insensitive to variation in the substrate quality and therefore the most efficient. Sainz *et al.* (2005) isolated the three individual trypsin isozymes in the shrimp *L. vannamei* and found that the one of middle molecular weight presented the lowest catalytic efficiency. For this reason, Sainz and Cordova-Murueta (2009) suggested that phenotypes composed by the other two isoenzymes would be best for digestion. Present results obtained in *P. argus* agree with this suggestion made for shrimps. To our knowledge, this is the first study demonstrating the effects of trypsin isoenzyme pattern on protein digestion in a crustacean species.

Due to the autoproteolytic stability of crustacean trypsins (Hehemann *et al.*, 2008), the isotrypsin patterns can be observed in enzymes extracted from feces (Córdova-Murueta *et al.*, 2003). This allows the classification of crustaceans according to their trypsin phenotype without sacrifice the animal, thus it is possible to perform further growout trials for verifying our *in vitro* results. Finally, the digestion cell method was successfully applied for the first time to a crustacean species and proved to be suitable for *in vitro* digestion studies. This system could be used in further studies to evaluate *in vitro* other aspects of the digestive process in spiny lobsters and other crustaceans.

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Capítulo 5

New members of the brachyurins family in lobster include a trypsin-like enzyme with amino acid substitutions in the substrate binding pocket

Perera, E., Pons, T., Hernández, D., Moyano, F.J., Martínez-Rodríguez, G., Mancera, J.M. ***FEBS Journal 277, 3489-3501*** **(2010)**

Resumen

Se estudio la diversidad de tripsinas en la glándula digestiva de la langosta *Panulirus argus* a nivel molecular. Se clonaron varias enzimas tipo tripsinas pertenecientes, al menos, a tres familias génicas diferentes. Las secuencias primarias tienen una alta identidad, y comparten los residuos importantes para la especificidad y actividad tripsina. Los modelos tridimensionales desarrollados para cada tripsina sugieren algunas diferencias respecto a su interacción con sustratos e inhibidores. Aunque la mayoría de las enzimas clonadas son tripsinas típicas, se encontró una enzima que no pudo ser clasificada debido a sustituciones de aminoácidos en las cercanías del centro activo. Los cambios más significativos son: Gly216 y Gly226 están sustituidas por Leu y Pro, respectivamente. Estos residuos deben impedir el acceso de sustratos con residuos grandes en posición P1, como la Arg o Lys. Por otra parte, estas sustituciones deben hacer el bolsillo de la enzima más hidrofóbico que en las tripsinas típicas. Estos resultados mostraron que las braquiurinas de crustáceos presentan especificidades aún no descritas. Las enzimas tipo tripsina de *P. argus* se estudiaron además desde el punto de vista de su expresión génica mediante Real-Time RT-PCR y desde el punto de vista evolutivo, en relación con el resto de las tripsinas conocidas de crustáceos. Es la primera vez que se da a conocer la secuencia de las tripsinas de una langosta espinosa, contribuyéndose a la comprensión de la evolución de estas enzimas en crustáceos.

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Abstract

Crustacean serine proteases (Brachyurins, EC 3.4.21.32) exhibit a wide variety of primary specificities and no member of this family has been reported for spiny lobsters. The aim of this work was to study the diversity of trypsins in the digestive gland of *Panulirus argus*. Several trypsin-like proteases were cloned and results suggest that at least three gene families encode trypsins in the lobster. Three-dimensional comparative models of each trypsin anticipated differences in the interaction of these enzymes with proteinaseous substrates and inhibitors. Most of the studied enzymes were typical trypsins, while one of them could not be allocated to any of the brachyurins groups due to amino acid substitutions found in the vicinity of the active site. Among other changes in this form of the enzyme, conserved Gly216 and Gly226 (chymotrypsin numbering) are substituted by Leu and Pro respectively, while retain all other key residues for trypsin specificity. These substitutions may impair the access of bulky residues to the S1 site while they make the pocket more hydrophobic. The physiological role of this form of the enzyme could be relevant since it was found to be highly expressed in lobster. Further studies on the specificity and structure of this variant must be performed to locate it within the brachyurins family. It is suggested that specificity within this family of enzymes is broader than it is currently believed.

Keywords: Trypsin; Comparative modeling; Substrate binding pocket; Brachyurins; *Panulirus*

Database: Nucleotide sequence data are available in the GenBank database (http://www.ncbi.nlm.nih.gov) under the following accession numbers: PaTry 1a (GU338026), PaTry 1b (GU338027), PaTry 2 (GU338028), PaTry 3 (GU338029), and PaTry 4 (GU338030). Model data are available in the PMDB database (http://www.caspur.it/PMDB) under the following accession numbers: PaTry1a (PMDB id: PM0076235), PaTry1b (PMDB id: PM0076234), PaTry2 (PMDB id: PM0076233), PaTry3 (PMDB id: PM0076232), PaTry4 (PMDB id: PM0076231).

1. INTRODUCTION

Serine proteases perform many important physiological functions like blood coagulation, fibrinolysis, cellular and humoral immunity, fertilization, embryonic development and digestion. As occur in most crustacea, trypsins are main proteases in the digestive tract of spiny lobsters, accounting for up to 60% of digestive proteolysis [1]. We recently have reported the existence of three major trypsin isoforms and other serine proteases in the digestive gland of *Panulirus argus* [1]. If this trypsin diversity in lobsters occurs along with differences in specificity, inhibitor interaction or regulation mechanism among variants of the enzyme, then the efficient protein digestion in these crustacea can be better explained, as well as their ecological success. Some studies are available at the molecular level on decapods trypsins mostly focused on nucleotide sequence [2, 3]. There is no previous report on trypsin sequence for any spiny lobster species.

Since the discovery of trypsin a plethora of studies have been conducted on mammalian trypsins and therefore they are biochemically and structurally well characterized. These enzymes have a similar fold of two β-barrels with the catalytic triad (His57/Asp102/Ser195, chymotrypsin numbering) between the two domains. Trypsin cleaves its substrates at the C-terminal side of arginine or lysine at P1 position. This primary specificity is mainly determined by three residues. Two Gly (216 and 226, chymotrypsin numbering) are located on the wall of the binding pocket and allow the access of bulky residues like Arg and Lys, while the basic side chain of these residues are stabilized by Asp189 (chymotrypsin numbering) near the bottom of the pocket. Also, mutagenesis studies have demonstrated that other regions far from the S1 site play important roles in substrate specificity [4, 5].

Since 1992 the NC-IUBMB has recommended the term brachyurins (EC 3.4.21.32) for serine endopeptidase found in crustacea [6]. Type Ia brachyurins possess broad specific, with activities similar to those of trypsin, chymotrypsin and elastase. Type Ib enzymes have drastically reduced activity toward Arg substrates, while retaining the other features of type Ia substrate specificity. Other group, type II brachyurins, has strict trypsin-like specificity. This variation, from wide to strict specificity, is very attractive for studying

structure-function relationships. The fact that the 3D structure of some decapod serine proteases have been elucidated by X-ray crystallography [7, 8] provides good opportunity for analyzing those relationships in new enzymes by comparative modeling. The aim of the present work was to study the diversity of trypsins in the digestive gland of *P. argus* with focus on: i) position of lobster trypsins within the brachyurins family and ii) features of lobster enzymes that suggest different specificities or interactions with substrates and/or inhibitors.

2. MATERIALS AND METHODS

2.1. Animals and total RNA extraction

Lobster juveniles were collected in the Golf of Batabanó, Cuba. Intermolt animals were placed on ice for 10 min to obtain a chill coma and then were dissected for collecting the digestive gland, stomach, intestine, gills, heart, and abdominal muscle. Before dissection, hemocytes were collected using the Citrate-EDTA buffer pH 4.6 as anticoagulant [28]. All samples were immediately frozen in liquid nitrogen. Total RNA extraction was performed by the Chomczynski method [29]. It was quantified by its Abs_{260} and its quality was accessed by $Abs^{260}/_{280}$.

2.2. Cloning and sequencing

Trypsin cDNAs from several crustaceans (see Fig 5 for species and accession numbers) were retrieved from GenBank/NCBI and then CLUSTALW was used for searching conserved sequences. The softwares GenRunner v3.05 and Oligo Analyzer v1.1.2 were used for primer analysis. Two pairs of degenerated primers were designed: *Fw1: 5'-CCAARATCATCCARCACGARG-3', Rv1: 5'- AGTCACCCTGGCANGMGTC -3'* and *Fw2: 5'-TTCTGCGGHGCBTCCATCTACA-3', Rv2: 5'- CYTCGTGYTGGATGATYTTGG-3'*. All primers for this study were purchased from Invitrogen (Paisley, UK) unless otherwise stated, and all kits were used following manufacturer's instructions.

Total RNA (5 µg) was reverse transcribed into first strand cDNA using oligo-dT primer and SuperScript™ III reverse transcriptase (Invitrogen, Paisley, UK). Using Platinum *Taq* DNA Polymerase (Invitrogen, Paisley, UK), PCR amplifications were done on total cDNA as follow: one cycle at 94°C for 2 min, 35 cycles at 94°C for 30 sec, 50 °C for 30 sec, 72 °C for 1 min, and one overextension cycle at 72 °C for 10 min. Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples without cDNA synthesis. PCR products were run on 1% agarose gels containing 0.5 µg/mL ethidium bromide and sized by the 1 Kb Plus DNA Ladder (Invitrogen, Paisley, UK). The *Rv2* primer is the complement and reverse of *Fw1* primer, so these primers amplified two adjacent fragments. With *Fw1* and *Rv1* primers a single 350 pb fragment was obtained whereas *Fw2* and *Rv2* have produced a single 200 pb fragment. Thereafter, PCR was done as above with *Fw2* and *Rv1* yielding the entire fragment as a single band of about 500 pb.

The three PCR products were cloned into plasmids using the TOPO TA Cloning® Kit (Invitrogen, Paisley, UK). Plasmids were extracted from Transformed One Shot® TOP10 Competent *E. coli* cells using the GenElute™ Five-Minute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Clones containing inserts of expected size were identified by PCR analysis (T3 and T7 primers of TOPO TA Cloning® Kit) and restriction enzyme analysis (EcoRI), followed by agarose gel electrophoresis, and sequenced from both directions using the Sequencing Service of University of Malaga, Spain. After retrieval, sequence chromatograms were checked using Chromas Lite 2.01 (Technelysium Pty., Queensland, Australia) and trimmed for vector sequence. Inserts were analyzed by NCBI/BLASTN homology search in GenBank for confirming trypsin identity. Variability observed in the two minor fragments (except those in primer regions) allowed to verify the sequence variability found in the longest one. These assemblages of fragments yielded three distinct partial trypsin cDNA sequences.

2.3. Obtaining 5´ and 3´ ends by RACE

Using total RNA as the template, the 5´ and 3´ ends of trypsin mRNAs were amplified using 5´ and 3´ RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Paisley, UK). Specific forward primers were designed to match with conserved sequence

in the three fragments at two different positions (Table 1) and used in combination with a PolyT-V primer to amplify the 3´ ends. For 5´ RACE amplifications, specific primers for each of the three fragments were designed (Table 1), and used in combination with RACE primers supplied in the kit. Primers were designed for achieving an overlapping between RACE clones and previously obtained partial cDNAs of around 150-200 pb. Cloning and sequencing of PCR products were performed as described above. Thereafter, specific primers were designed (Table 1) to amplify full-length trypsin cDNAs.

Table 1. Primers used in this study.

Name	Nucleotide sequence	Position*	Direction
Primers for 3' RACE			
Trys ext	5'-CACCTTCAACGACTATGTCCAGC-3'	419-441	Forward
Trys int	5'-CAAGCCCCCTCACCTTCAACG-3'	409-429	Forward
Primers for 5' RACE			
GSP1	5'-TGCGCTGGAAGAGC-3'	447-460	Reverse
GSP2 Try 1-2	5'-GTTCGTTCCCTTCATTCACCG-3'	304-324	Reverse
GSP2 Try 3	5'-GACGGATATGTCGTTGCTGATA-3'	378-399	Reverse
GSP2 Try 4	5'-GAGCAATGGCCTGGACATG-3'	432-451	Reverse
Primers for full-length trypsins			
Trys Fw	5'-CCAGAGACCAGCCATGAAG-3'	3-21	Forward
Try 1-3	5'-TTTTTTTTTTTGAATTCGCTTGG-3'	863-881	Reverse
Try 4	5'-TTTTTTTTTTGAACCTTTTAAAT-3'	863-881	Reverse
Primers for RT-qPCR			
PaTry 1 Fw	5'-AACAAGATCGTTGGTGGTGA-3'	96-115	Forward
PaTry 2 Fw	5'-CTGACGCCGAGCCTGGTA-3'	115-132	Forward
PaTry 3 Fw	5'-GGACATCTCCTTCGGCTT-3'	159- 176	Forward
All PaTrys Rv	5'-AGTGACCAGCACAGATAGC-3'	220-238	Reverse
PaTry 4 Rv	5'-GTGGATCCAGTGTTCGTCAT-3'	689-706	Reverse
All PaTrys Fw	5'-CCGTGCCCATCGTGTCTGA-3'	551-569	Forward
EF1α Fw	5'-CCAGTAGACAAACCACTTCG-3'	532-551	Forward
EF1α Rv	5'-CATACCTGGCTTCAAGATGC-3'	620-639	Reverse
β-act Fw	5'-CAGGAATTGCCGATAGGATGC-3'	571-591	Forward
β-act Rv	5'-TACTTGCGTTCAGGGGGAGC-3'	642-661	Reverse

* In cases of same primer for several trypsin variants, numbers correspond to hybridization position on PaTry1;for the other variants few nt displacements could occurred.

Table 2. Trypsinogen cDNAs and putative proteins of *Panulirus argus.*

Clone name	GenBank number	cDNA length	ORF	3´-UTR	Polyadenylation signal/Distance to PolyA tail	Encoded amino acids/ mature protein	Signal/Activation peptides	MW/pI of mature protein
PaTry 1a	GU338026	873	798nt	61nt	AATAAA/13nt	266aa/237aa	15aa/14aa	25kDa/4.07
PaTry 1b	GU338027	873	798nt	61nt	AATAAA/13nt	266aa/237aa	15aa/14aa	25kDa/4.07
PaTry 2	GU338028	870	798nt	58nt	AATAAA/10nt	266aa/237aa	15aa/14aa	25kDa/4.1
PaTry 3	GU338029	874	798nt	61nt	AATAAA/13nt	266aa/237aa	15aa/14aa	25kDa/4.1
PaTry 4	GU338030	875	802nt	57nt	ATTAAA/13nt	278aa/249aa	15aa/14aa	27kDa/4.6

Table 3. Amino acid sequences of trypsinogen signal and activation peptides of *Panulirus argus* and some other crustaceans.

Species	Signal peptide	Activation peptide	Accession number	Reference
Spiny lobster *Panulirus argus*	MKTLVFCLLLAGAFA	APSGKPKFRRGLNK	GU338026	This work
	MKTLVFCLLLAGAFA	APSGKPKFRRGLNK	GU338028	
	MKTLVFCLLLAGAFA	APSGKPKFRRGLNK	GU338029	
	MKTLVFCLLLAGAFA	APSGKPKFRRGLNK	GU338030	
Pacific white shrimp *Litopenaeus vannamei*	KSLILCVLLAGAFA	APSRKPTFRRGLNK	CAA60129	[2, 10]
	KSLVLCLLLAGAFA	APSRKPTFRRGLNK	CAA75310	
	KSLVLCLLLAGAFA	APSRKPTFRRGLNK	CAA75311	
Signal crayfish *Pacifastacus leniusculus*	MKTLVFCLLLVGALA	APSRRLRFPPNNYK	CAA10915	[3]
Chinese shrimp *Fanneropenaeus chinensis*	MKSLVLCLLLAGAFA	APSRKPTFRRGLNK	1134602	[27]
	MKTLVLCVLVAGAFA	APSRKPTFRRGLNK	1134625	
Salmon louse *Lepeophtheirus salmonis*	KFVCTLLFLVVAANA	VPPQIKYSESFMKVKSMRHRFGGR	AAL71875	[11]
	- FVCALFFLAVAASA	VPRIKYSDSFMKVKSMRHRFGGR	AAL71876	
	- FVCALFFLAVAASA	VPRIKYSESFMKVKSMRHRFGGR	AAL71877	

Table 4. Structure validation of the 3D models.

Criteria	Characteristic	PaTry1a	PaTry1b	PaTry2	Patry3	PaTry4	2f91 (A)	2ftl (E)
PROHECK	Most favored regions	91.3%	90.8%	91.8%	91.3%	89.6%	89.5%	85.5%
	Additional allowed	8.7%	9.2%	8.2%	8.7%	8.9%	9.5%	14.5%
	Generally Allowed	0%	0%	0%	0%	1.5%	0.5%	0%
	Disallowed regions	0%	0%	0%	0%	0%	0.5%	0%
WHATCHECK (Z-scores)	2nd generation packing quality	-0.83	-0.88	-0.46	-0.61	-1.56	-0.1	-0.6
VERIFY-3D	3D-1D score (≥0.2)	88.66%	86.13%	82.35%	94.96%	82.8%	96.14%	92.76%

2.4. Sequence analysis

Nucleotide sequences were analyzed for homology by BLASTN using the website (http://www.ncbi.nlm.nih.gov/) of the National Center for Biotechnology Information (NCBI). CLUSTALW (http://www.ebi.ac.uk/clustalw/) was used for fragment assemblage. Translation of the sequences was carried out with the Expasy Translate Tool (http://www.expasy.org/tools/dna.html). Homology analysis of putative protein sequences was done with BLASTP at the NCBI web site. The protein motifs' features were predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Theoretical isoelectric points and molecular weights of deduced proteins were further predicted using the ExPASy's Compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html). Prediction of signal peptide cleavage site was carried out by SIGNALP (http:// www.cbs.dtu.dk/services/SignalP/). Charge and hydrophobicity (Kyte-Doolittle hydropathy scale) distributions in mature trypsins were analyzed using the protein analysis tools of GeneRunner v3.05 software.

2.5. Comparative 3D modeling

Sequences and 3D structures of crayfish and bovine trypsins were retrieved from the UniProt/Swiss-Prot and the Protein Data Bank (PDB) databases, respectively. Position-Specific Iterated BLAST (PSI-BLAST) against the NCBI non-redundant (nr) database (http://www.ncbi.nlm.nih.gov) was used to identify *P. argus* (PaTry) related sequences. Sequence alignments with E-value less than 10^{-3} and with bit score greater than 100 were considered significant. The profile-alignment option of the CLUSTALX program [30] was used to compare *P. argus*, crayfish and bovine trypsin sequences. The aligned sequences were adjusted manually to minimize the number of gaps and insertions. These manual adjustments were based on the results from sequence similarities and structural alignment between crayfish (PDB: 2f91) and bovine (PDB: 2ftl) trypsin, using the CE method (http://cl.sdsc.edu/ce.html) [31]. In search for alternative structural templates from known structures in the PDB, and a sequence-to-structure alignment of PaTry sequences, we have used the METASERVER available at http://meta.bioinfo.pl/ [32]. Also, we complement the METASERVER predictions with those obtained by PHYRE [33] and I-TASSER [34]

in order to gain success in fold recognition approach. Based on the identified sequence-to-structure alignments we have predicted the PaTry 3D-models using MODELLER [35].

The predicted 3D-models of PaTry sequences were subjected to a series of tests for evaluating its internal consistency and reliability. Backbone conformation was evaluated by the inspection of the Psi/Phi Ramachandran plot obtained from PROCHECK analysis [36]. Packing quality of the 3D model was investigated by the calculation of WHATCHECK Z-score value [37]. Finally, sequence-structure compatibility was evaluated by VERIFY-3D [38]. All these programs were executed from the structure analysis and verification servers at UCLA (http://www.doe-mbi.ucla.edu/Services/SV/).

2.6. Phylogenetic analysis

The best-fit model of amino acid substitution was selected by testing alternative models of evolution using both Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) implemented in ProtTest [39]. The WAG+Γ (gamma shape parameter = 1.23) model of evolution was selected for further analysis. Phylogeny was reconstructed by analyzing amino acid sequences of mature crustacean trypsins using neighbor-joining (NJ) and maximum parsimony (MP) in the software MEGA 4.1 [40], and maximum likelihood (ML) using PHYML v2.4.4 [41]. Topology robustness was tested with 1000 bootstrap replicates in NJ, 500 replicates in MP, and 100 replicates in ML.

2.7. Tissue specific mRNA expression by RT-qPCR

Total RNA from equivalent amount of the different organs were purified as described in section 2.1 and additionally cleaned up with RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA quality was assessed by using the Agilent RNA 6000 Nano Assay Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and only non-degraded samples were used afterward. cDNAs were synthesized using the qScript™ cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD, USA) and then used as templates for RT-qPCR on a Mastercycler ep realplex (Eppendorf) using PerfeCTa™ SYBR® Green FastMix™ (Quanta BioSciences, Gaithersburg, MD, USA). Primers for the

P. argus trypsin cDNAs (Table 1) were designed from herein obtained sequences (Fig 1) to be cDNA specific. β-actin (β-act) and elongation factor 1-α (EF1-α) were used as housekeeping genes. The design of β-act specific primers (Table 1) was guided by known sequence of β-act in *P. argus* (GenBank accession no. GQ865599) whereas to design EF1-α specific primers (Table 1), a partial sequence (693 pb) of *P. argus* EF1-α transcript was obtained for this work (GenBank accession no. GU338031). All primers for RT-qPCR were synthesized by Biomers.net (Ulm, Germany). PCR optimal conditions were previously determined to be 4 pmol/μL of primers and extension at 60°C. By means of calibration curves (10 fold dilutions, corresponding to cDNA in reactions from 20 ng to 0.2 pg) all primer pairs were checked to produce similar efficiencies of 0.98-1.0, R^2 of 0.99-1.0, and slopes ranging from -3.3 to -3.4. Control reactions with RNA instead cDNA were included. Relative mRNA expression was determined by the comparative Ct method ($2^{-\Delta\Delta Ct}$) [42]. Five individuals were analyzed per organ and results were expressed as mean ± standard error.

3. RESULTS AND DISCUSSION

3.1. Characterization of cDNAs and trypsin-like deduced protein sequences

The three partial cDNA fragments initially identified and the 5´ and 3´ ends obtained, generated by assemblage three distinct cDNA sequences. Later, specific primers designed to flank the 5´ untranslated region (UTR) and 3´ UTR of the different cDNAs allowed the amplification of several full-length cDNAs. Eleven clones of expected size were sequenced. Three of them did not have suitable open reading frames (ORFs) and two clones contained incongruences when sequencing on both strands. Thus, all these five sequences were discharged. The remaining six cDNAs encoded different proteins homologous to PA (S1) peptidases (MEROPS nomenclature) and with high identity to crustacean trypsins. GenBank accession numbers, features of the isolated cDNAs, and their corresponding putative proteins are summarized in Table 2. One clone resulted identical to PaTry2 but two PCR consistent errors (A/G, C/T) [9] thus not further analyzed.

For all cDNAs, short (14-15 nt) 5´ UTR sequences were found with no major differences among clones. The 3´ UTR sequences of PaTry1a, PaTry1b and PaTry 3 were identical, and differed only in 5 nt substitutions from the 3´ UTR region of PaTry2. However, the 3´ UTR sequence of PaTry4 differed to those of the other trypsins in more than 36% of its nucleotides. All ORFs started at the first ATG codon of the 5´ terminal region and ended with TAG stop codon except PaTry4 that ended with a different stop codon (TGA). Also, the polyadenylation signal of PaTry4 was slightly different to which occurs in all other clones. PaTry4 was the largest and the least anionic of all trypsin found in *P. argus*. No cationic form of the enzyme was found in this work.

The coding regions of PaTry1a and PaTry1b were similar except for two nucleotide substitutions (T/A, G/A), that leaded to two amino acid substitution (V/D, D/N) in mature proteins (Fig 1). Differences between these clones were non standard PCR errors thus they were considered as genuine products of two closely homologous genes in early diversification or allelic variants at the same locus. The 17 substitutions between the coding region of PaTry1 and PaTry2 lead to only eight amino acidic changes, suggesting a close relationship among these transcripts. Klein and coworkers have reported two, three and four amino acid changes within each of the trypsin families I, II and III respectively, in the shrimp *L. vannamei* [2, 10]. However, 44 nt substitutions were observed between PaTry3 and both PaTry1 and PaTry2, leading to 23 and 26 amino acids changes respectively, thus this transcript may belong to a distinct gene family. The amino acid composition of trypsin I and II families in shrimp varied in 23 positions [2].

PaTry4 was the most divergent variant with more than 70 single nt changes respect to all other *P. argus* trypsin transcripts, mostly toward the 3´ region. PaTry4 differed in more than 50 amino acids of all other predicted trypsins, indicating this product belong to a third family. None of the *P. argus* trypsin cDNAs contained the Cla I cleavage sites reported by Klein and coworkers [2] to occur in all *L. vannamei* trypsin cDNAs. These authors have found Pst I cleavage sites only in one trypsin family of shrimp. Cleavage sites for Pst I occurred in all *P. argus* trypsins cDNA except in PaTry4.

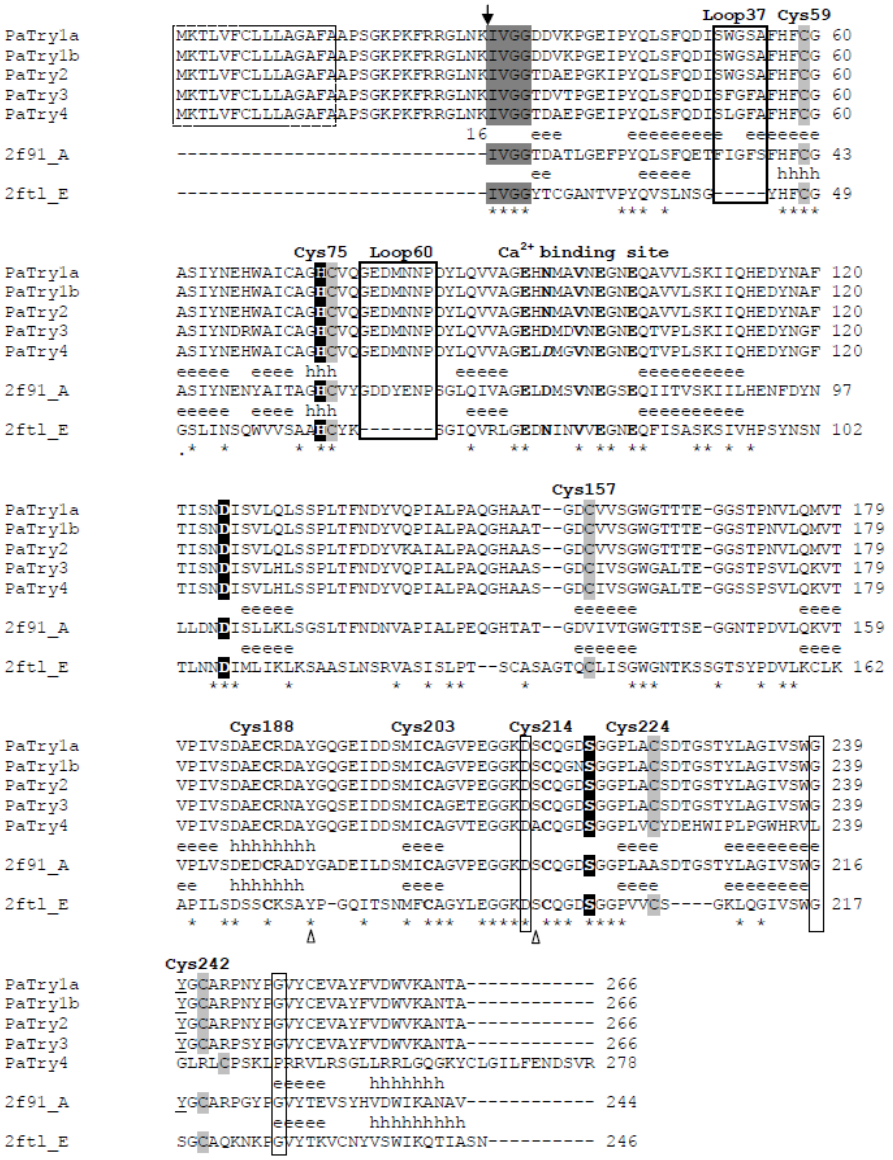

Fig 1. Sequence-to-structure alignment of *Panulirus argus* trypsinogens with crayfish (PDB code: 2f91A) and bovine (PBD code: 2ftlE) trypsins. Complete conserved residues are marked with asterisks at the bottom of sequences. Signal peptides are boxed with a dashed line. Activation peptide cleavage site is indicated by a black-head arrow. Conserved N-terminal residues of mature enzymes and cysteine residues in predicted disulfide bridges are indicated by dark and light grey shadow, respectively. The black shadowed white letters indicate the catalytic triad (His74, Asp125, Ser218), this residues are the equivalent to His57, Asp102 and Ser195 in chymotrypsin nomenclature; primary specificity determinants are boxed with a continuous line and secondary determinants are indicated by white head-arrows at the bottom of sequences. Residues forming the calcium binding site are in bold. Differences in two of the superficial loops are boxed and indicated according to Fodor and coworkers [8] nomenclature. Numbers start at the first residue of proteins.

Another distinctive feature of PaTry4 was its amino acid composition. PaTry4 contained less (around 1.3-1.8 times) Ala and Thr respect to the other *P. argus* trypsins. Leu content in PaTry4 (9.24%) almost doubled the ones in all other trypsins in Fig 5 except Astacide trypsins. Interesting, Arg content in PaTry4 (3.61%) was 4.3 times higher than in PaTry1 and PaTry 2, and 2.8 times higher than in PaTry3. Among crustacean trypsins in Fig 5, such a high content of Arg was only observed in *H. americanus*. The rest of *P. argus* trypsins share a very low Arg content with all other crustacean trypsins (0.5-1.6%). Altogether, present results suggest the existence of at least three gene families encoding trypsin enzymes in *P. argus* (PaTry1-2, PaTry3, and PaTry4). Further studies on the genomic sequence are needed.

All deduced proteins contained the same signal peptide of 15 amino acids (Fig 1), indicating all these proteins are secreted. It contained a high proportion of hydrophobic residues with an Ala as ending amino acid, as typical in eukaryotic signal sequences. Contiguous to the signal peptide, the same activation peptide of 14 amino acids occurred in all *P. argus* trypsins (Fig 1). These two regions have been shown to be conserved among crustaceans (Table 3) indicating that there are few differences in secretion and activation mechanisms of these enzymes. Among studied crustacea, significant differences in these parts of sequences have been only reported for the parasite copepod *L. salmonis* [11] (Table 3). Activation peptides in the spiny lobster, like those of other crustacea and insects, finished with Lys at P1 position. Besides, it lacked the repeated Asp residues of most vertebrates, whose are supposed to have evolved progressively for protection against autoactivation [12]. Results indicate that after secretion into the lumen of the digestive gland (tubules), *P. argus* trypsins may self-activate or other trypsin-like proteases in the digestive gland may be responsible for the activation. All PaTry shared a common N-terminal sequence (IVGG) (Fig1), which is conserved in trypsins.

Distributions of charged amino acid in *P. argus* mature trypsins PaTry1, PaTry 2 and PaTry 3 were similar to each other and to those in *P. leptodactylus* (Fig 2) and other crustacean trypsins. However, PaTry4 exhibited a distribution of charged amino acid toward the C- terminal of the mature enzyme (Fig 2) that has not been observed before in crustacean trypsins. This charge distribution toward the C- terminal region resembles the

one in cationic SalTRP-III of salmon [13]. However, hydrophobicity plots of the four trypsin sequence of *P. argus* (not shown) are similar.

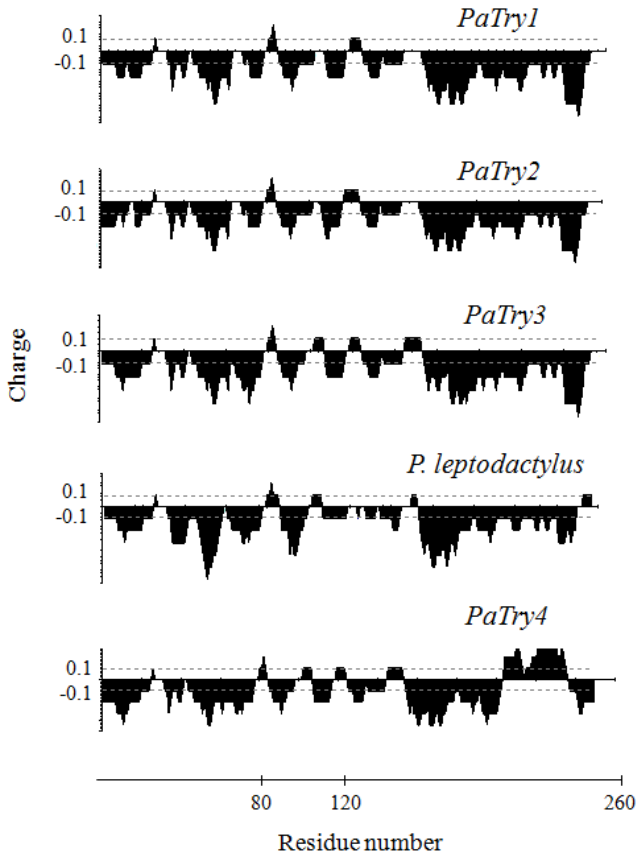

Fig 2. Distribution of charged amino acid in *Panulirus argus* and *Pacifastacus leptodactylus* mature trypsins. Amino acids were plotted using a 9-residue window.

3.1.1. Residues conferring trypsin specificity

The residues of the catalytic triad (His74, Asp125, and Ser218) equivalent to His57, Asp102 and Ser 195 in chymotrypsin nomenclature are conserved across all *P. argus* trypsin-like proteins (Fig 1). The region around catalytic Ser in all *P. argus* trypsin-like proteins (GDSGGP) is conserved in serine proteases. In lobster, the exception is variant PaTry1b where the negative charged Asp is substituted by the uncharged residue Asn (Fig 1). Since the carboxylate of this Asp 217 (194, chymotrypsin numbering) is involved in the formation of a salt bridge with N-terminal Ile of the mature enzyme for completing the formation of the oxyanion hole S1, this substitution may strongly affect the activity of this

variant, as it has been observed before [14]. However, it is interesting to note that this mutation increases the activity of trypsinogen constructs [15]. Pasternak and coworkers [16] solved the crystal structures of the BPTI in complexes with four variant trypsinogens and the activity of variant D194N resulted particularly high respect to trypsinogen. The physiological significance of this trypsin variant in lobster should be further studied. The sequence DIAL that usually contains catalytic Asp of serine proteases has been reported to be DISLL in *L. vannamei* [10] and resulted less conserved in lobster (DISVL). Among the three active site motifs, this is the least conserved in serine proteases and serine proteases homologs in the *Drosophila melanogaster* genome [17]. Yet, the sequence TAAHC that usually surround catalytic His in serine proteases is TAGHC in crayfish and CAGHC in both *P. argus* and *L. vannamei* trypsins.

Primary specificity residues are conserved (Fig 1). All *P. argus* trypsins present an Asp212 (189, chymotrypsin numbering) residue near the base of the substrate binding pocket to stabilize the positive charge of P1 Arg or Lys side chains. Besides, Gly239 and Gly249 (216 and 226, chymotrypsin numbering) are located on one wall of the pocket of all *P. argus* trypsins except PaTry4.

Concerning secondary specificity determinants, Try192 (Fig 1) is conserved among all *P. argus* (this work), *L. vannamei* [10] and *L. salmonis* [18] trypsins, whereas Ser 213 (Fig 1), who occurs in all the shrimps and most of the lobster trypsins, is replaced by Ala in the most divergent variant of *P. argus* (PaTry4). At equivalent position in bovine trypsin, Ser 190 can form hydrogen bound with a P1-Arg side chain and its substitution is thought to disrupt Arg vs. Lys preference [19]. This substitution has been reported for just one clone in the copepod *L. salmonis* [18] but it is typical of lepidopteran trypsins. Different to all other insects, lepidopteran trypsins have no preference for Arg or Lys in P1 position [20] although this effect could not be corroborated by kinetic assays in the lepidoptera *Sesamia nonagroides* [21].

3.2. Three-dimensional structure by comparative modeling

Despite the high sequence similarity between *P. argus* sequences and crayfish trypsin (PDB: 2f91), we used fold-recognition/ab initio methods to search for alternative structural templates in the PDB, and a sequence-to-structure alignment. The *P. argus* trypsin-like sequences have four more conserved cysteine residues (Cys71, Cys157, Cys224, Cys252) than crayfish trypsin and therefore, additional disulfide bonds could be established. According to all the structure prediction methods, METASERVER, PHYRE, and I-TASSER, the crayfish trypsin match ranked highest with scores greater than the threshold. Crayfish trypsin has three disulfide bonds (Cys42-Cys58, Cys168-Cys182, and Cys191-Cys220), which are also present in bovine trypsin (PDB ID: 2ftl). Based on the sequence-to-structure alignment (Fig 1), two of the conserved cysteine residues in *P. argus* trypsin-like sequences (Cys157, Cys224) are in equivalent positions to bovine trypsin Cys135 and Cys201 that engage in a four additional disulfide bond, absent in crayfish. Therefore, we calculated 3D models of *P. argus* sequences based on a consensus sequence-to-structure alignment derived by METASERVER, PHYRE and I-TASSER, and using MODELLER forcing this program to make four disulfide bridges (Cys59-Cys75, Cys157-Cys224, Cys188-Cys203 and Cys214-Cys242/Cys244). Four disulfide bridges have been suggested before for crustacean trypsins [2, 11]. In addition, we hypothesize that Cys71 and Cys252 in Patry1a, PaTry1b, PaTry2 and PaTry3 sequences, and Cys71 and Cys267 in PaTry4 are free cysteines. As in crayfish [8], there is no disulfide bridge connecting the two domains of lobster trypsins.

The 3D models were analyzed by different structure validation programs including PROCHECK, WHATIF and VERIFY-3D (Table 4). In general, quality values obtained for the 3D models are similar to those observed in the template structure. This result indicated a high quality of 3D models presented in this work for PaTry1 to PaTry4.

All the 3D models shown the conserved core structure of the chymotrypsin fold consisting of two six-stranded β-barrel domains packed against each other, with the catalytic residues (His74, Asp125, Ser218) located at the junction of the two barrels. Another conserved characteristic of lobster trypsins is the presence of calcium binding sites (Fig 3C). The

calcium binding motif does not occur in many invertebrate trypsins but its presence has been previously reported in decapods crustaceans [2, 3, 8]. To date it is not clear whether invertebrate trypsins depend on calcium ions for maximal activity or stability. Hehemann and coworkers [22] have proposed that despite the presence of calcium binding site, Ca^{2+} neither affected the activity nor the stability of crab trypsin because there are no accessible autolysis sites in the N-terminal domain, which need to be stabilized by Ca^{2+} coordination. The 'self-destruction' segment in the N-terminal domain of bovine trypsin is also absent in lobster.

From the analysis of the crystal structure of crayfish trypsin it is known that Loop37 and Loop60 (Fig 1 and Fig 3D) are remarkably different in comparison with those of vertebrate trypsins, and also they are important for inhibitor binding [8]. The phenylalanine and isoleucine residues in crayfish Loop37 interact with the C-terminal segment of the inhibitor SGTI, whereas Loop60 plays a role in the formation of the S1'-P1' interaction [8]. Besides the largest loops of *P. argus* (Fig 3D), considering the amino acid substitutions at equivalent positions in these loops (Fig 1), we suggest that different substrate/inhibitor interactions could exist for lobster trypsins and the crayfish enzyme.

It is known that trypsin specificity is governed by a network of structural interactions [4, 5]. Trypsin is only converted into a chymotrypsin-like enzyme when, in addition to the replacement of S1 residues, residues in the surface loops of trypsin are substituted by the analogous in chymotrypsin loops [5, 23]. Ma and coworkers [24] noticed that in trypsins the length of Loop 1 (L1) is not conserved while the length of Loop 2 (L2) is conserved. This agrees with studies in which trypsin with S1+L2 exchange is more active than the S1+L1 mutant [23]. Predicted differences in L1 length between PaTry4 and crayfish trypsin are represented in Fig 3D. However, in terms of amino acidic sequence the surface L1 has been shown to be similar among trypsin variants within species like the flat fish *Solea senegalensis* [25], salmon [13] and *P. argus* (this work) in contrast to L2 that notably varied. Several residues in the L2 differ between PaTry1 to PaTry 3 and PaTry4 (Fig 4).

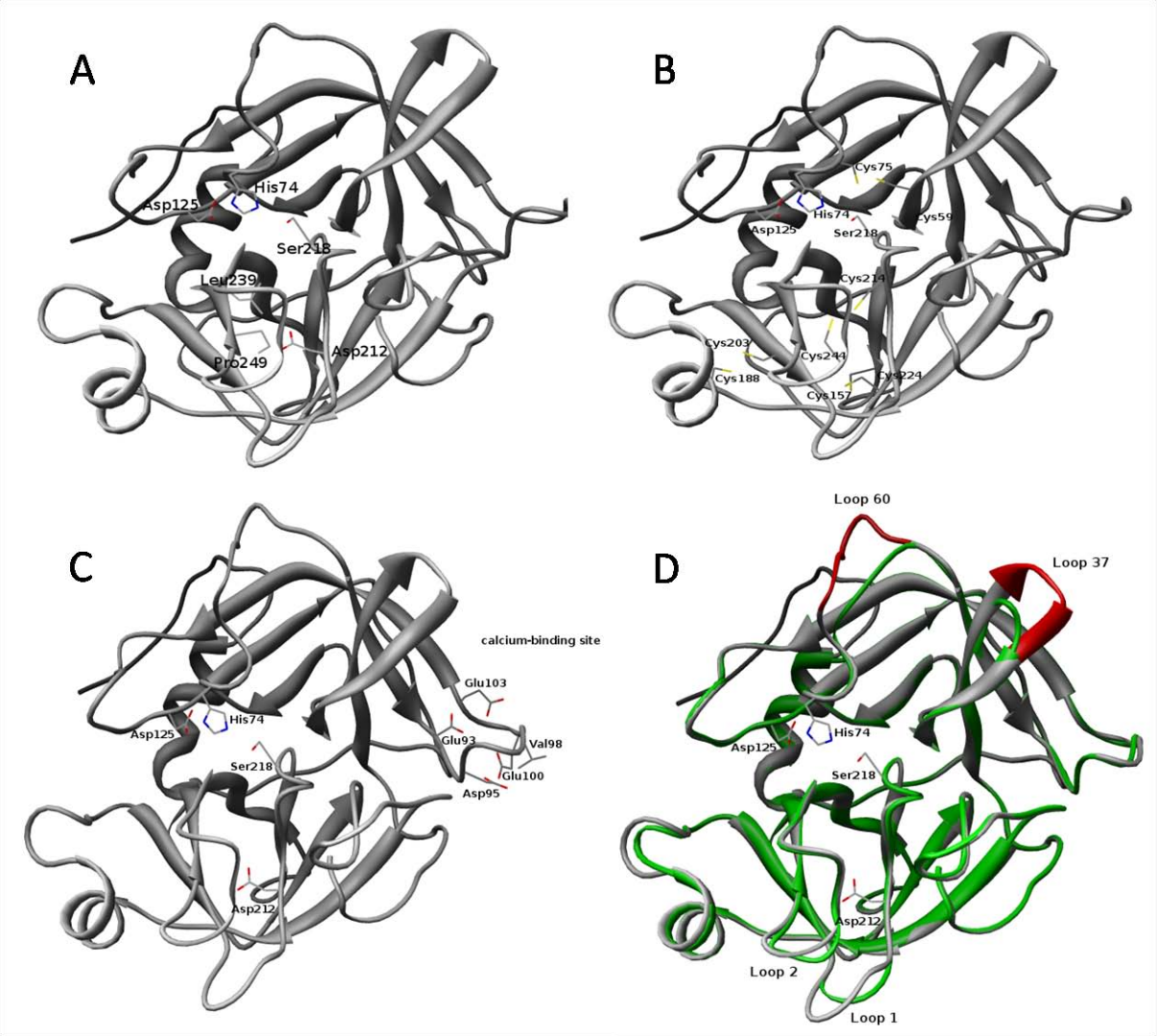

Fig 3. Three-dimensional model of PaTry4 showing the conserved catalytic triad. (A) Leu239 and Pro249 substitution in PaTry4 of glycines at equivalent positions (216 and 226, chymotrypsin numbering) in bovine and all other crustacean trypsins, (B) predicted disulfide bridges in lobster trypsins, (C) calcium-binding site configuration in lobster trypsins and (D) superposition of PaTry4 and crayfish trypsin (PDB code: 2f91A) sowing difference in superficial loops.

Conserved Gly216 and Gly226 (chymotrypsin numbering) are substituted by Leu and Pro respectively in PaTry4. These residues are predicted to be projected into the pocket (Fig 3A) and thus, these substitutions may impair the access of bulky residues to the S1 site. In addition, since hydrophobicity is correlated to aliphatic amino acid surface area (hydropathy index: Gly -0.4, Pro 1.6 y Leu 3.8), it is likely that these substitutions make the pocket of PaTry4 more hydrophobic. The combined effect of both steric restriction and hydrophobicity might confer elastase-like activity to this enzyme but conclusive studies are required.

Crayfish Tyr217 interacts with residue at P6 position of the inhibitor SGTI [8]. At equivalent position (240 in lobster) there is also a tyrosine residue in PaTry1a, PaTry1b, PaTry2 and PaTry3, but instead of tyrosine a serine or glycine residue appears in bovine and PaTry4 sequences, respectively (Fig 1). Other important difference of PaTry4 is the presence of His236 instead Val236 which is present at equivalent positions in bovine, crayfish and all other *P. argus* trypsins (Fig 4).

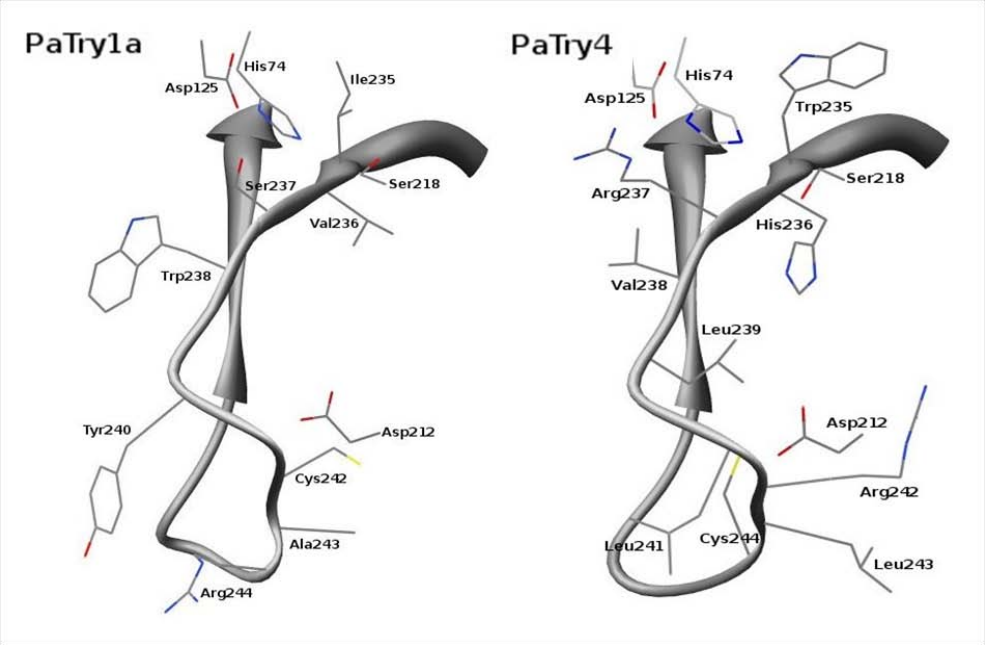

Fig 4. Distinctive features of L2 in PaTry4 in relation to all other *Panulirus argus* trypsins.

The Cys191–Cys220 (chymotrypsin numbering) disulfide bond is important in determining the geometry of the specificity pocket. This bound is conserved in lobster (this work) and crayfish [8] trypsins. The second cysteine in PaTry4 is displaced two residues toward the C-terminus, which may result in a slight enlargement of the S1 pocket. The crystal structure of crab collagenase has shown that the insertion of two residues following Gly216 (chymotrypsin numbering) creates an extended S1 site in which appears to be able to accommodate the Arg side chain in a shallower orientation [26]. Overall geometry of the pocket in PaTry4 could be intermediate between the fiddler crab collagenolitic serine protease [7, 26] and the crayfish trypsin [8]. Definitive structural studies are required.

In spite of changes in the active site of type Ia and Ib brachyurins causing differences in substrate specificity [6], they share a very high sequence identity while greatly differ from

brachyurins II (strict trypsins) where most *P. argus* enzymes can be included as new members. Although PaTry4 share a high identity with the rest of *P. argus* enzymes and other crustacean strict trypsins, this enzyme could not be allocated to any of the brachyurins types due to amino acid substitutions found in the vicinity of the active site that make it specificity unpredictable at this time.

Further determination of PaTry4 specificity could make this protein a model for better understanding structure-function relationship due to the naturally occurrence of point mutations in the specificity pocket.

3.3. Phylogenetic analysis

The phylogenetic tree obtained for crustacean trypsins by the ML, NJ and MP methods were essentially the same as shown in Fig 5. Major branches were poorly supported. However, two groups were distinguished as monophyletic, the one of crayfish (Astacidea) trypsins and a group that include trypsins from *P. argus* (Palinura), Brachyura, Penaeoidea, Caridea and Euphausiacea (Fig 5).

Although with low bootstrap values, NJ reconstruction allowed the second group to be divided into two subgroups, being one of them the one of *P. argus* trypsins (Fig 5). The close relationship among trypsins from Penaeidae and the ones from Caridea and Euphausiacea has been evidenced before [27].

It is interesting to note that in some groups, the topology reflect relationships among trypsins variants rather than among species. Conversely, trypsins from *P. argus* form a clade in spite of relatively low nodal support (Fig 5) probably due to long evolutionary distance of Palinura/ Astacidea trypsins from those of the other groups.

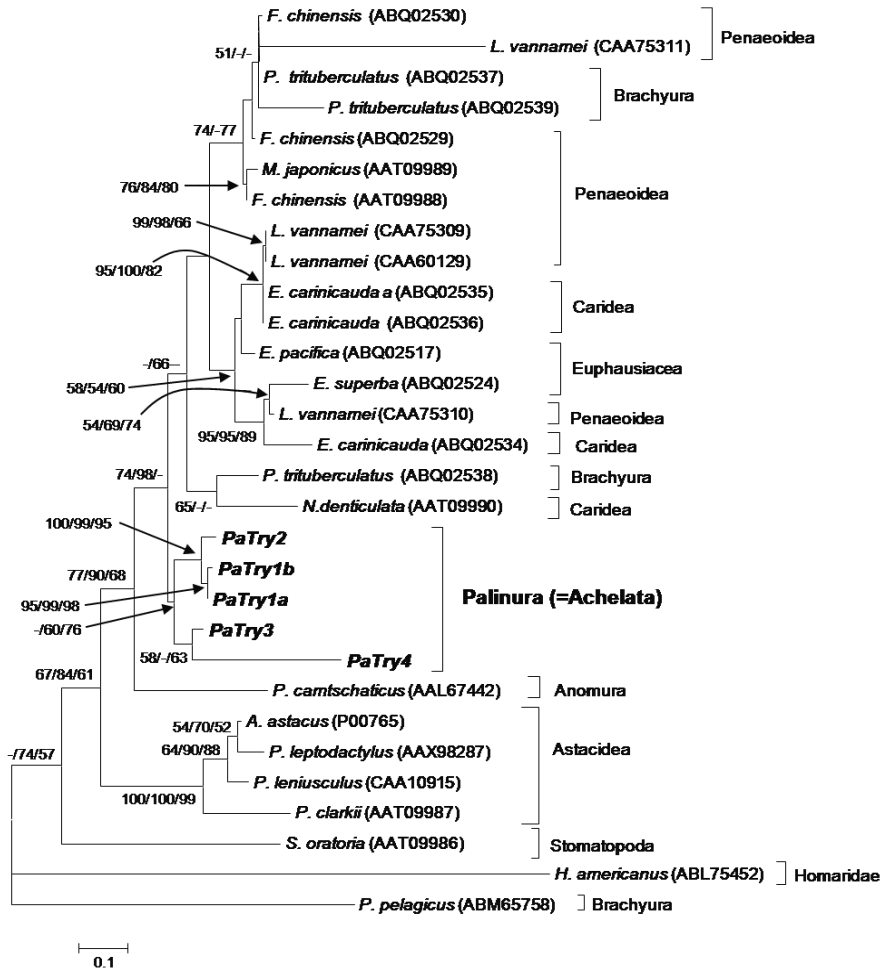

Fig 5. Phylogenetic relationship among crustacean mature trypsins, as derived from the ML, MP and NJ methods. Only bootstrap values higher than 50% are shown on each branch. Species and accession numbers are shown in the tree.

3.4. Tissue specific expression pattern of trypsin variants

Due to sequence differences, it was possible to construct primers for the selective recognition of the different trypsins in RT-qPCR assays. No expression of the trypsin variants reported here was found in hemocytes, gills, heart, and muscle, neither in digestive tissues (stomach, intestine) (not shown) other than the digestive gland (Fig 6). PaTry2 is the least expressed trypsin and PaTry3 the one of higher relative expression (Fig 6). PaTry4 was found to be not expressed in two of the five individuals analyzed. When present, this trypsin variant is highly expressed thus the physiological role of this serine protease could be relevant. Results indicate that *P. argus* trypsins are differentially regulated at the transcription level.

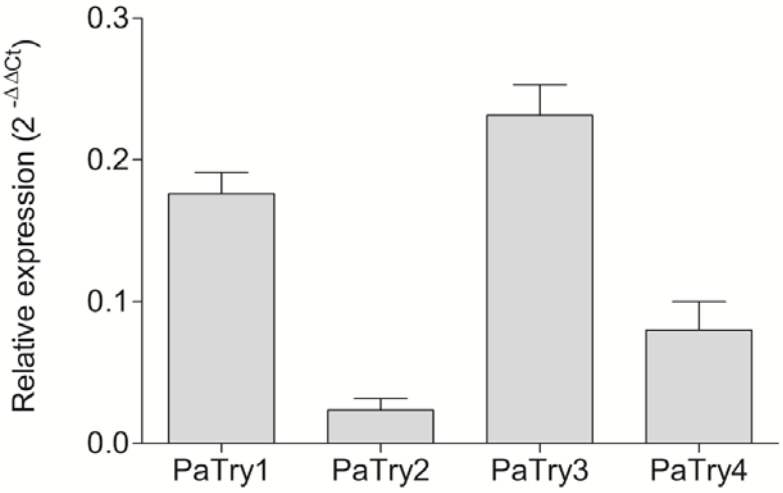

Fig 6. Expression of different trypsins in the digestive gland of the spiny lobster *Panulirus argus*. EF1α was used as housekeeping gene. Same results were obtained when using β-act as the housekeeping gene (not shown).

Brachyurins family is of great interest in terms of structure-function relationships and evolution of serine proteases. Reports of new members provide a more complete picture of the family and potentially can give rise to the description of novel enzymes. We suggest that specificity within this family of enzymes is broader than it is currently believed.

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Capítulo 6

Dietary protein quality differentially regulates trypsin enzymes at the secretion and transcription levels in the lobster (*Panulirus argus*) by distinct signaling pathways

Perera, E., Rodríguez-Viera, L., Rodríguez-Casariego, J., Fraga, I., Carrillo, O., Martínez-Rodríguez, G., Mancera, J.M., ***J Exp Biol 215, 853-862*** **(2012)**.

Resumen

Se estudiaron los efectos de la alimentación con dietas formuladas con diferente composición proteica (pescado, calamar y soja, como principales fuentes de proteína), en la secreción y expresión de tripsinas en la langosta *Panulirus argus*. La máxima secreción de tripsina se observó a las 4 h de haber ingerido el alimento. A este tiempo, las dietas basadas en harinas de pescado y calamar indujeron la secreción de tripsinas y estimularon la expresión de la isoforma más abundante. Sin embargo, las dietas basadas en harina de soja no fueron capaces de estimular la secreción y expresión de tripsinas en la glándula digestiva de la langosta. Ensayos *in vitro* mostraron que las proteínas intactas, y no los hidrolizados de proteínas, estimulan la secreción de tripsina, mientras que la señal para estimular la expresión de tripsinas parece ser la aparición de amino ácidos libres en la glándula. Los resultados sugieren que existe una cronología en la regulación de los procesos de secreción y expresión de tripsinas durante la digestión en la langosta. Se concluye que las enzimas tipo tripsina en *P. argus* están reguladas a nivel de transcripción y secreción por las proteínas de la dieta a través de dos mecanismos de señalización. Nuestros resultados indican que es posible incrementar la eficiencia de la digestión proteica en langostas espinosas mediante la selección de fuentes de proteínas apropiadas. Sin embargo, existen otros factores, por ejemplo la baja solubilidad de las proteínas del alimento seco, que pueden evitar estos incrementos.

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Abstract

The effects of pelleted diets with different protein composition (fish, squid or soybean meals as main protein sources) on trypsin secretion and expression were studied in the lobster *Panulirus argus*. Trypsin secretion was shown to be maximal 4 h after ingestion. At this time, fish and squid based diets induce trypsin secretion, as well as up-regulated the major trypsin isoform at the transcription level. While fish and squid based diets elicited a "normal" prandial response, soybean based diet failed in stimulate digestive gland to secrete trypsin into the gastric juice or induce trypsin expression above of the levels observed in fasting lobsters. *In vitro* assays showed that intact proteins rather than protein hydrolysates stimulate trypsin secretion in lobster. However, signal for trypsin transcription appears different to that for secretion and is probably mediated by the appearance of free amino acid in the gland, suggesting a stepwise regulation of trypsin enzymes during digestion. It is concluded that trypsin enzymes in *P. argus* are regulated at the transcription and secretion level by quality of dietary proteins through two distinct signaling pathways. Our results indicate that protein digestion efficiency in spiny lobsters can be improved by selecting appropriated protein sources. However, other factors like poor solubility of dietary proteins in dry diets could hamper further enhancements in digestion efficiency.

Keywords: *Panulirus*, Spiny lobster, Protein digestion, Digest, Regulation, Trypsin

1. INTRODUCTION

As occurred in most crustacea, trypsin enzymes play the central role in protein digestion in spiny lobsters (Johnston, 2003; Celis-Gerrero *et al*., 2004; Perera *et al*., 2008a). Some studies are available on the biochemical characterization of spiny lobster trypsins (Galgani and Nagayama, 1987; Celis-Gerrero *et al*., 2004; Perera *et al*., 2008a) and variations of activities throughout development and molt stages have been also reported (Johnston, 2003; Perera *et al*., 2008b). However, the effect of diet on these enzymes has been poorly studied and only recently the time course of trypsin activity after ingestion of different diets was reported for one spiny lobster species (*Jasus edwardsii,* Simon, 2009). Since few mechanistic studies have been performed, virtually nothing is known on the mechanisms behind protein digestion in spiny lobsters and especially those regulating trypsin enzymes as main digestive proteases.

In *P. argus* trypsin is a polymorphic enzyme (Perera *et al*., 2008a) and some trypsin cDNA showing different expression rates have been cloned (Perera *et al*., 2010a). Additionally, trypsin isoforms in lobster appear to differ in catalytic properties or specificity since differences in digestion efficiency have been found among three distinct isoenzyme patterns (Perera *et al*., 2010b). All these features of lobster trypsins provide several points for the regulation of their activity to occur and thus, make this species a good model for studying trypsin regulation mechanisms in crustacea. Despite the many studies on trypsin enzymes in other crustaceans, these mechanisms are fairly unknown and in general, limited information is currently available for non-insect invertebrates (Muhlia-Almazán *et al*., 2008).

Also, there is a great interest in the commercial growout of wild-caught spiny lobsters (Jeffs and Davis, 2003). Research effort over the past decades has considerably increases our knowledge about the nutritional requirements of spiny lobsters (Williams, 2007) but feed most species with formulated diets on a least-cost basis is still a challenge. Spiny lobsters growth performance is relatively poor when fed on dry formulated diets unless high inclusions of krill meal and/or krill hydrolysate are used (Smith *et al*., 2005; Barclay *et al*., 2006; Cox and Davis, 2009). It seems that spiny lobsters would not be able to

efficiently use proteins from common aquafeeds ingredients (e.g. fish, squid and soybean meals) in dry food. This conflict with the fact that they are equipped with a wide repertory of digestive enzymes specially proteases (Galgani and Nagayama, 1987; Celis-Gerrero *et al*., 2004; Perera *et al*., 2008a) and with previous digestibility studies (Ward *et al*., 2003; Irving and Williams, 2007; Perera *et al*., 2010b). Poor protein use could be related to some impairment in normal digestive progression when lobsters fed on pelleted diets (Simon and Jeff, 2008; Simon 2009).

The aim of present work was to assess the effect of different protein sources on trypsin expression and secretion after feeding in *P. argus*. Our results demonstrate that trypsin enzymes are differentially regulated at the transcription and secretion level by ingested proteins. Additionally, we provide evidence that intact proteins, instead small peptides or free amino acids, are the major signal eliciting a normal prandial secretory response in lobster, whereas transcriptional regulation of the major isoform appear to occur by a distinct mechanism.

2. MATERIALS AND METHODS

2.1. Animals and diets

Spiny lobsters *P. argus* (Latreille, 1804) (90–150 g) were collected in the Gulf of Batabanó, Cuba, and held under optimal laboratory conditions at the Center for Marine Research of the University of Havana as described before (Perera *et al*., 2005). Lobsters were fed daily with fresh fish (*Opisthonema oglinum*) *at libbitum* and experiments were carried out after animals were adapted for 2 week to captive conditions and fed intensively on food offered. Only intermolt specimens [determined according to Lyle and MacDonald (1983)] were used in experiments because in this stage a maximum of trypsin activity has been previously reported (Perera *et al*., 2008b). Soybean, fish and squid meals used as protein sources in diets were prepared as described earlier (Perera *et al*., 2010b) and experimental formulated diets (Table 1) were prepared by thoroughly mixing the dry ingredients with oil and then adding water until the consistency was that of stiff dough.

This was passed through a mince, dried at 60°C and broken into a convenient pellet size (~3mm x 10mm). Diets were stored at -20°C until used.

Table 1. Formulation (g/100g) of experimental diets.

	Squid	Fish	Soybean
Squid meal[a]	44	6.5	-
Fish meal (herring)[a]	8	44	8
Soybean meal[b]	-	-	61
Gelatin[b]	8	8	8
Whole wheat [c]	26.75	26.75	5.25
Shark oil [d]	1.9	0.2	6.4
Soybean lecithin[b]	2	2	2
Cholesterol [e]	2	2	2
Vitamin/mineral[f]	2	2	2
Vitamin C[b]	0.1	0.1	0.1
Vitamin E[b]	0.25	0.25	0.25
Dicalcium phosphate [c]	1	1	1
Calcium carbonate [c]	2	2	2
Metionine[g]	-	-	0.1
Stuff (Talc) [b]	2	5.2	2
Total	100	100	100

Diets were formulated for 45 % crude protein, 9 % crude fat, 25 % carbohydrates.
Pellets contain 10-12 % of water content.
[a]Prepared at the laboratory as detailed before (Perera *et al*., 2010b).
[b]Commercially available feedstuffs in Cuba
[c]Santa Cruz Fish Feed Factory, Camagüey, Cuba.
[d]From Fisheries Research Center Laboratory, La Habana, Cuba.
[e]Wako Pure Chemical Industries, Ltd., Osaka , Japan.
[f]Premix from DIBAQ-Aquaculture, Segovia, Spain, containing (per Kg of feed): Vitamin A 15000 UI, Vitamin D3 3000 UI, Vitamin E 180 mg, Vitamin K 15 mg, Vitamin B1 37.5 mg, Vitamin B2 37.5 mg, Vitamin B6 24.75 mg, Vitamin B12 0.045 mg, Vitamin H 1.14 mg, D-Pant. Acid 120 mg, Nicotinic Acid 225 mg, Vitamin C 300 mg, Folic Acid 11.24 mg, Inositol 112.5 mg, Zinc 75 mg, Selenium 0.3 mg, Magnesium 86.25 mg, Copper 2.25 mg, Manganese 22.5 mg, Iodine 7.5 mg, Iron 3 mg, Cobalt 0.3 mg.
[g]Merck KGaA, Darmstadt, Germany.

2.2. **In vivo** ***effects of food proteins on trypsin activity and expression***

It has been shown that the time course of digestive enzymes during digestion in the spiny lobster *J. edwardsii* is better reflected in the gastric juice, since only secreted enzymes can be measured (Simon, 2009). Thus, we first examine the time course of trypsin secretion in *P. argus* by sampling the gastric juice after ingestion. However, this was possible only for fresh fish as food, since the ingestion of dry pelleted diets resulted in dough with almost no free gastric juice until four hours after ingestion. Specimens of *P. argus* (N=8) were starved for 48 hours, fed with fresh fish (*O. oglinum*) and sampled every one hour for seven hours. Gastric juice samples were obtained through the oral cavity into disposable

insulin syringes as described by Vonk (1960) but instead a fire-polished glass cannula we used a needle whose sharp end was removed and protected with a plastic cannula. Gastric samples were immediately frozen in liquid nitrogen and then stored at -80 °C. Lobsters were handled with care and samples were rapidly taken (less than 1 min.) to avoid excessive stress.

Since hyperglycemic response to stress is well known in crustaceans (Kleinholz and Little, 1949), we tested in a preliminary trial the influence of this manipulation on glucose levels in haemolymph of *P. argus* (N=5). Hemolymph was taken from the sinus at the base of the third walking leg as described before (Perdomo *et al*., 2007) and glucose was measured using a clinical RapiGluco-Test (Helfa Diagnostic, La Habana, Cuba) according to manufacturer's instructions. This preliminary experiment also worth for confirming that trypsin activity in gastric juice does not vary due to manipulation.

In a second experiment, four groups of five lobsters were starved for 48 h and fed with three diets named according to the main protein source they contain (soybean, fish and squid). One group was led without food and served as control. After four hours of ingestion, lobsters were anesthetized by immersing them into ice-cold water and dissected for digestive gland and gastric juice extraction. Both samples for activity assays were immediately frozen in liquid nitrogen and stored at -80°C. Additional digestive gland samples for expression analysis were rapidly transferred to RNA*later*® Solution (Ambion, Life Technologies Corporation), led stand at 4°C for 24 hours and finally conserved at -20°C.

2.3. In vitro *effect of protein/ hydrolysates on trypsin activity and expression*

For assessing if digestion end-products or intact proteins can stimulate trypsin synthesis and/or secretion, the effects of protein (diet) hydrolysates and bovine serum albumin (BSA) on the digestive gland were studied *in vitro*. The incubation medium was *Panulirus* saline (PS) solution (Zhainazarov *et al*., 1997) composed of (in mM) 460 NaCl, 13 KCl, 14 Na_2SO_4, 13 $CaCl_2$, 10 $MgCl_2$, 2 glucose, and 10 *N*-2-hydroxyethylpiperazine-*N*`-2-ethane sulfonic acid (HEPES), but pH was adjusted to 6.6 instead to 7.4, according to the slightly

acidic pH in the digestive gland of crustaceans (Bickmeyer *et al.*, 2008). The solution was filtered using Whatman cellulose nitrate membrane filters (0.45 µm pore size) and aerated at room temperature (25°C) during 30 min before experiment.

The hydrolysates were obtained as follows. The formulated diets (soybean, fish and squid) were shaken in PS (0.5g/mL) for 15 min and then incubated with lobster gastric juice (3:1) during four hours at room temperature. Thereafter, the mixtures were centrifuged at 1000 x g for 5 min to eliminate insoluble materials. The supernatants were boiled during 5 min, in order to abolish enzyme activity and precipitate proteins, and centrifuged again for 30 min under similar conditions. Although after this treatment only free amino acids and peptides should remain in the supernatants they were filtered through Amicon filters with cut-off of 10 000 Da. The resultant solutions were stored at -20°C and referred as diet hydrolysates. Total amino acid composition of diets and free amino acids in hydrolysates were determined by HPLC at "Instituto de la Grasa", Sevilla, Spain. Peptide composition of filtered hydrolysates was assessed by Tricina-PAGE.

Digestive gland samples (30 mg) were taken from 48 h starved lobsters (N=5) as described above and washed in 1 mL of PS for removing hemolymph and digestive enzymes from the surface of tissue, and then transferred to 1.5 mL Eppendorf tubes containing 200 µL of filtered and aerated PS. After that, 100 µL of the following solutions were added to five samples (tubes) from each individual: soybean hydrolysate, fish hydrolysate, squid hydrolysate, BSA and PS. After 20 min of incubation, digestive glands were transferred to RNA*later*® Solution, led stand at 4°C for 24 hours and finally conserved at -20°C. The incubating media was frozen in liquid nitrogen and stored at -80 °C for determining the trypsin activity released.

2.4. Trypsin activity assay

Digestive glands were homogenized in reaction buffer (200 mM Tris–HCl pH 7.5) and centrifuged 1000 x g at 4°C for 30 min. Crude extracts of digestive glands or gastric juice were diluted with reaction buffer to measure enzyme activities at initial rates. Trypsin activity was measured using 1.25 mM N-benzoyl-DL-arginine *p*-nitroanilide (BApNA) in

200 mM Tris–HCl pH 7.5 as described before (Perera *et al*., 2008a). One unit of trypsin activity was defined as the amount of enzyme that catalyzed the release 1 µmol of *p*NA per minute, using the appropriate molar extinction coefficient. Under our assay condition the molar extinction coefficient of *p*NA was calculated to be 2.563 mM^{-1} cm^{-1}. Trypsin activity was expressed per mL or per gram of tissue as needed.

2.5. Determination of total soluble protein

Proteins were measured according to Bradford (1976) using BSA as standard.

2.6. Trypsins expression by RT-qPCR

All kits in this study were used following manufacturer's instructions. Total RNA was purified using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) including RNAse-free DNAse treatment. RNA was quantified by its Abs_{260} and its quality was assessed by its $Abs_{260/280}$ and using the Agilent RNA 6000 Nano Assay Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNAs were synthesized using the qScript™ cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD, USA) and then used as templates for RT-qPCR on a Mastercycler ep realplex (Eppendorf, Hamburg, Germany) using PerfeCTa™ SYBR® Green Fast-Mix™ (Quanta BioSciences, Gaithersburg, MD, USA) in white wells twin.tec real-time PCR plates 96 (Eppendorf, Hamburg, Germany). Four previously isolated lobster trypsins cDNAs (Perera *et al*., 2010a) with GenBank accession no. *PaTry1a* (GU338026), *PaTry2* (GU338028), *PaTry3* (GU338029) and *PaTry4* (GU338030) were studied. Specific primers (Biomers.net, Ulm, Germany) used for lobster trypsins were successfully employed before (Perera *et al*., 2010a) and are shown in Table 2. Efficiency of target amplification for each primer set was optimized in the previous study by using 4 pmol/µL of primers and 60 ^{o}C of annealing/extension, thus these conditions were employed here. By means of calibration curves (10-fold dilutions, corresponding to cDNA in reactions from 20 ng to 0.2 pg) all primer pairs were checked to produce similar efficiencies, except *PaTry4*. Reaction volume was 10 µL containing 4 µL cDNA (4 ng total cDNA), 5 µL of PerfeCTa SYBR Green Fast Mix (2X) and 0.5 µL of each primer. Cycling conditions were as follow: initial

denaturing/activation step for 5 min at 95 ^{o}C, 40 cycles of denaturing (95 ^{o}C for 30 s) and annealing/extension (60 ^{o}C for 45 s). Control reactions with DEPC water and RNA instead of cDNA were included to ensure absence of contamination or sample gDNAs. Specificity was checked by melting curve analysis. Additionally, PCR products were verified by nucleotide sequencing. All samples were run in duplicate. Replicate PCR reactions generated highly reproducible results. The intra-assay coefficient of variation (CV) for Ct values varied from 0 to 0.004 and the inter-assay CV from 0.011 to 0.027.

Table 2. Primers used in this study for RT-qPCR

Name	Nucleotide sequence	Position*	Direction
PaTry 1 Fw	5'-AACAAGATCGTTGGTGGTGA-3'	96-115	Forward
PaTry 2 Fw	5'-CTGACGCCGAGCCTGGTA-3'	115-132	Forward
PaTry 3 Fw	5'-GGACATCTCCTTCGGCTT-3'	159- 176	Forward
All *PaTry*s Rv	5'-AGTGACCAGCACAGATAGC-3'	220-238	Reverse
PaTry 4 Rv	5'-GTGGATCCAGTGTTCGTCAT-3'	689-706	Reverse
All *PaTry*s Fw	5'-CCGTGCCCATCGTGTCTGA-3'	551-569	Forward

* For primers used for several trypsin variants, numbers correspond to hybridization position on *PaTry1*. For the other variants few nt displacements could occur.

2.6.1. Standard curves and absolute quantification

Four full-length lobster trypsin cDNAs were amplified as detailed before (Perera *et al*., 2010b) and cloned into plasmids using the TOPO TA Cloning® Kit (Invitrogen Ltd, Paisley, UK). Plasmids were extracted from Transformed One Shot® TOP10 competent *Escherichia coli* cells using the GenElute™ Five-Minute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Clones containing inserts of expected size were identified by PCR analysis (T3 and T7 primers of TOPO TA Cloning® Kit) followed by agarose gel electrophoresis, and sequenced from both directions to ensure their correspondence with previously reported lobster trypsins (*PaTry1* to *PaTry4*). Plasmids containing the trypsin variants of interest were quantified using a spectrophotometer. Absolute calibration curves were constructed using the circular plasmid vectors (pCR®4-TOPO®) containing each of the four full-length lobster trypsin cDNAs, serially diluted with DEPC water from $3x10^7$ to 30 copies. Targets and calibrators showed similar amplification efficiencies and in all cases but *PaTry4*, near 100%. The number of transcript molecules was calculated from the linear regression of the standard curve and analyses were done with the Mastercycler ep realplex

software. Results were expressed as mean ± s.e.m, after being normalized to ng of total RNA.

2.7. Pattern of evolution of trypsin enzymes: preliminary assessment

The software MEGA5 was used for all tests performed (Tamura *et al.*, 2007). Pairwise comparisons between *PaTry 1* to *3* nucleotide sequences were carried out to calculate the ratio of nonsynonymous to synonymous substitution (*K*a/*K*s) as an indicator of the pattern of evolution (Hurst, 2002) of these enzymes. The best model of nucleotide substitution was obtained in MEGA5 and resulted the one of Tamura (1992) because transition-transversion and G+C content biases. Then, taken into account the calculated Transition/Transversion bias (R=1.21) and applying the Jukes-Cantor correction for multiple substitutions at the same site, we compute *K*a/*K*s ratio for each mature trypsin pair using the modified Nei and Gojobori method with 1000 bootstrap for variance estimation. Statistical significance of selection was tested by the Z-test and the Fisher's exact test. A codon based selection test using the likelihood method was used further for detecting positive selection sites.

2.8. Statistical analyses

All data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test, respectively, with $P \leq 0.05$. Data from time course of trypsin activity after ingestion and data from protein and activity comparison among diets, hydrolysates and BSA were subjected to one-way ANOVAs ($P \leq 0.05$). Expression data were analyzed by two-way ANOVA ($P \leq 0.05$) being diet and trypsin variant the two source of variation. In all cases, replications were individual lobsters and duplicate measurements were performed. The Tukey test ($P \leq 0.05$) was used to determine differences among means. The Software package Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA) was used for all tests performed and figures were generated by OriginPro 8 (OriginLab Corporation, Northampton, MA, USA).

3. RESULTS

3.1. Trypsin secretion after ingestion

A preliminary trial testing the effects of gastric juice sampling procedure on stress level showed that mean glucose levels in haemolymph did not vary due to manipulation ($F=0.32$, $P>0.05$, mean ± s.e.m.: 0.2 ± 0.027 mmol/L), and no variation in trypsin activity occurred in the gastric juice within the seven hours of sampling in non-fed lobsters ($F=1.18$, $P>0.05$, mean ± s.e.m.: 0.7 ± 0.04 U/mL).

Trypsin activity in the gastric juice of fasting lobsters was highly dependent of the protein concentration in the fluid and this relationship was better described by a straight line [Trypsin activity = 0.1525 (protein concentration) + 0.044] with a high determination coefficient ($R^2=0.94$). This indicates that 94% of trypsin activity in the gastric juice can be explained by proteins from digestive gland secretion. Dietary proteins remaining in the gastric chamber after 48 hours of fasting and non-trypsin enzymes in the gastric juice (Perera *et al*., 2008a) would account for the variation observed. Thus, the time course of trypsin activity in gastric juice after ingestion was followed as indicative of trypsin secretion by digestive gland. Trypsin activity in the gastric juice significantly varied ($F=4.16$, $P\leq0.01$) after ingestion. Following feeding, trypsin activity progressively decreased in gastric juice until significant lower levels after three hours (Fig. 1). However, between the 3th and the 4th hour after ingestion a significant trypsin activity enhancement was observed (Fig. 1), returning rapidly to prefeeding levels. Afterward, trypsin activity remained stable until seven hours post-feeding.

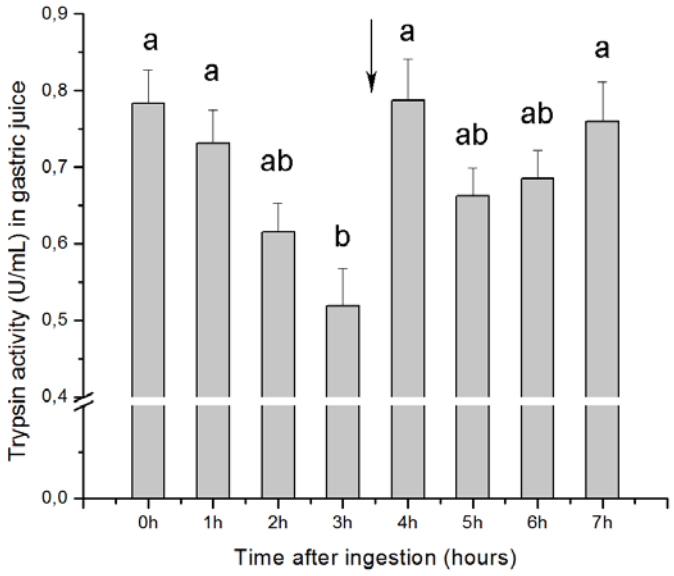

Fig. 1. Time-course of trypsin activity (mean ± s.e.m.) in gastric juice of *P. argus* after ingestion of fresh fish. Differences were found through time ($P \leq 0.05$). Letters above bars indicate statistical differences according to the Tukey test ($P \leq 0.05$). Arrow indicates pulse of trypsin secretion from the digestive gland to the gastric juice after meal ingestion.

3.2. Effects of dietary protein on trypsin secretion

Since trypsin secretion peaked four hours after ingestion, trypsin activity of gastric juice and digestive gland at this time was compared in *P. argus* feeding on diets of different protein composition. No differences were observed in trypsin activity of digestive glands of lobsters fed with the different diets respect to fasting animals (Fig. 2A). However a significant reduction ($F=3.96$, $P\leq0.05$) in protein content of gland was observed in individuals ingesting fish and squid diets (Fig. 2B). This reduction in soluble protein in the digestive gland matches with significant higher ($F=8.59$, $P\leq0.05$) levels of trypsin activity in gastric juice of these lobsters (Fig. 2C). The secretion elicited by fish and squid diets is comparable to those found after the ingestion of fresh fish (Fig. 1) whereas lobsters ingesting soybean based diet did not change trypsin activity in gastric juice (Fig. 2C).

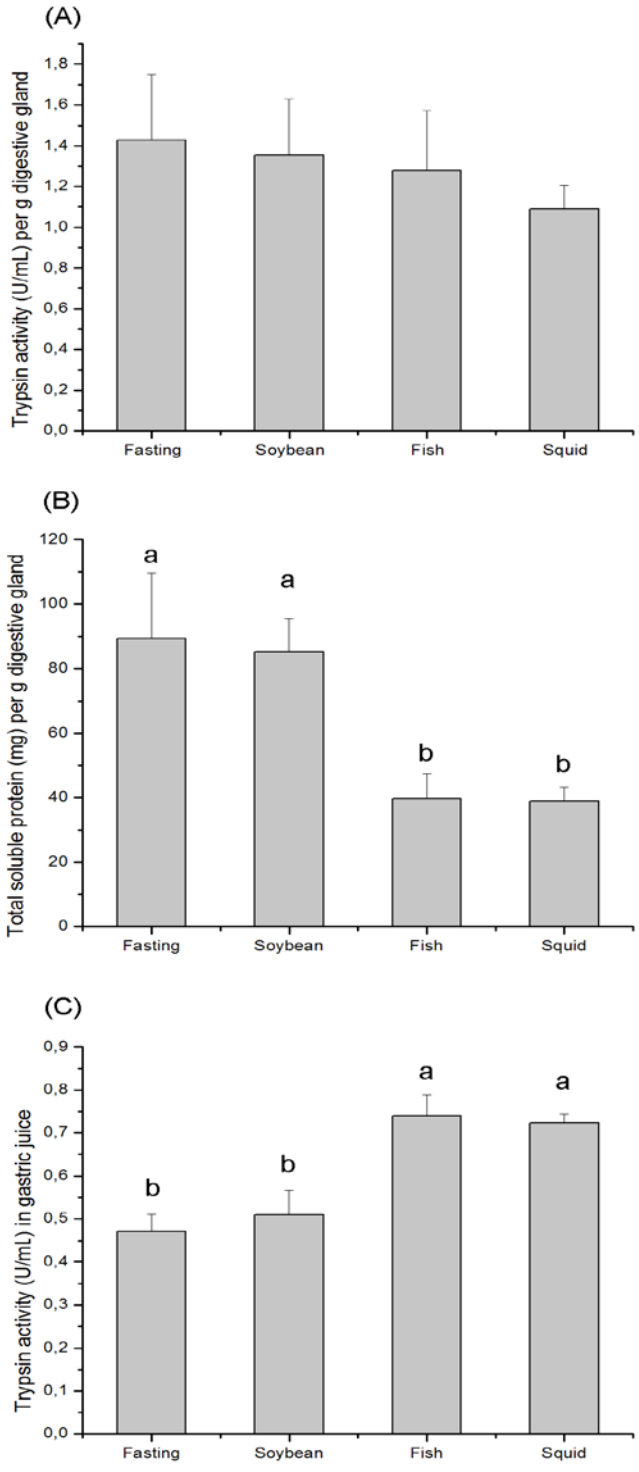

Fig. 2. Trypsin activity in the digestive gland (A), total soluble protein content in the digestive gland (B) and trypsin activity in the gastric juice (C) of *P. argus* four hours after ingestion. Values are mean ± s.e.m. Soybean, Fish and Squid refer to major protein source in dry formulated diets. Differences were found ($P \leq 0.05$) in protein content of digestive gland and trypsin activity in the gastric juice among diets. Different letters above bars indicate statistical differences according to the Tukey test ($P \leq 0.05$).

3.3. Effect of dietary protein on trypsin expression

Expression was studied by means of RT-qPCR for four trypsin variants (*PaTry1* to *4*) previously reported in *P. argus* (Perera *et al*., 2010a). For absolute quantification, standard curves were generated with plasmids containing the different trypsin cDNAs (Fig. 3) and checked to produce similar amplification efficiencies than their targets *PaTry 1* (E=0.99, R^2=1.0, Slope=-3.357), *PaTry 2* (E=0.97, R^2=0.999, Slope=-3.401), *PaTry 3* (E=1.02, R^2=0.999, Slope=-3.284). *PaTry 4* was not included in statistical analysis due to few individuals expressing this transcript in most treatments and because the little different amplification efficiency (E=0.92, R^2=1.0, Slope=-3.542) obtained. Significant differences in trypsin expression were found among diets ($F=3.10$, $P\leq0.05$) and among trypsin variants ($F=19.35$, $P\leq0.001$). *PaTry 3* was always found to be the most abundant transcript (Fig. 4). No variations were found in the expression of *PaTry 1* and *PaTry 2* among feeding treatments, whereas the most abundant variant (*PaTry 3*) significantly increased its expression in lobsters ingesting fish and squid diets (Fig. 4).

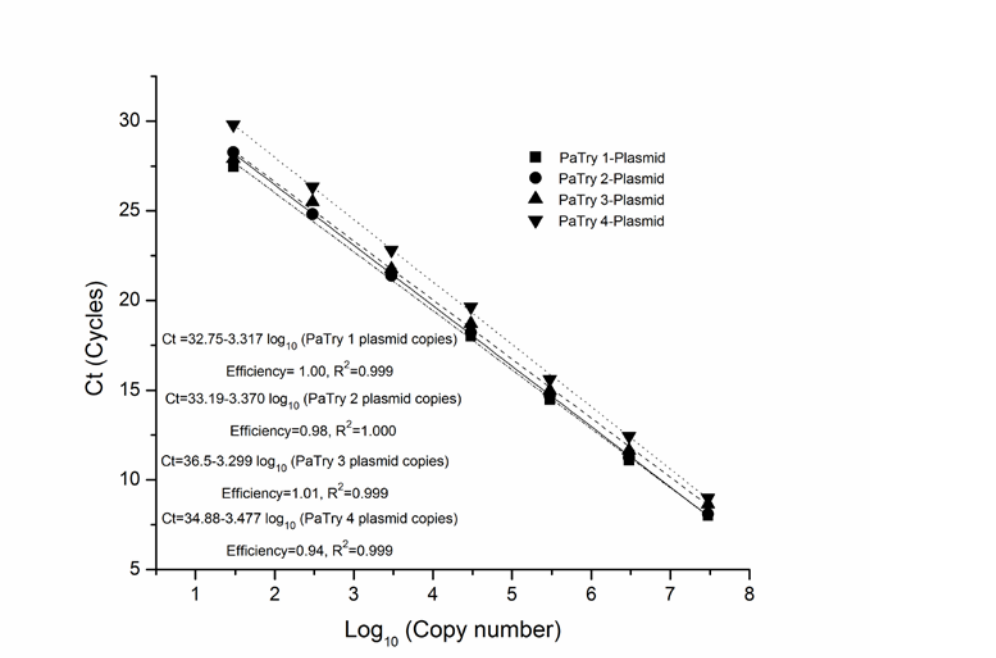

Fig. 3. Standard curves for absolute quantification in RT-qPCR of four trypsin variants in lobster. Data points are means of triplicate measurements. Equations as well as amplification efficiencies are shown in the graph. Target trypsins have similar amplification efficiencies than the standards (see text for details).

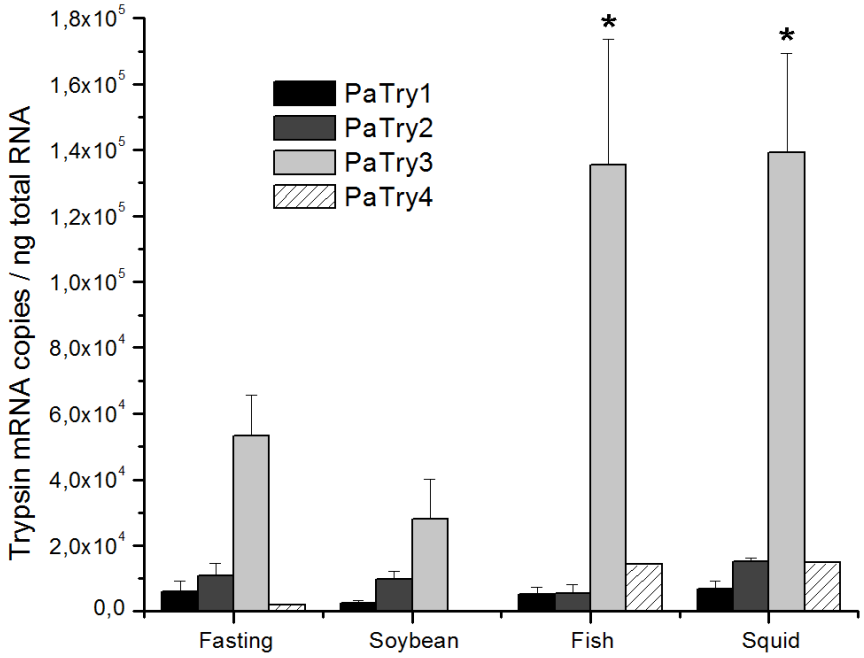

Fig. 4. Differential expression (mean ± s.e.m.) of four trypsin variants (*PaTry1* to *4*) in the digestive gland of *P. argus* four hours after ingestion. Soybean, Fish and Squid refer to major protein sources in dry formulated diets. Differences were found among diets ($P \leq 0.05$) and trypsin transcripts ($P \leq 0.001$). *PaTry3* was always significantly most abundant than *PaTry1* and *PaTry2*, and it is likely to be most abundant than PaTry4 (not included in statistical analysis due to few individuals expressing this form of the enzyme). *PaTry3* was the only trypsin responding to protein quality of diet. Asterisks above bars indicate statistical differences among diets for *PaTry3*, according to the Tukey test ($P \leq 0.05$).

3.4. Effects of diet hydrolysates and intact proteins on trypsin secretion in vitro

Under our assay conditions, incubation of lobster digestive glands with diet hydrolysates (<10kDa) did not produce significant changes in trypsin secretion into the incubating media (Fig. 5A). Digestive glands incubated with squid diet hydrolysate seem to slightly raise trypsin secretion, but a significant increase above controls could be detected only for glands incubated with BSA (Fig. 5A).

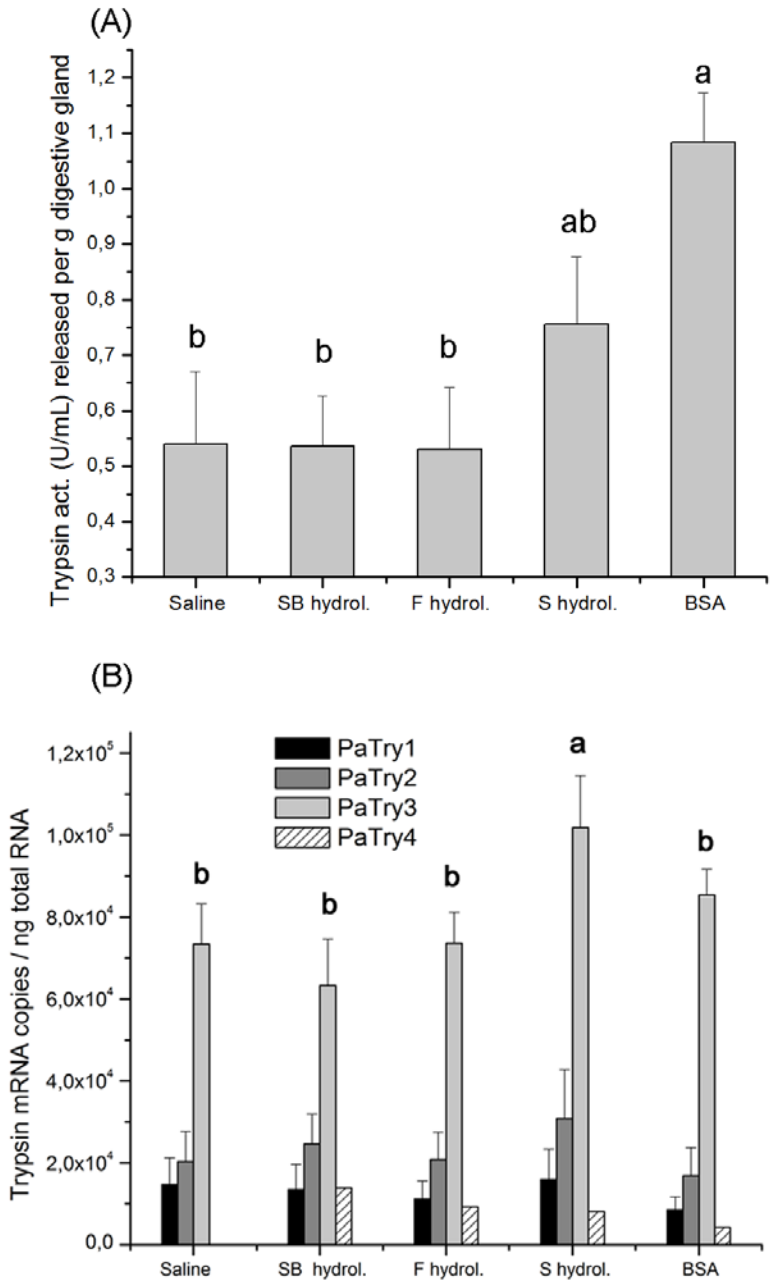

Fig. 5. Trypsin activity released (A) and expression (B) of four trypsin variants (*PaTry1* to *4*) in isolated digestive gland of *P. argus* after 20 minutes of exposure to diet hydrolysates and bovine serum albumin (BSA). Values are mean ± s.e.m. SB: soybean, F: fish and S: squid refer to major protein sources in dry formulated diets used for hydrolysates preparation (see Materials and Methods section for details). Differences were found in trypsin secretion ($P \leq 0.05$) and expression among treatments ($P \leq 0.05$). *PaTry3* was always significantly ($P \leq 0.001$) most abundant than *PaTry1* and *PaTry2*, and it is likely to be most abundant than *PaTry4* (not included in statistical analysis due to few individuals expressing this form of the enzyme). *PaTry3* was the only trypsin responding to treatments. Different letters above bars indicate statistical differences among treatments for *PaTry3* according to the Tukey test ($P \leq 0.05$).

3.5. Effects of diet hydrolysates and intact proteins on trypsin expression in vitro

PaTry 3 resulted the only trypsin variant induced by dietary stimulation, resembling the results obtained *in vivo*. Only squid diet hydrolysate increased the copy number for *PaTry 3* transcripts (Fig. 5B). Total amino acid composition of the test diets was similar except for some differences in Val and Lys content (Table 3). However, free amino acids composition differed among diet hydrolysates reflecting differences in digestion of animal vs. vegetal protein sources. The Val and Glu+Gln content of soybean hydrolysate were lower and the content of Arg and Lys were higher than in the other digests (Table 3). Electrophoresis analysis of hydrolysates shown strong differences in peptide composition between the soybean and the fish/squid digests (Fig. 6).

Table 3. Amino acid composition of experimental diets and corresponding hydrolysates. Values are means of triplicate measurements in percent ± standard error.
ND Not detected, * Not determined.

	Diets (total amino acids in %)			Hydrolysates (free amino acids in %)		
	Squid	Fish	Soybean	Squid	Fish	Soybean
Asp+Asn	10.24 ± 0.50	9.97 ± 0.34	12.72 ± 0.43	ND	ND	0.51 ± 0.05
Glu+Gln	1.18 ± 0.14	1.51 ± 0.36	1.24 ± 0.20	2.40 ± 0.17	1.48 ± 0.05	0.90 ± 0.02
Ser	6.49 ± 0.05	6.47 ± 0.05	7.56 ± 0.05	3.17 ± 0.19	3.69 ± 0.03	2.52 ± 0.03
His	2.86 ± 0.05	2.82 ± 0.11	2.92 ± 0.19	7.62 ± 0.54	7.43 ± 0.22	6.23 ± 0.09
Gly	10.89 ± 0.48	12.22 ± 1.03	9.37 ± 0.57	1.50 ± 0.07	1.27 ± 0.08	1.38 ± 0.07
Thr	5.85 ± 0.05	5.63 ± 0.15	4.92 ± 0.12	2.25 ± 0.09	1.03 ± 0.07	0.85 ± 0.02
Arg	8.46 ± 0.03	8.53 ± 0.15	8.95 ± 0.21	14.07 ± 0.94	13.86 ± 0.65	19.43 ±0.22
Ala	9.16 ± 0.05	9.44 ± 0.28	7.34 ± 0.09	8.28 ± 0.26	7.97 ± 0.41	6.72 ± 0.07
Pro	1.80 ± 0.28	2.14 ± 0.20	3.28 ± 0.62	13.26 ± 0.26	14.05 ± 0.27	11.82 ± 1.02
Tyr	4.21 ± 0.12	3.90 ±0.22	4.14 ± 0.01	11.45 ± 3.55	8.22 ± 1.77	6.75 ± 0.10
Val	2.80 ± 0.18	2.94 ± 0.20	6.02 ± 0.98	2.34 ± 0.08	2.06 ± 0.22	0.76 ± 0.23
Met	2.78 ± 1.05	2.20 ± 0.57	1.89 ± 0.21	3.48 ± 0.00	3.71 ± 0.18	4.45 ± 0.20
Cis	0.97 ± 0.07	0.95 ± 0.05	1.25 ± 0.05	0.88 ± 0.01	0.71 ± 0.12	0.86 ± 0.04
Ile	4.91 ± 0.13	4.75 ± 0.24	4.57 ± 0.06	5.13 ± 0.19	5.52 ± 0.11	5.38 ± 0.08
Trp	0.49 ± 0.04	0.35 ± 0.02	0.43 ± 0.00	*	2.54 ± 0.08	2.21 ± 0.05
Leu	10.45 ± 0.15	10.02 ± 0.30	9.71 ± 0.14	10.60 ± 0.73	12.68 ± 0.65	11.32 ± 0.11
Phe	5.81 ± 0.05	5.78 ± 0.06	6.00 ± 0.12	5.87 ± 0.10	6.05 ± 0.00	7.60 ± 0.12
Lys	10.64 ± 0.07	10.39 ± 0.27	7.69 ±0.23	7.69 ± 0.62	7.73 ± 0.58	10.32 ± 0.19

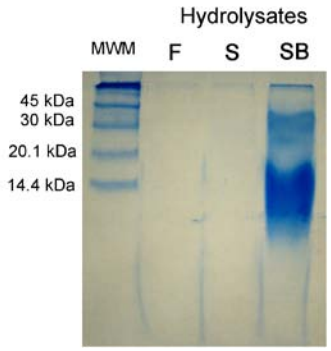

Fig. 6. Tricina-PAGE showing peptide composition of fish (F), squid (S) and soybean (SB) hydrolysates. Note the absence of detectable amounts of peptides in the fish and squid hydrolysates, while soybean hydrolysate is rich in peptides of less than 12 kDa. MWM: low molecular weight markers (Pharmacia).

3.6. Pattern of evolution of trypsin enzymes: preliminary assessment

*K*a/*K*s ratio for *PaTry1*/*PaTry2* and *PaTry1*/*PaTry3* pairs resulted 0.70 and 0.66 respectively, while *K*a/*K*s for *PaTry2*/*PaTry3* pair was 1.03. Both Z-test and Fisher's exact test of selection gave no statistical indication of positive selection. However, the maximum likelihood approach revealed 25 codons evolving under positive selection (*dN*-*dS*>0). From these sites, the 72% contain unique substitutions in *PaTry3*. Interestingly, the 55% of these *PaTry3* substitutions occur in important regions for enzyme function (Table 4).

Table 4. List of positive selected sites in important functional motifs.

Sites	Change *PaTry1/2* to *PaTry3*	Location
52	W to F	Loop 37
54	S to F	Loop 37
95	N to D	Ca^{2+} binding site
105	A to T	Near the Ca^{2+} binding site
107	V to P	Near the Ca^{2+} binding site
190	D to N	Loop 3
195	G to S	Loop 3
206	V to E	Loop 1
207	P to T	Loop 1
246	N to S	Loop 2

Site numbers start at the fist residue of lobster trypsinogens as in Perera *et al*. (2010a).
Loop 37 is used according to the nomenclature in crayfish trypsin (Fodor *et al*., 2005)
Loops 1, 2 and 3 are according to vertebrate nomenclature (Perona and Craik, 1995, Hedstrom, 1996)

4. DISCUSSION

Trypsin activity has been positively correlated with digestion efficiency and growth in fishes (Rungruangsak-Torrissen *et al*., 2006; Savoie *et al*., 2011) but conflicting results have been obtained in crustacea (Le Vay *et al*., 1993). Effect of dietary protein on trypsin activity has been reported in shrimp larvae (Le Moullac *et al*., 1994), juveniles (Muhlia-Almazán *et al*., 2003) and adults (Le Moullac *et al*. 1996) but in general, the effect of proteins on trypsin enzymes have been poorly studied in crustacea in comparisons to vertebrates and other groups of invertebrates.

In most previous studies performed with crustacea on this topic, enzyme activity or expression have been measured after many days of conditioning to experimental diet [e.g. 20 days (Le Moullac *et al*., 1996), 3 weeks (Muhlia-Almazán *et al*., 2003; Simon, 2009)], depending on the goal of each study. Present work was focused on short-term changes in trypsin secretion and expression without conditioning and preceded for two days of fasting, in order to assess the direct effect of feeding. Conditioning allows for many changes in the organism to the new nutritional status and thus, it is difficult to separate the direct effect of food ingested from overall changes (e.g. endocrine, metabolic) that indirectly may also affect digestive enzymes. In addition, in our study the time-response analysis led us to select the time (4h) where trypsin activity in gastric juice showed the maximum enhancement after ingestion for further comparisons among diets.

4.1. Effect of dietary proteins on trypsin secretion

Trypsin secretion has been reported in fasting mammals (Konturek *et al*., 2003), shrimps (Lehnert and Johnson, 2002) and insects (Moffatt *et al*., 1995) [except in batch digesters such as mosquitoes (Lehane *et al*., 1995)]. In accordance, significant amount of trypsin activity was observed in fasting lobsters. After ingestion of fish, trypsin activity in the gastric juice of *P. argus* dropped gradually, as reported before for the lobster *J. edwardsii* (Simon, 2009) and assumed to be due to the drinking of water (Simon and Jeffs, 2008). In the case of dry diets, food absorbed most of the gastric fluid and it was impossible to obtain samples until 4 h after ingestion. This would be a great problem for the use of dry

diets in *P. argus* since proteins most be dissolved before being attacked by proteolytic enzymes. Difficulties in digesting dry formulated diet in the foregut of lobsters have been reported before (Simon and Jeffs, 2008; Simon, 2009). Recent studies have pointed out that solubility of dry diets would command protein digestion efficiency in lobsters (Simon, 2009; Perera *et al*., 2010b).

Previous studies have found different secretory patterns in the digestive gland of crustaceans: i) three phases (at 0–15 min, 1–2 h and 3.5–5 h after a meal) in the lobster *Homarus gammarus* (Barker and Gibson, 1977), ii) two peaks within 6 h of feeding in the crayfish *Astacus leptodactylus* (Hirsch and Jacobs, 1928) and the shrimp *Litopenaeus vannamei* (Muhlia-Almazán and García-Carreño, 2002), and iii) only one phase of secretion 1–4 h after feeding in the prawn *P. semisulcatus* (Al-Mohanna *et al*., 1985). In spiny lobsters the time course of digestive enzyme activities after feeding have been reported only for one species (*J. edwardsii*) and all studied enzymes showed a common peak in gastric juice 4h after ingestion (Simon, 2009). Our results compare well with this previous report and indicate that in *P. argus* a significant amount of trypsin enzymes from the digestive gland have attained the gastric chamber after 4 h of digestion.

In our study no statistical differences in trypsin activity of digestive gland were found in *P. argus* after 4 hour of ingestion. Studies on the effects of diet on digestive enzyme activities in crustacea have yielded contradictory results and this has been attributed mainly to the use of digestive gland as the examined tissue. After collection, glands are usually disrupted to obtain homogenates where stored and secreted enzymes are mixed. In spite of this limitation, it is interesting to note that the non-significant trend observed in the digestive gland for trypsin activity advises more enzyme secretion in fish and squid fed lobsters respect to fasting or soybean fed specimens. High amount of trypsin enzymes remaining in the gland after 4 h of digestion could explain the lack of significance in our results. In contrast, soluble protein content of the gland significantly decrease after feeding fish and squid diets (but not with soybean), perhaps as a result of secretion of other enzymes in addition to trypsin. Consequently, by examining the gastric juice as suggested by Simon (2009), we found that soybean based diets lack stimulatory capacity on lobster digestive gland while fish and squid diet elicited a secretory response similar to those observed with

fresh fish. These results can be taken as definite evidence that the nature of ingested protein differentially affects the released of trypsin enzymes from the digestive gland of *P. argus*. Conversely, in adult shrimps Le Moullac *et al*. (1996) found that only casein increased trypsin activity while squid meal and fish soluble concentrate had no effect. The authors explained their result because the high level of squid and fish proteins tested could saturated the response of shrimps, although the use of digestive gland homogenates in that study would account for some differences with our as explain above. Our results in *P. argus* suggest that in contrast to soybean proteins, the use of fish and squid proteins in dry feeds is not limited by the digestive response of lobsters, but probably by diet solubility hampering digestibility as suggested before (Simon and Jeff, 2008; Simon, 2009; Perera *et al*., 2010b).

4.2. Effect of dietary proteins on trypsin expression

Our results indicate that trypsin enzymes in *P. argus* are also regulated transcriptionally by dietary proteins, as reported before for shrimps (Le Moullac *et al*. 1996; Muhlia-Almazán *et al*., 2003), mosquito (Noriega *et al*., 1994) and rats (Lhoste *et al*., 1994; Hara *et al*., 2000). Interestingly, the most abundant transcript in *P. argus* (*PaTry3*) was the only one for which dietary up-regulation could be demonstrated while all other trypsins appear to be expressed in a constitutive fashion. To our knowledge, it is shown for the first time for a crustacean species that different trypsin isoforms differ in their responsiveness to dietary proteins. Differences in trypsins isoform expression have been found in *Daphnia magna* but in response to protease inhibitors in diet (Schwarzenberger *et al*., 2010).

In our study *PaTry4* transcript, which correspond to a trypsin-like enzyme of probably wide specificity (Perera *et al*., 2010a), was not included in statistical comparisons due to few individuals expressing this variant and because a slightly small amplification efficiency for this transcript was found respect to the others. It is known that even small differences in the amplification efficiencies will lead to differences in cycle threshold (Ct) whether or not the concentrations of the starting templates differ (Platts *et al*., 2008). This variant seems to be also up-regulated upon feeding but definite studies are required both to

confirm this observation and to definitively determine the true specificity and physiological role of this enzyme.

It is worthy to note that in the present work, RNA extractions were carried out from tissue biopsies with no regard for the different cell types contained in the digestive gland. Trypsin synthesis in crustacean digestive gland has been demonstrated only in the F cells (Lehnert and Johnson, 2002) while expression values are given herein on a total RNA basis. Therefore, our results are thought to underestimate actual trypsin expression in F cells of *P. argus*.

4.3. Signals for trypsin secretion and expression

Since early studies (Hirsch and Jacobs, 1928) it is known that digestive enzymes in crustaceans are secreted upon feeding but the mechanism remains unknown. In dogs (Meyer and Kelly, 1976) and humans (Thimister *et al*., 1996) amino acids and peptides are more effective than intact proteins in stimulating pancreatic exocrine secretion. However, trypsin secretion is more stimulated by intact proteins than hydrolyzed proteins or amino acids in rats (Green and Miyasaka, 1983), fishes (Cahu *et al*., 2004) and insects (Blakemore *et al*., 1995; Lehane *et al*., 1995). In order to study the possible signals for trypsin induction/secretion in *P. argus*, *in vitro* assays were performed whereby digestive glands were exposed to an intact protein (BSA) and diet hydrolysates (<10kDa). Our results showed that trypsin secretion did not change upon hydrolysates incubation, while 0.01 % BSA significantly stimulates trypsin secretion. Trypsin secretion in insect opaque zone preparations occurs at concentrations of BSA as low as 0.0001% (Blakemore *et al*., 1995). It seems that in *P. argus*, and likely in most crustaceans, the signals for trypsin secretion are similar to those in rats, fishes and insects (intact proteins) and this information has been not available to any crustacean until now.

Postprandial enzyme secretion in mammals is mostly (70%) stimulated by CCK through neural pathway (Konturek *et al*., 2003). Although the action mechanism is not so well understood as in mammals, CCK is also recognized as major regulator of trypsin secretion in fishes (Buddington and Krogdahl, 2004). There are strong evidences for the presence of

CCK-like peptides in crustaceans (van Wormhoudt *et al*., 1989; Favrel *et al*., 1991; Resch-Sedlmeier and Sedlmeier, 1999) although their role on digestive enzyme secretion has been not clarified. However, in our *in vitro* assays BSA was able to elicit enzyme secretion acting directly over the digestive gland (prandial mechanism), suggesting that as occur in insects (Lehane *et al*., 1995; Noriega *et al*., 1996; Noriega and Wells, 1999; Lu *et al*., 2006; Brandon *et al*., 2008; Graf *et al*., 1998) peripheral signals (neural and endocrine) are not obligatory though may have modulator roles.

We found that squid diet hydrolysate was able to induce the major trypsin (*PaTry3)* transcription after feeding while intact proteins like BSA had no effect. This result indicate that induction signals appears to be different to those for secretion and is probably mediated by the appearance of free amino acid in the gland [Note the absence of significant amount of small peptides in squid and fish hydrolysates (Fig. 6)]. This result suggests a stepwise regulation of trypsin enzymes during digestion.

Lys, Arg and Met are considered as the most limiting amino acids for growth of crustaceans (Akiyama *et al*., 1992). Digestibilities of these amino acids (and His and Tyr) appear to be high in the three tested diets [but soybean diet was supplemented with free Met, Table 1]. It is known that these amino acids are largely as free amino acids in casein (Savoie, 1994) and soybean hydrolysates (Henn and Netto, 1998). On the other hand we suggest a low digestibility for Ser, Gly and Thr in the three diets and for Val in soybean diet. However, no major disparity were found in the amino acid composition of the squid and fish hydrolysates and the difference in their capacity for stimulating trypsin expression could result from differences in amino acid concentration. The release of free amino acids from squid meal doubles by far the one from fish meal during early digestion (Perera *et al*., 2010b). Although previous studies have also suggested a specific effect of squid proteins on digestive enzymes (Le Moullac *et al*., 1996; Perera *et al*, 2005), other results in lobster (Perera *et al*., 2005) and other crustaceans (Cruz-Ricque *et al*., 1989) have pointed to a more general metabolic effect of squid proteins.

Since current results suggest that free amino acid can increase trypsin expression in the digestive gland of *P. argus* (and potentially protein digestion efficiency), further studies

are welcome on the selection of rapidly digestible protein sources. However, we argue the use of high inclusion of protein hydrolysates to pay off the poor digestibility of dietary proteins. Yet, the effect of protein hydrolysates other than in attractiveness of diets is not yet well understood. High inclusion of protein hydrolysate in diets depressed growth in fishes (Zambonino-Infante and Cahu, 2007) and crustaceans (Córdova-Murueta and García-Carreño, 2002) while moderate levels have positive effects in fish (Zambonino-Infante and Cahu, 2007; Savoie *et al*., 2011), cephalopods (Aguila *et al*., 2007) and crustaceans (Córdova-Murueta and García-Carreño, 2002). Squid meal, which is rich in small molecular weight proteins (Córdova-Murueta and García-Carreño, 2002; Perera *et al*., 2010b) also promote better growth rates in crustaceans at low supplementation levels (Córdova-Murueta and García-Carreño, 2002). Conversely, high levels of krill hydrolysates still enhance growth in shrimps (Córdova-Murueta and García-Carreño, 2002) and spiny lobsters (Smith *et al*., 2005; Barclay *et al*., 2006).

We found that high inclusion of soybean meal in diet did not induce trypsin expression or secretion in *P. argus.* These observations indicate that this diet lack particular signals for efficient protein digestion or some components of soybean meal block the signal transduction into de producing/secreting cells. Soybean lectins are known to bind to the brush border membrane of the Atlantic salmon distal intestine (Hendriks *et al*., 1990). In contrast, fish and squid based diets induce trypsin secretion to the same extent than fresh fish did, a response mediated by the recognition of intact proteins, while major trypsin isoform is transcriptionally induced after feeding through a distinct signaling pathway, probably mediated by free amino acids.

4.4. Preliminary insight on the evolution of trypsins in P. argus

The very marked differences found in expression among trypsins in *P. argus* drive us to perform a preliminary analysis on the pattern of evolution for three *P. argus* trypsins (*PaTry1 to 3*). Previous phylogenetic reconstruction grouped all these enzymes together respect to all other crustacean trypsins (Perera *et al*., 2010a) and nucleotide composition does not differ among *PaTry1* to *3* (not shown), suggesting these genes are under purified selection. Analysis of *K*a/*K*s for *PaTry1*/*PaTry2* and *PaTry1*/*PaTry3* pairs sustains this

hypothesis (*K*a/*K*s<1) although values are unusually high, whereas neutral selection is suggested by *K*a/*K*s for *PaTry2*/*PaTry3* pair (*K*a/*K*s~1). Both Z-test and Fisher's exact test gave no statistical indication of positive selection. However, since averaging *K*a/*K*s for all sites ignores the type of selective pressure that applies to individual amino acids, we used the maximum likelihood approach for detecting positively evolving sites in a background of purifying/neutral selection.

Results indicate that most sites of *P. argus* trypsins are under purifying selection and thus that these proteins are subject to functional constraints. In addition, most nonsynonymous changes resulted in the substitution of similar amino acids, with slight change in charge or hydrophobicity while conserving the volume of residues, in accordance with the importance of three-dimensional architecture on the function of these enzymes. However, it is worthy to note that several positively evolving sites are within (or in close vicinity to) functionally relevant motifs (Table 4). The loop 37 in crayfish trypsin is known to be important in hydrophobic interaction with extended subsites of inhibitors (Fodor *et al*., 2005) and probably proteinaseous substrates. In *P. argus* trypsins, Trp and Ser residues of *PaTry1* and *PaTry2* loop 37 are both replaced by the more hydrophobic Phe in *PaTry3*. In the calcium binding site, Asn residues in *PaTry1* and *PaTry2* are replacing by the more charged Asp in *PaTry3*. Two, one and two positive selected sites occur within loop1, loop2 and loop 3, respectively. These three loops determine trypsin specificity (Hedstrom, 1996). It is not possible, however, to anticipate the impacts of all these substitutions on enzyme activity but one can hypothesize that *PaTry3* hold distinct functional features.

Trypsin isoform sequences from other spiny lobster species are required to truly determine if some trypsins are under selective pressure at certain sites to undergo adaptive evolution (or evolving independently) while other trypsins are evolving in a concerted fashion as occur in *Drosophila* (Wang *et al*., 1999).

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Capítulo 7

Lobster (*Panulirus argus*) hepatopancreatic trypsin isoforms and their digestion efficiency

Perera, E., Rodríguez-Casariego, J., Rodríguez-Viera, L., Calero, J., Perdomo-Morales, R., Mancera, J.M. ***Biol. Bull.*** 222: (En prensa)

Resumen

Se conoce que los crustáceos presentan varias isoformas de tripsina en su sistema digestivo. Mientras que el número de isoformas reportadas continúa creciendo, especialmente las derivadas de secuencias de cDNA, se desconoce el papel o contribución de cada isoforma en la digestión de proteínas. Entre los invertebrados, es en insectos donde se ha logrado una mayor comprensión del papel funcional de las distintas tripsinas. En un estudio anterior se demostró que los diferentes patrones electroforéticos de tripsinas (fenotipos) de la langosta *P. argus* difieren en la eficiencia en la digestión. Por este motivo, en este trabajo se utilizó este crustáceo para profundizar en las bases bioquímicas de tales diferencias. Los resultados demuestran que la isoforma presente en todos los individuos de *P. argus* presenta una alta eficiencia catalítica y es la más reactiva hidrolizando sustratos proteinaceos nativos. Por otra parte, se observó que una de las isoformas presente en los individuos menos eficientes tiene la menor k_{cat}, la menor k_{cat}/K_m y es la menos competente digiriendo proteínas nativas. Una de las interrogantes básicas en biología es cómo las diferencias genéticas entre individuos se traducen en diferentes desempeños fisiológicos. Este trabajo es el primero en demostrar en un crustáceo que la variación en la digestión de proteínas entre individuos con diferentes fenotipos de tripsina recae en las características bioquímicas de las isoformas presentes. Los resultados obtenidos contribuyen a la comprensión del polimorfismo de las tripsinas y la digestión de proteínas en crustáceos.

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Abstract

It is well known that crustaceans exhibit several trypsin isoforms in their digestive system. While the number of crustacean trypsin isoforms continues increasing, especially those derived from cDNA sequences, the role of particular isoenzymes in digestion remains unknown. Among invertebrates, significant advances in the understanding of the role of multiple trypsins have been obtaining only on insects. Since it has been demonstrated that trypsin isoenzyme patterns (phenotypes) in lobster differ in digestion efficiency, we used this crustacean as a model for assessing the biochemical basis of such differences. We demonstrated that the trypsin isoform known to be present in all individuals *P. argus* has a high catalytic efficiency (k_{cat}/K_m) and is the most reactive toward native proteinaseous substrates, while one of the present isoforms in less efficient individuals has a lower k_{cat}, a lower k_{cat}/K_m and is less competent digesting native proteins. One of the fundamental questions in Biology is how genetic differences produce different physiological performances. This work is the first to demonstrate the presumption that crustacean trypsin phenotypic variation in protein digestion relies on the biochemical properties of the different isoforms. Results are relevant for understanding trypsin polymorphism and protein digestion in lobster.

Keywords: Catalytic efficiency; Digestion; Isoforms; Lobster; *Panulirus*; Purification; Trypsin

1. INTRODUCTION

Trypsin and trypsin-like enzymes have been extensively studied in a wide variety of taxa due to their central role in digestion and other physiological process (e.g. blood coagulation, fibrinolysis, cellular and humoral immunity, fertilization and embryonic development) (Di Cera, 2009). Vertebrate trypsins have been intensely studied since the 1930s (See Perona and Craik, 1995; Hedstrom, 1996, 2002; Di Cera, 2009, for reviews). Although invertebrate trypsins have received less research attention, a growing body of information is also available on these enzymes (see Carrillo-Farnés *el al*., 2007; Muhlia-Almazán *et al*., 2008; Sainz and Córdova-Murueta, 2009 for reviews). Invertebrate trypsins share the common characteristics of the Clan PA, family S1A, although they exhibit some distinct features like apparent independence of calcium ions, activity toward native proteinaseous substrates, high number of isoforms, and poor stability at acid pH. In the case of crustaceans, much of this information has been obtained in studies on crude extracts or partially purified enzymes.

However, some trypsins could be purified to homogeneity from crustacean species. Besides species-specific differences, the number of isolated isoforms from crustaceans differed among studies mainly due to the purification strategy employed. This is a result of the difficulties for separating so many similar proteins in its active form from complex crude extracts. Gel filtration and/or affinity chromatography at the beginning of the purification process and single or successive anion exchanges as final steps have been the most successful procedures for isolation of different trypsin isoforms in crustaceans (Kim *et al.,* 1992; Sjödahl *et al*., 2002; Fodor *et al*., 2005; Hehemann *et al*., 2008). Also, a method used to circumvent the difficulties in the chromatographic separation of crustacean trypsins is the cutting of bands directly from polyacrylamide gels (Sainz *et al*., 2004). In some crustacean species, other purification flowcharts yielded single or very few isoforms and/or partially purified enzymes (Wu *et al*., 2008; Guizani *et al*., 1992; Dendinger and O' Connor, 1990).

The high amount of trypsin-like enzymes in the digestive gland of crustaceans and the intricacies of trypsin isoforms purification have lead to few isoforms characterized at the

biochemical level. This harms our physiological understanding of trypsin diversity in crustaceans, despite published works on trypsin cDNAs, gene organization and expression (Klein *et al*., 1996, 1998; Hernández-Cortes *et al*., 1999; Perera *et al*., 2010a). In addition, although biochemical differences have been found among some crustacean trypsin isoforms, these have been not related to the physiological process to which they are involved. Do differences among isoforms have a physiological meaning or an adaptive value? Our previous studies at the biochemical and molecular levels in *P. argus* have corroborated the presence of several trypsins (Perera *et al*., 2008; Perera *et al*., 2010a), and have provided some indication of digestion advantage of some isoforms (Perera *et al*., 2010b; Perera *et al*., 2011). According to our previous results, specimens exhibiting some isoenzyme patterns seem to be more efficient in digestion than individuals with others isoenzyme patterns. This individual variation remains poorly understood because these enzymes have been not purified from its natural source and no information on their biochemical properties is available.

In this study we isolate five trypsin isoforms in *P. argus* by using conventional chromatography, showing differences in stability, dependence of calcium ions and effect of inhibitors among isoforms. Also, we found kinetic and activity differences among isoforms that match with previous results on digestion efficiency (Perera *et al*., 2010b). Present results led us to understand better the trypsin polymorphism in lobster and approach, for the first time, to the biochemical basis of the variation in protein digestion efficiency in lobster.

2. MATERIALS AND METHODS

2.1. Animals, samples and reagents

Intermolt lobsters were collected and dissected as described before (Perera *et al*., 2008). Digestive glands were frozen in liquid nitrogen and stored at -80°C until were used. All chemicals used were reagent grade.

2.2. Purification of trypsin-like enzymes from digestive glands of lobsters

Our main goal with the present study was to understand the lobster individual variability in protein digestion in relation with the trypsin isoenzymes present. However, as the purification work was not possible from individual digestive glands because the high amount of starting material required, digestive glands from 30 individuals were pooled to ensure the presence of all major isoforms. Digestive glands were homogenized with a blender and centrifuged at 10 000 x g for 60 min at 4°C. After removing the surface lipid layer, the supernatant was made into an acetone powder by using -20°C acetone. The dried powder was suspended by gentle shaken overnight (4°C) in 25 mM Tris-HCl pH 7.5, 0.1 M NaCl, 0.01 mM diethyldithiocarbamate, and then centrifuged at 10 000 x g for 60 min at 4°C. The supernatant was subjected to 30% ammonium sulfate precipitation for eliminating particulate materials and centrifuged as above. This supernatant was referred as clarified crude extract.

All chromatographic steps were performed using a GradiFrac system (Pharmacia-LKB, Sweden) coupled to a HPLC pump K-1001 (Knauer, Germany). Six milliliters of clarified crude extract (~70 mg/mL) was fractionated by gel filtration on Sephadex G-75 column (2.6 x 66 cm) previously equilibrated with 10 mM methyl piperacine pH 5.0 (Buffer A) containing 0.1 M NaCl. The flow rate was 0.3 ml/min and protein elution was monitored at 280 nm. Fractions of 4 mL were collected for analysis and those with *N*-benzoyl-DL-arginine *p*-nitroanilide (BA*p*NA) activity were pooled and applied to an anion exchange column (1 mL, MonoQ^{TM} 5/50, GE Healthcare Bio-Science) equilibrated in Buffer A with 0.1 M NaCl. The column was washed with 10 mL of the same buffer, and then with 15 mL of Buffer A containing 0.19 M NaCl. Trypsin isoforms were separated with 110 mL of a linear gradient from 0.24 to 0.42 M NaCl in Buffer A at 0.5 mL/min. Fractions of 1 mL were collected for analysis of trypsin activity using BA*p*NA as the substrate and casein zymography (García-Carreño *et al*., 1993). A single band under both native-PAGE and SDS-PAGE (Laemmli, 1970), as well as in the zymography, was taken as a fraction containing a homogeneous enzyme. The relative molecular weight of the purified enzymes was determined by 14% SDS-PAGE using low molecular weight markers (14-94 kDa, Amersham Bioscience). All electrophoresis were conducted using a P81 Puffin^{TM},

8x10x0.75 cm devise (Owl). After electrophoretic analysis, those fractions showing higher purity of putative different isoforms were pooled separately for further characterization.

2.3. Protein determination

Protein concentration was determined by the Bradford method using BSA as standard (Bradford, 1976).

2.4. Enzyme activity, effect of inhibitors, optimal pH and stability

Amidase activity of trypsin was evaluated using BA*p*NA (0.9 mM, final concentration) as the substrate (Erlanger *et al*. 1961) in the assay buffer (0.1 M Tris-HCl, pH 7.0, 100 mM NaCl, and 20 mM $CaCl_2$). Reaction mixtures consisted of 20 µL of enzyme, 190 µL of assay buffer and 40 µL of substrate. Under the same assay conditions, preference for Arg or Lys in P1 position was assessed by using the substrates S2222 (Bz-Ile-Glu(ɣ-OR)-Gly-Arg-*p*NA) and S2251 (H-D-Val-Leu-Lys-*p*NA) (Chromogenix AB, Sweden), both at 0.1 mM of final concentration. In addition, chymotrypsin and elastase activities were evaluated by using 0.1 mM Suc-$(Ala)_2$-Pro-Phe-*p*NA (SA*p*NA) and 1.2 mM Suc-Ala-Ala-Ala-*p*NA, respectively. Bovine enzymes (48 nM bovine chymotrypsin and 25 nM bovine elastase) were included as controls. The *p*NA released was measured kinetically at 405 nm for 10 min at 37°C in a microplate reader ELx808 IU (BioTek) using the software KC4 (BioTek). The molar extinction coefficient of *p*NA was determined empirically under the same assay conditions as 2662 ± 22 M^{-1} cm^{-1} (n=5). One unit of activity was defined as the amount of enzyme that catalyzed the release 1 µmol of *p*NA per minute.

The effect of inhibitors [10 µM soybean trypsin inhibitor (SBTI), 10 mM benzamidine, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 10 µM aprotinin, 1 mM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), 1 mM ethylene-diaminetetraacetic acid (EDTA), 0.1 mM leupectin, 1 mM NEM)] was evaluated by incubating the isolated enzymes with inhibitors for 30 min before measuring trypsin activity using BA*p*NA as describe above. TLCK incubations were done at pH 6 to ensure inhibitor stability and then rose to pH 7.0 before enzyme assays. Percent of inhibition was calculated as: % inhibition= $[1-(V_i/V_0)]$ x100,

where V_i: enzyme activity in the presence of inhibitor and V_0: enzyme activity without inhibitor.

Optimal pH for the different isoforms was determined by evaluating trypsin activity with BA*p*NA at different pH values, using the following buffers: 50 mM glycine-HCl (pH 3 to 4), 50 mM sodium cacodilate (pH 5 to 6), 50 mM Tris-HCl (pH 7 to 9), and 50 mM glycine-NaOH (pH 10 to 11). The stability at acidic pH was evaluated by incubating the enzymes at different pHs for 1 h with and without 20 mM $CaCl_2$, before rinsing the pH to 7.0 for measuring trypsin activity.

2.5. Kinetic characterization of trypsin isoforms

Due to low amount of isolated lobster trypsins, concentration of active isoforms was determined indirectly as follows: first, the concentration of active bovine trypsin was determined by active-site titration with 4-nitrophenyl guanidinobenzoate (NPGB) (Chase and Shaw, 1967). Nominal concentration of bovine trypsin was determined spectrophotometrically at 280 nm using an extinction coefficient (E1%280) of 14.4 (Labouesse and Gervais, 1967) and a molecular weight of 23.3 kDa. Active-site-titrated bovine trypsin was in turn used to obtain the active concentration of SBTI (Knight, 1995; Bieth, 1995) assuming an equimolar binding between the enzyme and the inhibitor, $E_0/K_i^{app} \sim 100$, $[S_o] \leq K_m$ and after the equilibrium between SBTI and bovine trypsin was reached. Active-site-titrated bovine trypsin at 1 µM was incubated for 15 min at 37 ºC with increasing volumes of SBTI (5 µM nominal concentration of stock solution). The proteolytic activity of bovine trypsin was determined using BA*p*NA as substrate as described earlier. The fractional activities (V_i/V_o) were plotted against volumes of inhibitor, and the active concentration of SBTI was obtained from the intercept with the x-axis (Knight, 1995). This is the point of equivalence, where the concentration of enzyme equals the concentration of active inhibitor. The concentration of active inhibitor was calculated as: $[I_o]_{act}=(([I]_{act}*V_m)/V_i))*dil.$, where $[I_o]_{act}$ is the active inhibitor concentration, $[I]_{act}$ is the concentration of inhibitor equivalent to the concentration of the enzyme (x-intercept), V_m is the assay volume (250 µL), V_i is the volume of inhibitor added at the point of equivalence, and dil. is the dilution of the inhibitor starting solution.

Finally, the active concentrations of *P. argus* trypsins were determined by active-site-titration against titrated SBTI. The procedure was essentially as above, but fixing the concentration of titrated SBTI (13µM) while increasing the volumes of the lobster enzymes. The concentrations were obtained from the x-axis intercepts (points of equivalence) of plots between reaction velocities against volumes of enzyme (Williams and Morrison, 1979). The concentrations of active enzymes were calculated as: $[E_o]_{act}=(([E]_{act}*V_m)/V_i))*dil.$, where $[E_o]_{act}$ is the active enzyme concentration, $[E]_{act}$ is the concentration of active enzyme equivalent to the concentration of inhibitor (x-intercept), V_m is the assay volume (250 µL), V_i is the volume of enzyme added at the point of equivalence, and dil. is the dilution of the enzyme starting solution.

An amide substrate (BA*p*NA) was used for determining binding affinities (K_m) and turnover numbers (k_{cat}). Final active enzyme concentration for the assays was set to 0.1 µM. The enzyme activity was assayed with different concentrations of BA*p*NA (in mM): 0.009, 0.018, 0.045, 0.09, 0.18, 0.27, 0.36, 0.45, 0.54, 0.72, and 1.08, with at least four concentrations above and below the expected K_m value. The kinetic parameters V_{max} and K_m were evaluated by adjusting the data to the Michaelis-Menten equation. Values of turnover number (k_{cat}) were calculated from the following equation: $V_{max}/[E] = k_{cat}$, where [E] is the active enzyme concentration. The software GraphPad Prism ver. 5.0. (GraphPad Software, Inc.) was used for analysis of kinetic data.

2.6. Activity of trypsin isoforms toward native protein substrates

The hydrolytic activity of isolated trypsins on native bovine serum albumin (BSA) and myoglobin was evaluated in 0.1 M Tris-HC1, pH 7.0, 100 mM NaCl, 20 mM $CaCl_2$ by means of a Coomassie blue dye binding micro-assay (Sarath *et al.*, 2001) using 0.2 µM of active trypsins and 0.25 mg/mL of substrates in the final reaction mixture. Additionally, active trypsin isoforms (0.2 µM, final concentration) were incubated for 5 h at 37°C with 0.5 mg/mL solutions of native bovine serum albumin (BSA) and myoglobin in 0.1 M Tris-HC1, pH 7.0, 100 mM NaCl, 20 mM $CaCl_2$, and the mixtures were analyzed for degradation on 12% SDS-PAGE (Laemmli, 1970). Untreated protein substrates were used as controls.

3. RESULTS

3.1. Purification of trypsin-like enzymes from digestive glands of lobsters

During the obtaining of the extracts a rapid and strong melanization occurred as assessed visually by their darkening. It is known that high phenoloxidase activity is present in *P. argus* hemocytes (Perdomo-Morales *et al*., 2007) and plasma (Perdomo-Morales *et al*., 2008) due to prophenoloxidase and hemocyanin, respectively, which exert their activity upon proteolytic activation. Digestive glands are highly irrigated organs, and are the site for hemocyanin synthesis, thus we conceive that these proteins were responsible of the melanization observed. Since some intermediates of the melanization reaction (e.g. reactive quinones) may modifying protein structure and function (Whitehead *et al*., 2001), we used diethyldithiocarbamate (Amin *et al*., 2010) to prevent melanization of extracts by inhibiting phenoloxidase activity. A low concentration of inhibitor (0.01 M) was enough for abolishing all phenoloxidase activity (assessed using L-DOPA as substrate, Perdomo-Morales *et al*., 2007) and hence melanization of the extract as judge by visual inspection. Also, preliminary enzyme assays with heat-activated extracts (6h at 37°C) indicated that activation of trypsins occurred quickly during homogenization and that no activation of trypsins in the extract is necessary.

In the present study, five enzymes with trypsin activity were purified from the hepatopancreas of the spiny lobster *P. argus* by gel filtration and anion exchange chromatographies. Trypsins eluted between two major protein peaks in the gel filtration chromatography (Fig. 1), leading to a 16-fold purification of trypsin activity (Table 1). Most contaminating proteins eluted at the void volume (100-150 mL) and along with the lower molecular weight fraction (250-375 mL). Slight amidase activity emerged from the Sephadex G75 column in the void volume (110-140 mL) but not collected as assumed to be due to high molecular weight esterases in the extract (Perera *et al*., 2008), which are also able to hydrolyze BA*p*NA. Enzymes with trypsin activity eluted as a single peak (175-225 mL) and these fractions were collected and pooled together to 50 mL.

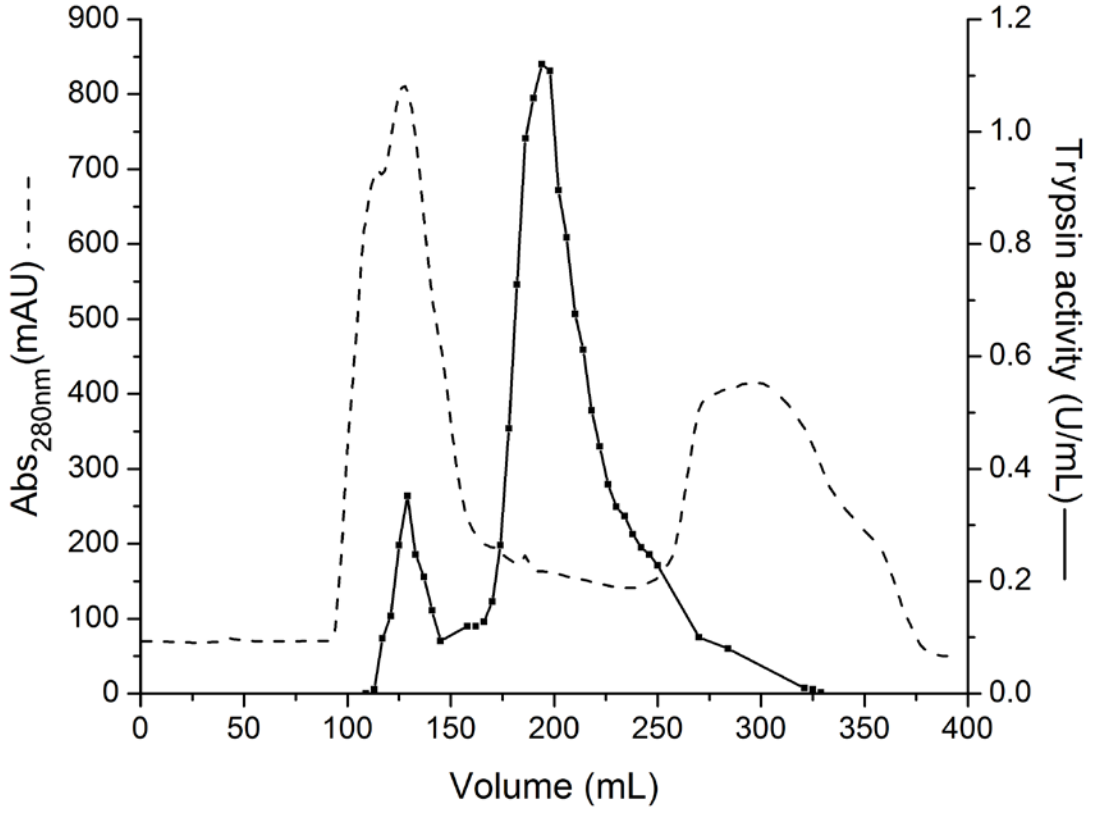

Fig 1. Gel filtration chromatography profile of digestive gland extract of *Panulirus argus*. Six milliliters of clarified crude extract (~70 mg/mL) obtained from digestive glands of 30 individuals *P. argus* were applied to a Sephadex G-75 column (2.6 x 66 cm) previously equilibrated with 10 mM methyl piperacine pH 5.0, 0.1 M NaCl. The flow rate was 0.3 mL/min and protein elution was monitored at 280 nm (dashed line). Fractions of 4 mL were collected and a 20 µL aliquot from each fraction was assayed for trypsin activity using BA*p*NA (solid line). Fractions of higher trypsin activity (175-225 mL) were pooled and used for further purification.

Pooled fractions from gel filtration were further subjected to anion exchange chromatography and four major peaks of trypsin activity were initially observed. Each fraction was analyzed by casein zymography before pooling those containing putative identical enzymes, and as a result five tryptic enzymes were revealed (Fig 2). The enzymes were named based on their elution order as PaT1 to PaT5. The different enzymes were purified from 58- to 351- folds with low yields (0.7-2.6) (Table 1).

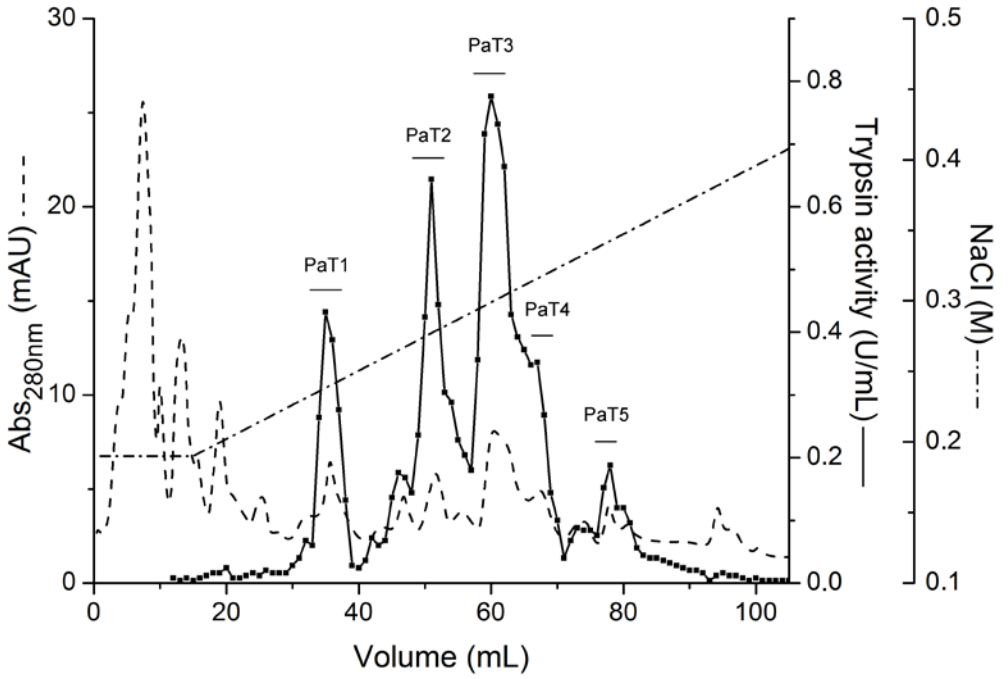

Fig 2. Separation of *Panulirus argus* trypsin isoforms by anion exchange chromatography. The pooled fractions from Sephadex G-75 chromatography (Fig. 1) were applied to a 1 mL MonoQ™ 5/50 column and eluted with 110 mL of a linear gradient from 0.24 to 0.42 M NaCl (dash and dot line) in 10 mM methyl piperacine pH 5.0. Protein elution was monitored at 280 nm (dashed line). Fractions of 1 mL were collected and a 20 µL aliquot from each fraction was assayed for trypsin activity using BA*p*NA (solid line). Lobster trypsins were named according to their elution order as PaT1 to PaT5.

Table 1. Purification table of *Panulirus argus* trypsin-like enzymes.

	Total protein (mg)	Total activity (ΔOD/min)	Specific activity (ΔOD /min/mg)	Yield	Fold
Crude extract	411.23	132,29	0.32	100	1.00
Shephadex G75	6.56	33.80	5.15	25.54	16.02
MonoQ					
PaT1	0.11	3.39	31.93	2.56	99.26
PaT2	0.07	1.54	22.54	1.17	70.07
PaT3	0.12	2.31	18.65	1.75	57.99
PaT4	0.05	0.95	17.77	0.72	55.23
PaT5	0.01	1.03	113.07	0.78	351.48

3.2 Purity and molecular weight of isolated enzymes

A single band under native-PAGE (Fig. 3A) and SDS-PAGE (Fig. 3C) was taken initially as criteria for a homogeneous enzyme. However, the more sensitive casein zymography revealed cross-contamination of some apparently homogeneous enzymes, thus was used as definite homogeneity criteria (Fig. 3B). The molecular weights of the enzymes were PaT1 (36.26 kDa), PaT2 (35.99 kDa), PaT3 (34.94 kDa), PaT4 (35.64 kDa) and PaT5 (36.17 kDa) as estimated by SDS-PAGE (Fig 3C).

3.3. Activity and inhibition studies confirming trypsin identity

Beside their activity on BA*p*NA, purified enzymes were from 3- to 5-times more active toward S-2222 than to S2251 (not shown), indicating that all enzymes showed the trypsin distinctive preference for Arg over Lys in P1 position. No enzyme exhibited activity toward neither the chymotrypsin (SA*p*NA) nor the elastase (Suc-Ala-Ala-Ala-*p*NA) substrates tested, thus all isolated enzymes have restricted trypsin activity.

Additional evidence of trypsin identity of purified enzyme was obtained by assessing the effect of various proteinase inhibitors on their activity. Inhibitors of serine proteases like SBTI and aprotinin strongly inhibited the activity of all enzymes, as did the trypsin inhibitor TLCK (Table 2). Other inhibitors like PMSF (serine) and Benzamidine (trypsin) were also effective inhibitors (Table 2). Although the activities of all enzymes were affected by these inhibitors, some differences were found among the isoenzymes. PaT5 was less inhibited by big (SBTI) and small (aprotinin) proteinaseous inhibitors, while it was the most affected by small synthetic inhibitors (PMSF, TLCK) (Table 2). PaT1 was the most inhibited isoform by leupeptin (Table 2). All trypsin enzymes studied were inhibited to some extent by low concentrations of EDTA. PaT5 was inhibited by EDTA to a greater extent, compared with the other trypsin isoforms (Table 2).

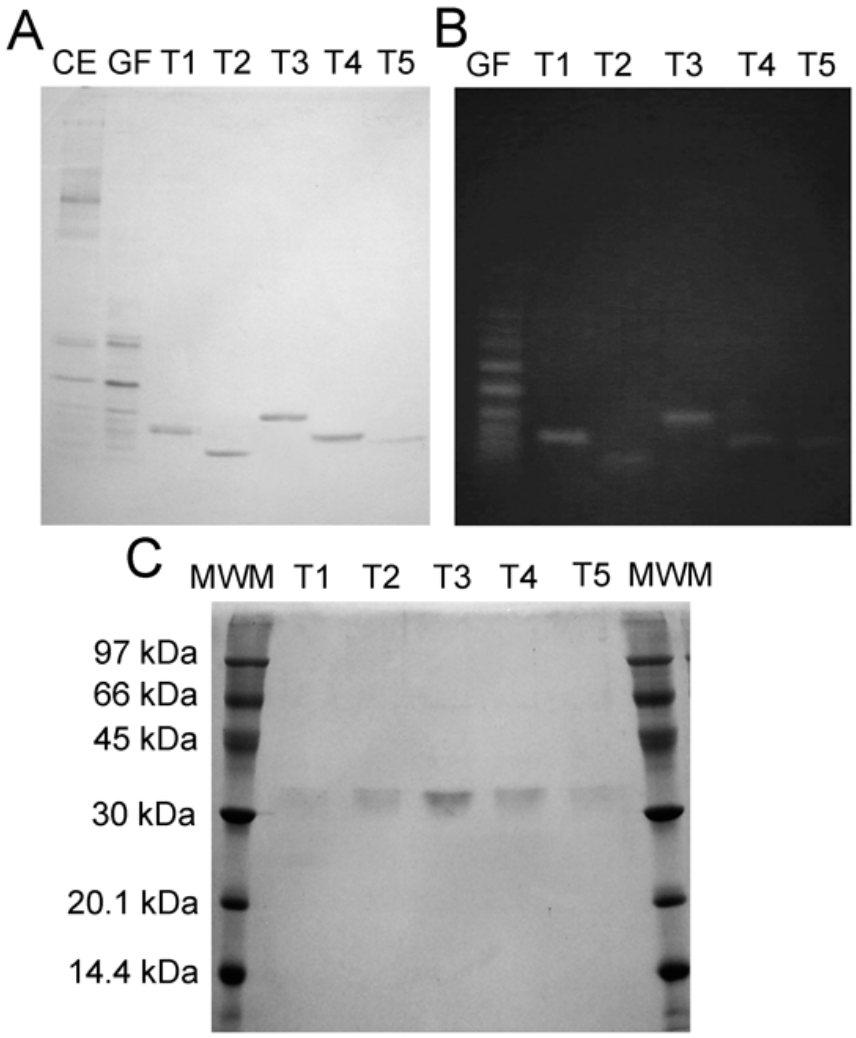

Fig 3. Purity of isolated *Panulirus argus* trypsin isoforms assessed by electrophoresis. A: 14% Native-PAGE of crude extract (CE), gel filtration pool (GF) and isolated *P. argus* trypsins (T1 to T5). B: 14% Casein zymogram of gel filtration pool (GF) and isolated *P. argus* trypsins (T1 to T5). C: 14% SDS-PAGE of the isolated *P. argus* trypsins (T1 to T5). MWM: Molecular weight markers. In the three panels, T1 to T5 correspond to the lobster trypsin isoforms named PaT1 to PaT5.

Table 2. Effect of different inhibitors on the activity of *Panulirus argus* isolated trypsin-like proteases. Enzyme samples were pre-incubated with each inhibitor for 30 min, followed by assays using BA*p*NA as the substrate. Reported values are means of triplicate determinations and are expressed as % of inhibition relative to control incubations in the absence of inhibitor (100%).

Inhibitor	Final concentration	Target protease	% Inhibition				
			PaT1	PaT2	PaT3	PaT4	PaT5
SBTI	10 µM	Serine	99.4	100	100	100	92.2
Aprotinin	10µM	Serine	100	97.2	92.2	96.1	91.3
PMSF	1 mM	Serine	79.2	83.3	76.5	78.4	82.6
Benzamidine	10 mM	Trypsin	90.7	86.1	89.6	88.2	95.3
TLCK	1 mM	Trypsin	97.1	93.1	97. 8	96.1	100
Leupeptin	0.1 mM	Serine/Cysteine	98.7	91.6	93.5	94.1	91.3
NEM	1 mM	Cysteine	9.4	8.3	5.9	7.8	4.3
EDTA	1 mM	Metallo	14.3	18.8	14.6	14.3	37.5

3.4. Effects of pH on activity and stability of trypsin isoforms: effect of calcium ions

The effect of pH on the activity of the five trypsins is presented in Fig. 4. Optimal pH for most isoforms ranges from 7 to 8. However, maximal activity for PaT4 was observed at pH 6-6.5 and it doubles the activity of the other isoforms at pH 5 (Fig 4). The effect of pH on stability of purified trypsins is presented in Fig. 5. In general, three patterns were observed: i) poor stability at acidic pH coupled with strong protective effect of calcium (PaT1, PaT2 and PaT5), ii) relatively high stability at acidic pH with slight protective effect of calcium (PaT3), and iii) relatively high stability at acidic pH with no effect of calcium (PaT4).

3.5. Kinetic parameters of trypsin isoforms

Proper determination of kinetic parameters of isolated trypsins requires the knowledge of its active concentrations, which are usually obtained directly by titration with NPGB (Chase and Shaw, 1967). However, several milligrams of enzyme are required for this kind of spectrophotometric “burst assays” (Chase and Shaw, 1967). Therefore, due to the low amounts of lobster trypsins obtained, their active concentrations were obtained by titrating

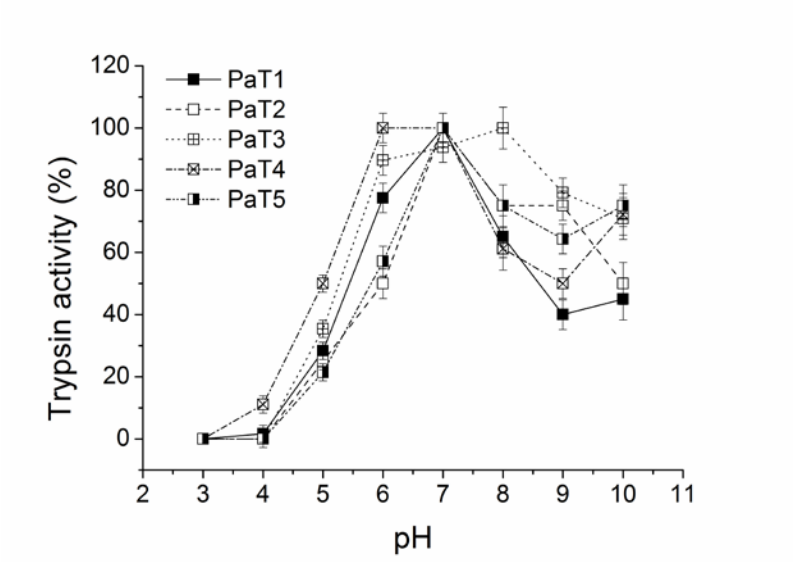

Fig 4. Optimal pH for *Panulirus argus* trypsin isoforms. Points represent mean ± standard deviation of triplicate measurements.

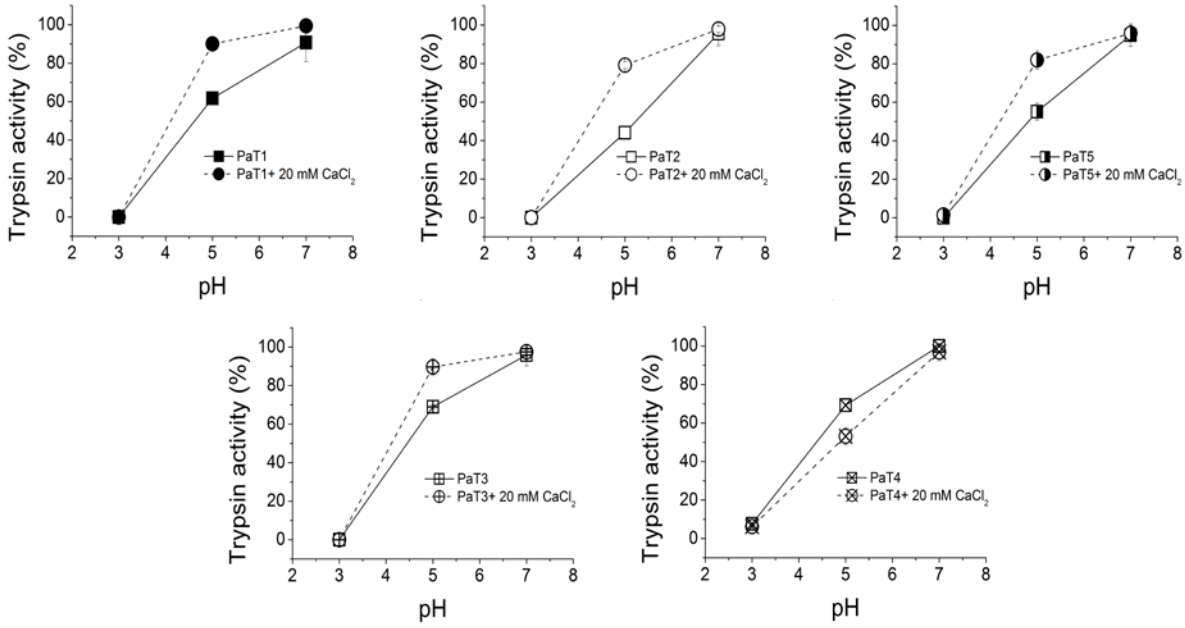

Fig 5. Effect of calcium ions on stability of *Panulirus argus* trypsin isoforms at different pHs. Enzymes were incubated for 1 h at the different pHs and then assayed for trypsin activity at pH 7 using BA*p*NA as substrate. PaT1, PaT2, and PaT5 isoforms present poor stability in acidic media and strong protective effect of calcium on stability. PaT3 isoform presents relatively high stability in acidic media and slightly effect of calcium. PaT4 isoform presents relatively high stability in acidic media and no effect of calcium. Points represent mean ± standard deviation of triplicate measurements.

with a tight binding inhibitor (SBTI) in a three step procedure. First, bovine trypsin was titrated with NPGB and the active concentration obtained was 148.2 ± 0.001 µM (3.45 mg/mL) (n=5) from a solution with 9.37 mg/mL of nominal concentration (36.8% of active enzyme).Then, the proteolytic activity of bovine trypsin at fixed concentration of 1 µM was determined against increasing concentrations of SBTI. The lineal portion of the plot between fractional activities vs. inhibitor volume was fitted to a line, and the intercept with the x-axis was used to calculate the concentration of the active SBTI (Fig. 6). Finally, titration of lobster trypsins was accomplished with known concentration of SBTI (13 µM) (Fig. 6).

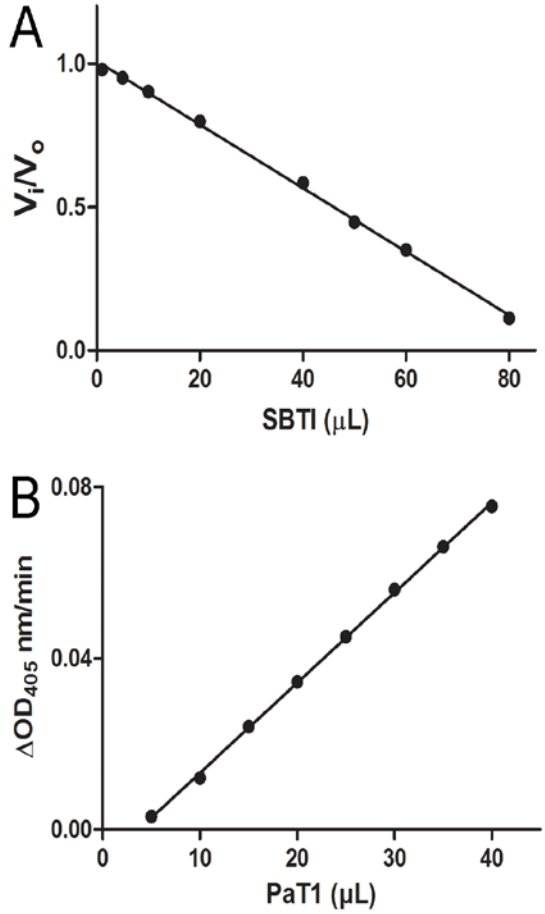

Fig 6. Titration of *Panulirus argus* trypsins. A: Soybean trypsin inhibitor (SBTI) titration with active-site-titrated bovine trypsin [bovine trypsin was previously titrated with 4-nitrophenyl guanidinobenzoate (NPGB) (not shown)]. The protease activity of bovine trypsin at 1 µM was determined using BA*p*NA as substrate after incubation at 37ºC for 15 min with increasing volumes of SBTI. Fractional activity (V_i/V_o) was determined from the ratio between inhibited (V_i) and un-inhibited reactions (V_o). B: Titration of lobster trypsins (only PaT1 is shown) with titrated SBTI. Increasing volumes of lobster trypsins were incubated with a fixed concentration (13 µM) of titrated-SBTI as determined in A. Trypsin activity was determined as above. In both A and B, fitted line were interpolated and active concentrations were calculated from the intercept in the x-axis, which represent the point where active enzyme concentration equals active inhibitor.

The concentrations of active lobster trypsins were: PaT1 (0.87 µM), PaT2 (1.14 µM), PaT3 (2.02 µM), PaT4 (1.92 µM), and PaT5 (0.40 µM). In the presence of all trypsin isoforms, the contribution of each enzyme to total trypsin activity was: PaT1 (14%), PaT2 (18%),

PaT3 (32%), PaT4 (30%), and PaT5 (6%). These values are biased because cross-contaminated fractions were discharged, but provide a preliminary estimation of the relative role of each isoform to overall activity. The *in vivo* contribution of the isoforms would vary among individuals given the phenotypes described (Perera *et al*., 2010b). For example, individuals with phenotype B (Perera *et al*., 2010b) lack PaT2 and as a result the contribution of PaT4 could be greater than 37%.

Using BA*p*NA as substrate, the kinetic parameters were determined for all isoforms and are shown in Table 3. No mayor differences were found in K_m among PaT1 to PaT3, while PaT4 and PaT5 only differed slightly (Table 3). The low turnover number (k_{cat}) of PaT4 resulted in the lowest catalytic efficiency (k_{cat}/K_m) (Table 3).

3.6. Activity of trypsin isoforms toward native protein substrates

The activity of isolated trypsins on native protein substrates was evaluated using two model proteins (BSA and myoglobin). First, the hydrolytic activity of isolated enzymes (0.2 µM, final active concentration) toward these protein substrates was evaluated by means of a Coomassie blue dye binding micro-assay (Sarath *et al*., 2001). In this method, Coomassie Brilliant Blue tends to bind to larger polypeptides and not to smaller polypeptides released by proteolytic activity, thus the fall in optical density is taken as indicator of digestion. Trypsin isoforms differed in activity toward BSA and myoglobin, being PaT1 the most reactive, while PaT4 was the less efficient (Fig. 7).

Table 3. Kinetic parameters for hydrolysis of BA*p*NA by *Panulirus argus* trypsins, and of trypsins from other taxa. Values of K_m and k_{cat} are means ± standard errors of triplicate assays. The low catalytic efficiency of PaT4 is underlined.

		K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Reference
Crustaceans	Spiny Lobster (*Panulirus argus*)				
	PaT1	0.12± 0.010	0.57±0.052	4.82	This work (37°C)
	PaT2	0.12± 0.014	0.62±0.074	5.02	
	PaT3	0.11± 0.006	0.57±0.041	4.98	
	PaT4	0.08± 0.010	0.16±0.042	<u>2.02</u>	
	PaT5	0.19± 0.015	1.15±0.087	6.04	
	Slipper Lobster (*Thenus orientalis*)				
		0.093	0.91	9.78	Johnston *et al*., 1995 (37°C)
	Krill (*Euphausia pacifica*)				
	TRY-EP	0.09	0.84	9.5	Wu *et al*. 2008 (37°C)
Insects	Mosquito (*Aedes aegypti*)				
	AaSPVI	0.01	0.16	13.5	Rascón *et al*., 2011(24 °C)
	AaSPVII	0.02	0.35	18.5	
	AaET	0.06	2.03	32.3	
Fishes	Chum Salmon (*Oncorhynchus keta*)				
	ST-1	0.07	7.70	110	Toyota *et al*., 2007 (35°C)
	ST-2	0.05	6.74	135	
	ST-3	0.04	3.86	96.5	
	Anchovy (*Engraulis japonicus*)				
	aT-I	0.04	1.94	52	Ahsan and Watabe, 2001 (25°C)
	aT-II	0.03	3.70	112	
	Anchovy (*Engraulis encrasicholus*)				
	Trypsin A	0.83	1.55	1.86	Martínez *et al*., 1988 (25 °C)
	Trypsin B	0.66	3.20	4.84	
Mammals	Bovine				
	BvT	1.0	1.30	1.20	Rascón *et al*., 2011(24 °C)

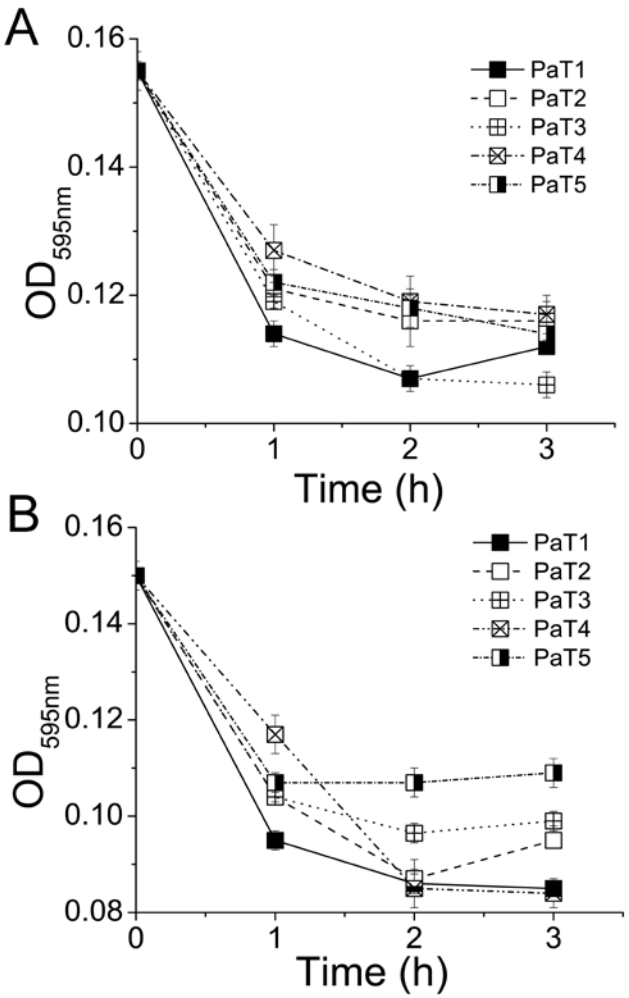

Fig 7. Digestion of native bovine serum albumin (A) and myoglobin (B) by *Panulirus argus* trypsin isoforms followed by the Coomassie blue dye binding micro-assay (Sarath *et al*., 2001). Coomassie Brilliant Blue bind to larger polypeptides and not to smaller polypeptides released by proteolytic activity, thus the fall in optical density is an indicator of digestion rate. Differences were found in the digestion efficiency among isoforms being PaT1 the most reactive toward both protein substrates, while PaT4 was the less efficient. Differences were more evident at 1 h of digestion. Points represent mean ± standard deviation of triplicate measurements.

Using the same substrates, the cleavage ability of isolated trypsins was assessed by SDS-PAGE analysis of the digestion products. Due to the low amount of *P. argus* trypsins obtained, just partial digestions of BSA and myoglobin were observed as evidenced by low concentration (stain) of digestion products. In spite of this technical limitation, some differences among isoforms could be noticed. Some digestion products were observed in all trypsin digests (i.e. Fig 8, lane 5, white arrows) but PaT1, PaT2 and PaT5 hydrolyzed more intensively the secondary band of BSA (just below BSA in the control lane); in lanes corresponding to PaT3 and PaT4 digests of BSA, this band appeared similar to the one in the control lane (Fig 8, lane 5, upper black arrow), indicating poor degradation. Other differences are highlighted in Fig 8.

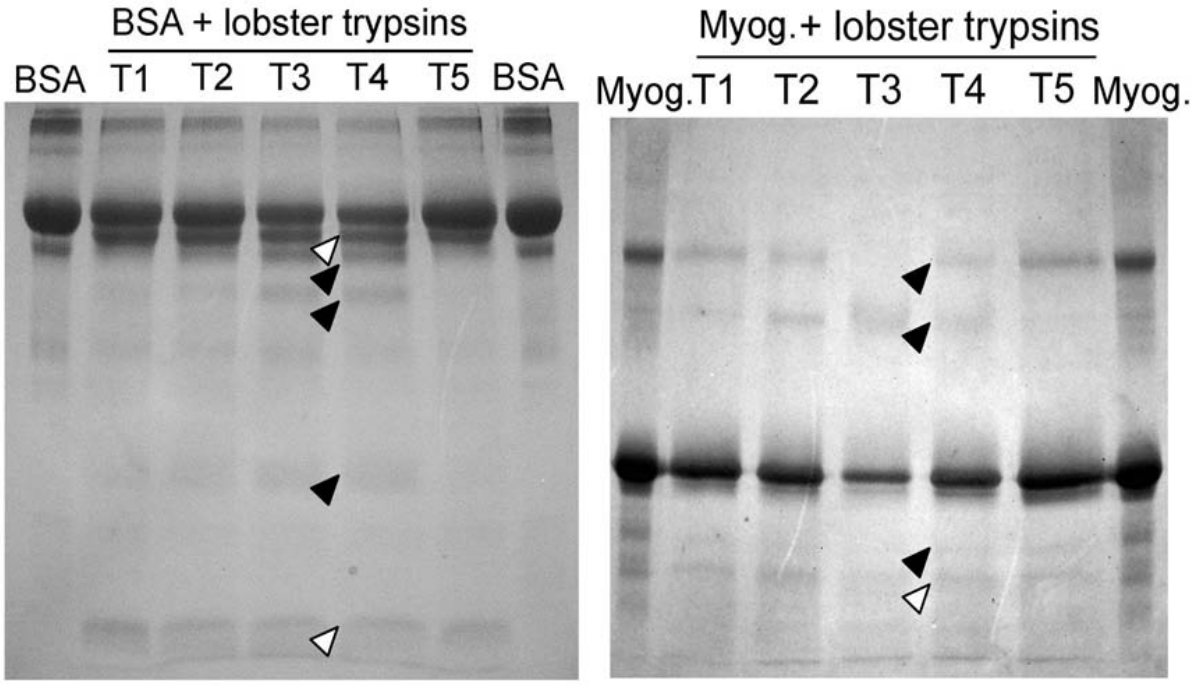

Fig 8. 12% SDS-PAGE showing differences in cleavage ability among *Panulirus argus* trypsin isoforms in digesting native bovine serum albumin (BSA) and myoglobin (Myog.). White arrow heads indicate protein bands common to all digests. Black head arrows indicate differences in peptide product presence or concentration in at least one isoform digest. This experiment was repeated twice with identical results.

4. DISCUSSION

4.1. Purification and biochemical features of lobster trypsin isoforms

In this work, five trypsin-like enzymes were purified to homogeneity by a combination of size exclusion (Fig. 1) and anion exchange chromatography (Fig. 2), as judged by native-PAGE (Fig. 3A), casein zymography (Fig. 3B) and SDS-PAGE (Fig. 3C). The different isoenzymes were purified from 58- to 351- folds (Table 1). The attainment of highly purified enzymes for accurate biochemical characterization was only possible by the collection of just a few fractions from each peak after being analyzed by casein zymography, leading to a considerable loss of yield down to 0.7-2.6 (Table 1). This procedure has been claimed before as essential for the separation of highly similar trypsins (Ahsan and Watabe, 2001). Other factors affecting yield are the high esterase activity in the crude extract (Perera *et al*., 2008) and some inactivation of trypsins at working pH (see below), but the latter could not be avoided because a higher pH precluded isoform separation during anion exchange in our experimental conditions.

Crustacean serine proteases (Brachyurins, EC 3.4.21.32) exhibit a wide variety of primary specificities (Barret *et al*., 1998). Type Ia brachyurins possess trypsin-, chymotrypsin- and elastase-like activities. Type Ib enzymes have reduced activity towards Arg substrates, while retaining the other features of type Ia substrate specificity. Type II brachyurins has strict trypsin-like specificity. Due to this variation in specificity, the trypsin identity of purified proteases was assessed by using different substrates. No activity was detected toward chymotrypsin and elastase substrates, thus all isolated enzymes have restricted trypsin activity (type II brachyurins, Barret *et al*., 1998) and hydrolyzed native proteinaseous substrates (Figs. 7 and 8). In addition, all enzymes showed the trypsin distinctive preference for Arg over Lys in P1 position, being from 3 to 5 times more active toward S-2222 than to S2251. Although these substrates also differed in position other than P1, our results compare well with those reported for other trypsins. The preference of trypsins for Arg over Lys is known to be 2- to 10-fold (Craik *et al*., 1985). Among invertebrates, the exceptions are trypsins from Lepidoptera with no preference between Arg and Lys at that position (Lopes *et al*., 2006).

Additional evidences for the trypsin identity of isolated enzymes were obtained by the use of inhibitors, since all enzymes were affected by serine-proteinase and trypsin inhibitors (Table 2). However, isoforms slightly differed in their interaction with inhibitors. PaT5 was less inhibited by big (SBTI) and small (aprotinin) proteinaseous inhibitors, while it was the most affected by small synthetic inhibitors (PMSF, TLCK) (Table 2), suggesting different structural features. Differences in susceptibility to SBTI and aprotinin suggest variations in enzyme-inhibitor interactions in sites far from S1. Also, since TLCK and PMSF react with the active site His and Ser residues, respectively, the strongest effect of these inhibitors on PaT5 could be related with the higher catalytic efficiency of this form of the enzyme (see below). PaT1 seems to better accommodate the argininal residue at the terminal carbon of leupeptin (Table 2), respect to the other trypsins.

The estimated molecular weights of trypsin-like enzymes in *P. argus* vary from 35 to 36 kDa (Fig 3C), similar to those reported for other crustaceans [24-31 kDa in *Procambarus clarkii* (Kim *et al*., 1992), 35 kDa in *Thenus orientalis* (Johnston *et al*., 1995), 33 kDa in *Euphausia pacifica* (Wu *et al*., 2008)]. These sizes are above our previous estimations (25 kDa) by bioinformatics analysis of lobster cDNAs (Perera *et al*., 2010a) and differences may be explained by errors inherent to both methods (bioinformatics and SDS-PAGE) and by post-translational modifications like glycosylation. Different to vertebrate trypsins, glycosylation has been recognized before in crustacean trypsins (Johnston *et al*., 1995; Sainz *et al*., 2004).

All trypsin-like enzymes studied were inhibited to some extent by low concentrations of EDTA. PaT5 was the most affected by EDTA (Table 2) supporting that this enzyme holds distinct structural features. The fact that crustacean trypsins have a calcium binding loop (Fodor *et al*., 2005; Perera *et al*., 2010a) as vertebrate trypsins (Perona and Craik, 1995; Hedstrom, 1996), disagree with reports on independence of calcium (Muhlia-Almazán *et al*., 2008). It has been proposed that crab trypsins have no accessible autolysis sites in the N-terminal domain, thus not requiring calcium coordination for stabilization (Hehemann *et al*., 2008). While this hypothesis is suitable for explaining the autoproteolytic stability of crustacean trypsins, calcium ions appear to still have an effect on the activity and stability in acidic media. We found in *P. argus* trypsins some inhibition in the presence of EDTA

(Table 2) and a clear effect of 20 mM $CaCl_2$ on stability in acidic media for all isoforms, except PaT4 (Fig. 5). Interestingly, while calcium ions have no effect on the stability of PaT4 at acidic pH (Fig. 5), under optimal conditions this enzyme requires calcium coordination for maximal activity as much as the others isoforms. EDTA inhibition of PaT4 was similar to those obtained for PaT1 to PaT3 (Table 2). Likewise, calcium ions affected the activity of the three isotrypsins of the shrimp *Litopenaeus vannamei* (Sainz *et al*., 2004).

Optimal pH for most isoforms ranges from 7 to 8, as typical for trypsins, and activity remains high in a broad alkaline range (Fig 4). This behavior has been observed before for *P. argus* trypsins on crude extracts (Perera *et al*., 2008) and on other purified crustaceans trypsins (Sainz *et al*., 2004; Wu *et al*., 2008). In contrast, a sudden reduction of activity occurs below pH 6, probably due to some functional impairment of the catalytic histidine (His). The pKa value of the catalytic His in the slipper lobster (*T. orientalis*) was found to be 5.7 in the free enzyme and 4.7 in the Michaelis complex (Johnston *et al*., 1995). Surprisingly, maximal activity for PaT4 occurs at pH 6-6.5, and it doubles the activity of the other isoforms at pH 5 (Fig 4), which match with its relatively high stability in acidic media irrespective of the presence of calcium (Fig 5).

Kinetic constants obtained for trypsin-like enzymes in *P. argus* compare well with those reported for other invertebrate trypsins (Table 3). No mayor differences were found in K_m among PaT1 to PaT3, while PaT4 and PaT5 only differed slightly. Positively charged P1 arginine side-chain of the amide substrate may orientate equally well into the binding cleft of the different isoforms. Among all isoforms, PaT4 showed the lowest turnover number (k_{cat}) resulting in the lowest catalytic efficiency (k_{cat}/K_m), whereas PaT5 presented the highest k_{cat} leading to the highest efficiency. PaT5 is a minor component of lobster trypsins and thus, its true contribution to protein digestion is not clear. From these results, it can be postulated that differences in catalytic efficiencies among lobster trypsin isoforms rely on some disparity in k_{cat}. This kinetic parameter reflects multiple chemical steps subsequent to substrate binding (Copeland, 2000). Although, it is known that the acylation step is rate determining for simple amide substrates (Brouwer and Kirsch, 1982; Ayala and Di Cera, 2000) like BA*p*NA, deacylation can be rate-limiting for peptide substrates of lobster

trypsins as occurred in their vertebrate counterparts (Hedstrom *et al*., 1992). In Chum Salmon, both K_m and k_{cat} differed among trypsin isoforms but major differences were found also in k_{cat} (Toyota *et al*., 2007), and the same is true in other fishes (Martínez *et al*., 1988; Ahsan and Watabe, 2001), and in the mosquito *Aedes aegypti* (Rascón *et al*., 2011) (Table 3).

Cold adapted crustaceans have trypsins with higher catalytic efficiency than those in the tropical lobster *P. argus* (Table 3). The structural features of trypsins and other enzymes from cold and temperate organisms determining high catalytic efficiencies (e.g. more flexible three-dimensional structure) have been discussed extensively (Adekoya *et al*., 2006; Papaleo *et al*., 2008; and reference therein). An increased structural flexibility is thought to reduce the activation energy necessary for generating reaction intermediates, resulting in a more efficient substrate turnover. However, difference in catalytic efficiency between bovine and salmon (cold adapted) trypsins is thought to result from strong differences in K_m (Sekizaki *et al*., 2000) probably due to variation in the electrostatic potential of the S1-binding pocket (Gorfe *et al*., 2000). The comparison of the physiological efficiencies among different crustaceans and fish trypsins shows that fish trypsins trend to be more efficient than their crustacean counterparts, even if the comparison is done between cold adapted species like krill and salmon (Table 3). Still, trypsins from tropical *P. argus* are more efficient than the bovine counterpart (Table 3).

Invertebrates exhibit a high number of isoforms for digestive trypsins. While lower vertebrates like fishes still have a varied number of isoforms, mammals are known to have few of them. One cationic and one anionic trypsin are known in humans (Figarella *et al*., 1975), bovines (Puigserver and Desnuelle, 1971), porcine (Voytek and Gjessing, 1971) and dogs (Ohlsson and Tegner, 1973). Although differences in trypsin isoforms expression and activity have been recently found in *Daphnia magna* in response to protease inhibitors in diet (Schwarzenberger *et al*., 2010), experimental evidence on the physiological role of multiple trypsins in crustaceans is scarce.

4.2. Lobster trypsin isoforms and digestion efficiency

The isolated trypsins were related to the ones composing the main three trypsin phenotypes in *P. argus* (Perera *et al*., 2010b), according to their electrophoretic mobility in native-PAGE and casein zymography, in an attempt to explain previous results on digestion efficiency. Individuals with phenotype A (PaT1/PaT2/PaT4, isoforms named as in this work) and individuals with phenotype C (PaT1/PaT2) exhibit higher efficiency digesting several protein sources than lobsters with phenotype B (PaT1/PaT4) (Perera *et al*., 2010b). In that work, we hypothesized that the isoform of middle electrophoretic mobility (here named PaT4) is less efficient during digestion and here we demonstrate that this isoenzyme is kinetically the less efficient (lowest k_{cat}/K_m). This is the first attempt to relate the kinetic properties of crustacean trypsins with the efficiency of the physiological process they are involved.

However, extrapolation of kinetic results to *in vivo* conditions must be made with extreme caution. For most enzymes, the *in vivo* concentrations of substrates are typically 0.1-1.0 K_m (Copeland, 2000) and hence, kinetic constants usually give little information on the *in vivo* performance. Yet, digestive trypsins work *in vivo* at a very high substrate concentration ([S] >> K_m) and thus, comparison of k_{cat} (maximal velocity under saturating substrate concentration) in this case could provide a raw approximation to physiological rates.

The other major constraint for extrapolation of our kinetic results to *in vivo* conditions comes from the fact that proteases act over proteins those are actually collections of substrates with different kinetics (Sarath *et al*., 2001). For this reason, we further compared the digestion of two model proteins (BSA and myoglobin) by the different isoforms (Fig. 7). PaT1 was the most reactive isoform toward both protein substrates, while protein digestion by PaT4 proceeded at the slowest rate. The slight differences observed in cleavage ability among isoforms (Fig. 8) also point to the poor digestion efficiency of PaT4. Hence, by the use of synthetic (BA*p*NA) and protein substrates, the present study demonstrate, for the first time, the presumption that the lobster trypsin phenotypic variation in protein digestion (Perera *et al*., 2010b) relies on the biochemical properties of the isoforms present, and especially in the contribution of the less efficient isoform (PaT4) to

the overall trypsin activity. Although trypsins are by far the main proteases in crustaceans, it should be noted that protein digestion does not relies solely on trypsin enzymes and thus, other proteases could underlie the digestion efficiency variations in lobster.

On the other hand, while K_m, k_{cat} and k_{cat}/K_m of PaT1 barely differed from those of PaT2 and PaT3 (Table 3), PaT1 was the most efficient digesting the native protein substrates (Fig. 7). Thus, results with proteinaceous substrates complemented those of the kinetic characterization by providing a better approximation to the physiological performance of digestive trypsins. Since PaT1 has been reported to be present in all individuals *P. argus* (Perera *et al*., 2008; Perera *et al*., 2010b), this finding rises interesting questions on trypsin evolution in crustaceans. From the evolutionary point of view, it is worth to note that the same isoenzyme patterns are present in other crustaceans like shrimps (Sainz *et al*., 2004).

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Capítulo 8

Discusión general

La fisiología digestiva de las langostas espinosas ha sido objeto de pocos estudios, existiendo muchos aspectos de la bioquímica y fisiología digestiva de estos crustáceos que se desconocen. Además, se continúan asumiendo conocimientos obtenidos en otros grupos como los camarones peneidos y langostas del género *Homarus* como si fueran ciertos en *Panulirus argus*. Lamentablemente, los esfuerzos realizados durante las últimas dos décadas en el desarrollo de dietas formuladas para la engorda de langostas espinosas en cautiverio (ver revisión de Williams, 2007) no han estado acompañados de estudios encaminados a la comprensión de cómo las langostas espinosas digieren las diferentes dietas evaluadas.

Las langostas espinosas son carnívoras durante todo su ciclo de vida. En la actualidad se desconoce la alimentación natural de las larvas filosomas pues los estudios de contenido estomacal son difíciles de realizar por su condición de oceánicas y su pequeño tamaño. No obstante, las filosomas de muchas especies sólo han podido ser cultivadas utilizando, entre otros alimentos vivos, nauplios de *Artemia*, gónadas de mejillón y larvas de peces (Kittaka, 1997); por tanto se asume que desde las primeras etapas larvales las filosomas se alimentan de organismos del zooplancton. Los estudios de contenido estomacal de post-puérulos han revelado que las principales entidades en la alimentación de esta etapa del ciclo de vida son los copépodos, anfípodos, isópodos, holoturias, foraminíferos y esponjas (Lalana y Ortiz, 1991). Por su parte, las investigaciones referentes a la alimentación de juveniles y adultos mostraron que las principales entidades en la dieta de *P. argus* son una amplia variedad de moluscos y crustáceos (Colinas y Briones, 1990; Herrera *et al*., 1991; Cox *et al*., 1997). Teniendo en cuenta los hábitos alimenticios de las langostas espinosas anteriormente expuestos, es de suponer que el sistema digestivo de estos crustáceos esté equipado con una batería enzimática que le permita la eficiente digestión de presas con una variada composición bioquímica y un alto contenido proteico.

Las enzimas digestivas presentes en las larvas filosomas han sido estudiadas en algunas especies, mostrando el predominio de peptidasas como la tripsina (Johnston *et al*., 2004a,b). La enzimas con actividad tripsina son las proteasas más importantes en puérulos de *Jasus edwardsii* (Johnston, 2003), post-puérulos y juveniles de *J. edwardsii* (Johnston, 2003) y *P. argus* (Capítulo 2 y 3), así como en adultos de *Panulirus japonicus* (Galgani y Nagayama, 1987), *J. edwardsii* (Johnston, 2003), *Panulirus interruptus* (Celis-Guerrero *et*

al., 2004) y *P. argus* (Capítulo 2 y 3). Nuestros resultados en *P. argus* obtenidos mediante el uso de inhibidores confirman que la mayor parte de la proteólisis digestiva recae en serino-proteasas como la tripsina y quimotripsina, y en las metalo-proteasas, presumiblemente exopeptidasas (Capítulo 2).

En la presente Tesis Doctoral se caracterizaron por primera vez las principales enzimas digestivas (tripsinas, quimotripsinas, esterasas, lipasas y amilasas) de *P. argus*. Los resultados mostraron que *P. argus* posee una amplia variedad de enzimas digestivas tanto en la glándula digestiva como en el jugo gástrico. Estas enzimas fueron caracterizadas a partir de extractos crudos y mostraron características similares (pH óptimo, temperatura óptima, fuerza iónica óptima, estabilidad y en el caso de las proteasas, susceptibilidad a inhibidores) a enzimas del mismo tipo en otros crustáceos (Capítulo 2). Con estos resultados se muestran por primera vez las bases bioquímicas de la digestión en *P. argus*. Los estudios precedentes sobre enzimas digestivas en otras especies de langostas espinosas sólo contemplaron un tipo de enzimas (Galgani y Nagayama, 1987; Celis-Guerrero *et al*., 2004) o sólo analizaron las variaciones en la actividad enzimática (Johnston, 2003; Simon, 2009a); por este motivo nuestros resultados convierten a *P. argus* en la especie de langosta espinosa con más información disponible respecto a sus enzimas digestivas.

No obstante, algunos aspectos de la bioquímica digestiva de *P. argus* necesitan estudios futuros. Así, deberíamos continuar con la identificación de varias serino-proteasas que no pudieron ser clasificadas como tripsinas o quimotripsinas y probablemente pertenezcan al grupo de serino-proteasas colagenolíticas, así como determinar su contribución a la digestión de proteínas en esta especie. Las serino-proteasas colagenolíticas fueron descritas con anterioridad en la langosta espinosa *P. japonicus* (Iida *et al*., 1991) y han sido objeto de varios estudios en otros crustáceos como el krill (Hellgren *et al*., 1991) y varias especies de cangrejos (Tsu *et al*., 1997; Rudenskaya *et al*., 2000). También, la identificación de las enzimas con actividad lipasa en *P. argus* (Capítulos 2 y 3) ha estado limitada por problemas técnicos, tales como el uso de sustratos no específicos y las dificultades en revelar la actividad de estas enzimas en geles de poliacrilamida. Además, la importancia de las enzimas tipo tripsina en la activación de otras proteasas en el sistema digestivo de las langostas también necesita estudios futuros.

La duplicación de genes ha jugado un papel importante en la evolución (Ohta, 1991). Los genes duplicados inicialmente son idénticos, y pueden permanecer de esa manera permitiendo la síntesis de una mayor cantidad del producto génico, o bien algunas copias pueden sufrir mutaciones y convertirse en pseudogenes. Además, las secuencias de algunas copias pueden divergir dando lugar a proteínas con diferencias en su estructura y función (Li y Graur, 1991). En diversos grupos taxonómicos los genes de enzimas digestivas se organizan en familias génicas que surgieron por sucesivos eventos de duplicación (por ejemplo: 6 genes para tripsinas en humanos incluyendo un pseudogen, 8 genes para tripsinas en *Drosophila*, etc.). La organización de los genes de tripsinas en familias multigénicas ha sido estudiada con anterioridad en crustáceos (Klein *et al*., 1998), aunque no existía información en *P. argus*.

A nivel de proteína, la presencia de isoformas para las enzimas tipo tripsina y quimotripsina ha sido descrita anteriormente para langostas espinosas en la especie *P. interruptus* (Celis-Gerrero *et al*., 2004) y en otros crustáceos como el camarón *Litopenaeus vannamei* (Sainz *et al*., 2004). En correspondencia con estos estudios, en *P. argus* se encontraron tres isoformas fundamentales con actividad tipo tripsina y tres enzimas tipo quimotripsina (Capítulo 2). Adicionalmente, se demostró la presencia de una gran variedad de enzimas con actividad esterasa, así como de dos isoformas fundamentales para la amilasa.

En algunas especies de crustáceos se ha reportado la presencia de hasta 6 enzimas con actividad amilasa, aunque en la langosta espinosa *P. interruptus* sólo se pudo detectar una isoforma (Van Wormhoudt *et al*., 1995). Existen evidencias de que las langostas espinosas presentan una baja capacidad para digerir y aprovechar los carbohidratos de la dieta (Johnston *et al*., 2003; Ward *et al*., 2003) y que no los utilizan de manera significativa en el metabolismo energético (Perera *et al*., 2005). Aunque existen algunos estudios sobre digestibilidad y utilización de carbohidratos en langostas espinosas (Radford *et al*., 2005; 2007; Simon 2009b,c), los datos aportados en el Capítulo 2 de la presente Tesis Doctoral (alta actividad amilasa en el jugo gástrico de *P. argus*, presencia de isoformas y estrecho rango de pH en el cual estas enzimas son activas), sugieren que la comprensión de la digestión de carbohidratos en langostas espinosas aun está en un estado muy incipiente, y

que son necesarios futuros estudios sobre este aspecto de la fisiología digestiva de *P. argus*.

Las principales enzimas digestivas de *P. argus* mostraron ser polimorficas, detectándose este fenómeno para las enzimas tipo tripsina, las esterasas y las amilasas (Capítulo 2). En el caso de las tripsinas se detectaron tres patrones electroforéticos (fenotipos) fundamentales, tres fenotipos fundamentales para las amilasas y una amplia gama de fenotipos para las enzimas con actividad esterasa. Debemos destacar que de 13 proteasas encontradas en el hepatopáncreas y jugo gástrico de *P. argus*, sólo las enzimas tipo tripsina mostraron ser polimórficas, aunque la cantidad de enzimas con actividad exopeptidasas no pudo ser estudiada en geles de poliacrilamida. Este resultado concuerda con el papel central que juegan las tripsinas en la digestión de proteínas en crustáceos y genera interrogantes sobre la relevancia fisiológica del polimorfismo en estas enzimas. ¿Son todos los fenotipos igual de eficientes en la digestión proteica? ¿Hay correspondencia entre el fenotipo más eficiente y el más abundante en la población estudiada? Estos aspectos se abordaron en el Capítulo 4.

En *P. argus* las tres isoformas principales de tripsina detectadas determinan tres fenotipos. La mayor parte de los individuos presentan las tres isoformas, pero un número variable de individuos carecen de alguna de las dos isoformas de mayor movilidad electroforética (Capítulo 2 y 4). Esta distribución de isoformas en *P. argus* es similar a la descrita para las mismas enzimas en el camarón *L. vannamei* (Sainz *et al*., 2004; 2005).

A diferencia de los neutralistas, que tratan de explicar el polimorfismo por la acumulación de mutaciones neutrales producto de la interacción entre las mutaciones y las fluctuaciones de los tamaños poblacionales (deriva genética), la mayoría de los especialistas coinciden en que las presiones selectivas son la fuerza motora de la evolución a nivel molecular y de fenotipo, aunque no niegan el efecto de la deriva (Kirpichnikov y Muske, 1980). En muchos casos se ha podido demostrar diferencias en la actividad, resistencia a inhibidores, estabilidad, etc. de isoformas. De las enzimas digestivas, un caso bien conocido es el de las dos amilasas de *Drosophila*, que difieren en la habilidad de hidrolizar el almidón, seleccionándose la forma más activa cuando las larvas se cultivan en un medio rico en almidón (Kirpichnikov y Muske, 1980). Sin embargo, aunque se han descrito diferencias

cinéticas entre las diferentes isoformas de tripsina del camarón *L. vannamei* (Sainz *et al*., 2004), el efecto de estas diferencias en la digestión de proteínas no ha sido estudiado en ninguna especie de este grupo. Recientemente se ha descrito que las diferencias en susceptibilidad a inhibidores entre isoformas de tripsina en *Daphnia* pueden dar lugar a diferencias en la eficiencia digestiva ante la presencia de inhibidores en la dieta (Schwarzenberger *et al*., 2010).

La ventaja digestiva de presentar determinadas isoformas de tripsina sólo ha sido estudiada con profundidad en salmónidos (ver revisión de Rungruangsak-Torrissen y Male, 2000), aunque también se ha sugerido para camarones peneidos (Sainz y Córdova-Murueta, 2009) teniendo en cuenta las características cinéticas de las diferentes isoformas (Sainz *et al*., 2004). De esta forma, el presente estudio es el primero en demostrar (*in vitro*) que los fenotipos de tripsina en un crustáceo difieren en la eficiencia con que digieren diferentes fuentes de proteína (Capítulo 4). Las langostas que presentan las tres principales isoformas de tripsina mostraron ser las más eficientes y este resultado no está relacionado con una mayor actividad total pues la misma se homogenizó entre los fenotipos. Por su parte, los fenotipos conformados por dos isoformas solamente también difieren en la eficiencia *in vitro,* lo cual corrobora que la acción coordinada entre diferentes isoformas resulta, al menos para algunas fuentes de proteína, en diferentes eficiencias digestivas.

La evaluación del crecimiento en langostas con diferente fenotipo pudiera corroborar los resultados *in vitro* de la presente Tesis Doctoral, y a su vez aportar información valiosa desde el punto de vista teórico y práctico. En primer lugar, estos ensayos proporcionarían el escenario ideal para profundizar en los efectos de las diferencias entre fenotipos de las enzimas tipo tripsina. Por ejemplo, la cuantificación de aminoácidos libres (o solamente lisina) en la hemolinfa durante la digestión pudiera detectar *in vivo* diferencias pequeñas en la eficiencia digestiva que pueden tener un impacto grande en el crecimiento (Rungruangsak-Torrissen y Male, 2000). En segundo lugar, de demostrarse diferencias en el crecimiento, se estaría estableciendo el primer carácter susceptible a selección en langostas espinosas con fines acuícolas. Actualmente, por la imposibilidad de producir grandes cantidades de postlarvas a través de la reproducción controlada y el cultivo larval, los estudios en este sentido son nulos. Sin embargo, los avances recientes (Barnard *et al*., 2011; Goldstein y Nelson, 2011) son prometedores y abren la posibilidad de manejar la

reproducción de algunas especies para obtener una descendencia con características deseables.

Los resultados enzimáticos obtenidos en esta Tesis Doctoral, respecto a la diversidad de isoformas y polimorfismo para las enzimas tipo tripsina (Capítulo 2, 3 y 4), se corroboraron a través de estudios moleculares (Capítulo 5). Las tripsinas de *P. argus* presentan una alta homología con las tripsinas de otros crustáceos y comparten las características estructurales de los demás miembros del Clan PA, familia S1 (http://www.merops.co.uk). La mayoría de las enzimas tipo tripsinas encontradas se corresponden con típicas tripsinas (braquiurinas tipo II); sin embargo, se detectó una enzima tipo tripsina con características estructurales muy particulares, que hace suponer una amplia especificidad para esta enzima. El análisis de los modelos moleculares obtenidos para las tripsinas de *P. argus* mostró que las mismas presentan una estructura similar a las de otros crustáceos como la del langostino (Fodor *et al.*, 2005), aunque difieren en algunos elementos estructurales superficiales que pudieran determinar diferencias en su interacción con sustratos e inhibidores (Capítulo 5). Además, los estudios moleculares realizados en *P. argus* (Capítulo 5) pusieron de manifiesto que la riqueza de isoformas de tripsina es mayor que la inferida a partir del uso de electroforesis en geles de poliacrilamida (Capítulo 2). La presencia de diferentes tripsinas en *P. argus* proporciona múltiples opciones para la regulación de las variaciones en la actividad total (Capítulo 3), según las necesidades fisiológicas de las langostas durante su ciclo de vida y tipo de alimentación.

Antes de los trabajos que conforman la presente Tesis Doctoral, sólo se contaba con un estudio para una langosta espinosa no tropical (*J. edwardsii*), donde se estudiaron las variaciones de las enzimas digestivas durante el desarrollo (Johnston, 2003). Las variaciones en la alimentación a lo largo del ciclo de vida de *P. argus*, que van desde cambios en la contribución relativa de determinadas entidades en la dieta, a la incorporación gradual de entidades de mayor tamaño (Herrera *et al.*, 1991), deben corresponderse con variaciones ontogénicas en las capacidades digestivas (enzimas digestivas). Estos cambios le permitirían a los especímenes adaptarse a la composición bioquímica del alimento según las necesidades energéticas y nutricionales de cada estadio de desarrollo. Los resultados de esta Tesis Doctoral mostraron que la actividad de las

principales enzimas digestivas varía acorde a los estados de desarrollo. Las actividades tripsina, quimotripsina y amilasa se incrementaron con la talla de los organismos, mientras que las actividades esterasa y lipasa mostraron una relación inversa con el estado de desarrollo (Capítulo 3). Los resultados sugieren que existe un control endógeno y programado de la actividad total de las principales enzimas digestivas. Los bajos valores de los coeficientes de determinación de las relaciones entre las actividades enzimáticas y la talla también sugirieren que otros factores influyen significativamente en la actividad de las enzimas digestivas. El análisis multivariante realizado indicó que el tipo de alimento ingerido en cada etapa del desarrollo puede modular estas variaciones (Capítulo 3).

Las modificaciones en la actividad de las principales enzimas digestivas durante el ciclo de la muda no habían sido estudiadas con anterioridad para ninguna langosta espinosa y su análisis arrojó evidencias adicionales sobre los factores involucrados en la regulación de dichas actividades. En general, las variaciones encontradas en las enzimas digestivas de *P. argus* (Capítulo 3) se corresponden casi idénticamente con las variaciones en la tasa de ingestión de la misma especie durante el ciclo de la muda (Lipcius y Herrnkind, 1982), lo cual sugiere que la ingestión de alimento es el factor externo fundamental en el control de la actividad total estas enzimas en el tracto digestivo. Sin embargo, no podemos olvidar la existencia de factores endógenos implicados directamente en el ciclo de la muda, que pudieran estar involucrados en el control de las tasas de síntesis de enzimas digestivas en *P. argus*. La 20-hydroxiecdisona inhibe la expresión de tripsina en algunos crustáceos (Shechter *et al*., 2007).

La mayoría de los cambios que ocurren a nivel morfológico y fisiológico en crustáceos durante el ciclo de la muda, si no todos, están determinados por cambios en la expresión coordinada de genes involucrados en procesos como degradación y síntesis de la cutícula, degradación y síntesis de proteínas (incluyendo enzimas digestivas), síntesis de matriz extracelular, metabolismo energético, así como activación proteolítica e inmunidad (Seear *et al*., 2010; Kuballa *et al*., 2011). A pesar de las grandes variaciones encontradas en la actividad de estas enzimas, la composición de isoformas en *P. argus* no varió en relación con el estadio de la muda (o desarrollo) (Capítulo 3), lo cual indica que la regulación es cuantitativa (en contraposición a la regulación cualitativa, ver Schulte, 2004). La regulación cuantitativa puede operar a nivel de expresión génica, síntesis de enzimas, o

secreción de las mismas. La expresión basal de las isoformas de tripsina en *P. argus* es diferente (Capítulo 5), lo cual sugiere que al menos la expresión génica es un punto importante de regulación, aspecto que también se demostró en esta Tesis Doctoral (Capítulo 6).

Además, los diferentes niveles de expresión entre las isoformas observadas en *P. argus* hacen suponer que existan diferencias en la importancia relativa de las mismas en la digestión de proteínas. Los resultados de digestibilidad (Capítulo 4) sugirieron que la isoforma de tripsina de movilidad electroforética media es la menos eficiente en la digestión. Este aspecto se abordó en el presente estudio mediante la purificación de las diferentes isoformas y su posterior caracterización cinética. Los resultados mostraron que esta isoforma (denominada PaT4) presenta la menor K_{cat} y, en consecuencia, la menor eficiencia catalítica (K_{cat}/K_m) (Capítulo 7).

Las constantes cinéticas usualmente brindan poca información sobre el funcionamiento de las enzimas *in vivo* debido a que las concentraciones *in vivo* de sustrato son aproximadamente de 0.1-1.0 K_m (Copeland, 2000). Sin embargo, las tripsinas digestivas funcionan *in vivo* a concentraciones altas de sustrato ([S] >> K_m), al menos en las etapas iniciales de la digestión, por lo que la K_{cat} (velocidad máxima en condiciones saturantes de sustrato) provee una buena aproximación a la velocidad de reacción *in vivo* y, por tanto, a la eficiencia digestiva en condiciones fisiológicas. De esta manera, y por primera vez para un crustáceo, se demuestra con resultados experimentales la suposición de que las diferencias en la eficiencia digestiva entre los diferentes fenotipos de tripsina están dadas por diferencias cinéticas de las diferentes isoformas.

No obstante, como las proteasas actúan sobre proteínas con muchos enlaces susceptibles a hidrólisis, cada uno con una cinética diferente, los resultados cinéticos se corroboraron mediante el uso de dos proteínas modelo (BSA y mioglobina) (Capítulo 7). La hidrólisis de estas dos proteínas por la isoforma PaT4 resultó más lenta, lo cual se corresponde con la baja K_{cat}/K_m encontrada usando BA*p*NA como sustrato sintético. Por otra parte, se demostró que la isoforma de mayor capacidad de hidrólisis de proteínas nativas (PaT1) se corresponde con la isoforma presente en todos los individuos de *P. argus* (Capítulo 7). Los resultados de la presente Tesis Doctoral demuestran que las variaciones fenotípicas en la

actividad tripsina están influenciadas por las características cinéticas de las isoformas presentes en cada individuo.

Durante mucho tiempo se consideró que los bajos crecimientos de langostas espinosas obtenidos con dietas formuladas se debían a bajas tasas de ingestión relacionadas con la escasa atractibilidad o palatabilidad del alimento (Glencross *et al*., 2001; Williams *et al*., 2005; Johnston *et al*., 2007). Sin embargo, el hecho de que proporcionar alimento varias veces al día no mejora las tasas de ingestión ni el crecimiento (Thomas *et al*., 2003; Cox y Davis, 2006; Simon y Jeffs, 2008) no apoya la anterior hipótesis. Resultados obtenidos posteriormente parecen concluir que, a diferencia de los camarones que son capaces de incrementar la tasa de ingestión en respuesta a baja digestibilidad de la dieta (Kureshy y Davis, 2002), la ingestión en langostas espinosas está limitada por una baja capacidad gástrica (2.5-3% de la biomasa húmeda), baja tasa de llenado (1-2 h) y vaciamiento (10 h) gástrico, así como por el largo tiempo necesario para que las langostas recobren el apetito (18-48 h) (Simon y Jeffs, 2008).

En la actualidad, y por las razones antes mencionadas, se acepta que debido a la imposibilidad de incrementar el consumo de alimento en langostas espinosas, la única manera de mejorar las tasas de crecimiento es mediante la optimización del aprovechamiento de la ración diaria (digestibilidad en primera instancia), especialmente del componente proteico de la misma. Lamentablemente se han realizado sólo dos estudios de digestibilidad *in vivo* de proteínas en especies de langostas espinosas (*J. edwardsii*: Ward *et al*., 2003; *P. ornatus*: Irvin y Williams, 2007). Aunque estos estudios mostraron que las langostas son capaces de digerir fuentes de proteína de origen animal y vegetal, Simon (2009a) mantiene que la razón fundamental de los bajos crecimientos obtenidos en diferentes estudios es la baja digestibilidad de la dieta. Nuestros resultados *in vitro* (Capítulo 4) apoyan esta hipótesis, ya que si bien las langostas son capaces de digerir diferentes fuentes de proteínas y producir cantidades significativas de aminoácidos libres y péptidos pequeños, se observó digestión incompleta de la mayoría de las fracciones proteicas que componen las harinas evaluadas. A esto se añade que existen problemas de solubilidad de las proteínas de las harinas de origen animal, como las de pescado y calamar.

Este estudio es el primero que emplea métodos *in vitro* para evaluar la digestibilidad de proteínas en una langosta espinosa (Capítulo 4) y los resultados obtenidos lograron explicar algunas contradicciones resultantes de los estudios *in vivo* precedentes (por ejemplo: baja digestibilidad de la harina de calamar). Por otra parte, la mayor liberación de aminoácidos y péptidos pequeños de la harina de calamar durante las etapas iniciales de la digestión, respecto al resto de las fuentes de proteínas animales y vegetales evaluadas (Capítulo 4), apoya la hipótesis de que el efecto promotor del crecimiento de la harina de calamar en crustáceos reside en componentes de bajo peso molecular (Córdoba-Murrueta y García Carreño, 2002). Estos componentes podrían provocar efectos metabólicos (Cruz-Ricque *et al*., 1989; Perera *et al*., 2005), secretagogos (Le Moullac *et al*., 1996; Perera *et al*., 2005) o estimular la expresión de enzimas digestivas (Capítulo 6).

Por otra parte, el análisis de la distribución de las enzimas digestivas en el tracto digestivo de *P. argus* permitió sugerir la importancia relativa de las diferentes cavidades en la digestión (Capítulo 2). De esta forma, la mayor actividad tripsina, quimotripsina y amilasa en el jugo gástrico de *P. argus*, respecto a la encontrada en la glándula digestiva, sugirió que la digestión de las proteínas del alimento comienza en la cámara gástrica justo después de la ingestión. Por el contrario, a pesar de la gran cantidad de proteínas no relacionadas con la digestión en la glándula digestiva de *P. argus*, las actividades (específicas) esterasa y lipasa fueron muy superiores en la glándula respecto al jugo gástrico, indicando que la digestión significativa de los lípidos de la dieta comienza más tarde, cuando las partículas del alimento pasan de la cámara gástrica al hepatopáncreas. Teniendo en cuenta la relevancia relativa de la cámara gástrica en la digestión de proteínas en *P. argus* (Capítulo 2), las limitaciones en la solubilidad de las proteínas de la dieta (Simon 2009a; Capítulo 4) y las evidencias de bajo procesamiento de los piensos en la cámara gástrica de las langostas espinosas (Simon, 2009a; Capítulo 4), se decidió estudiar el proceso de secreción de tripsinas hacia la cámara gástrica así como su relación con el tipo de alimento ingerido (Capítulo 6).

Aunque un estudio previo en otra especie de langosta (*J. edwardsii*) postula que no existen limitaciones en la secreción de enzimas a la cámara gástrica tras la ingestión de alimento peletizado (Simon, 2009a), nuestros resultados indican que para el caso de las tripsinas, este proceso está modulado por la calidad del alimento ingerido, en particular por la fuente

de proteína contenida en el alimento (Capítulo 6). Además, la fuente de proteína mostró también regular las enzimas tipo tripsina a nivel trancripcional en la glándula digestiva de *P. argus* (Capítulo 6), como ha sido descrito con anterioridad en camarones (Le Moullac *et al*. 1996; Muhlia-Almazán *et al*., 2003), mosquitos (Noriega *et al*., 1994) y ratas (Lhoste *et al*., 1994; Hara *et al*., 2000).

En el momento de máxima secreción después de la ingestión (4h), las dietas cuyo componente proteínico mayoritario fue la harina de pescado o calamar indujeron la secreción de tripsinas a la cámara gástrica en niveles similares a los observados tras la ingestión de alimento fresco. Además, estas fuentes de proteína modularon positivamente la expresión génica de la isoforma de tripsina más abundante. Por su parte, las dietas con harina de soja como componente mayoritario no tuvieron efectos sobre la expresión o secreción de tripsinas en *P. argus*, a pesar de contener una cantidad basal de harina de pescado. Estos resultados indican que deben existir, por tanto, valores umbrales para las señales encargadas de promover estas respuestas fisiológicas tras la ingestión.

Si bien es universalmente aceptado que las características del alimento pueden afectar las enzimas digestivas, los mecanismos involucrados han sido estudiados en pocos grupos. En los perros (Meyer y Kelly, 1976) y humanos (Thimister *et al*., 1996), los aminoácidos y péptidos son más eficientes que las proteínas intactas en estimular la secreción exocrina del páncreas. Sin embargo, la secreción de tripsina es más estimulada por proteínas intactas en ratas (Green y Miyasaka, 1983), peces (Cahu *et al*., 2004), insectos (Blakemore *et al*., 1995; Lehane *et al*., 1995) y la langosta *P. argus* (Capítulo 6). En la presente Tesis Doctoral se proporcionan las primeras evidencias del tipo de señales del alimento que median la secreción de tripsinas en un crustáceo. Sin embargo, futuros estudios en otras especies de crustáceos serán necesarios para determinar si este grupo de organismos, que presentan hábitos alimentarios tan diversos, comparte las mismas señales para la secreción de enzimas digestivas.

Adicionalmente, nuestros resultados también indican que el efecto del alimento sobre la expresión génica de tripsina en *P. argus* está mediado por la aparición de aminoácidos libres y péptidos pequeños en la glándula digestiva. El hecho de que en *P. argus* los procesos de secreción y transcripción de tripsinas estén afectados por dos tipos de señales

diferentes (proteínas intactas la secreción y aminoácidos libres la expresión), que aparecen en el tracto digestivo de manera sucesiva, pudiera indicar que existe una cronología en la regulación de las enzimas tipo tripsina durante la digestión. La importancia de la regulación coordinada en el tiempo de estos dos procesos pudiera ser tan relevante como la regulación de cada uno de ellos por separado. Un estudio en la langosta *J. edwardsii* mostró que, mientras las langostas alimentadas con mejillones presentan dos picos de secreción (4 y 18 h), las langostas que ingieren alimento peletizado no presentaron el segundo pico de secreción (Simon, 2009a). El autor sugiere que las dificultades de la glándula digestiva para procesar el alimento peletizado (exceso de material indigestible en el lúmen de los túbulos e intensificación de la digestión intracelular) pudieran comprometer la síntesis de nuevas enzimas digestivas. Nuestros resultados indican que el uso de fuentes de proteína de origen vegetal disminuye la capacidad de síntesis de tripsinas en la glándula digestiva. Sin embargo, las fuentes de proteína de origen animal en nuestro estudio estimulan la expresión génica por lo que es de suponer que la síntesis de nuevas enzimas esté, en teoría, garantizada. Sin embargo, debieran estudiarse los efectos a largo plazo de la alimentación con alimento peletizado sobre la expresión génica de las principales enzimas digestivas. La alimentación de *J. edwardsii* con piensos durante seis meses provoca una disminución significativa en la actividad total y específica de las enzimas digestivas tanto en la glándula digestiva como en la cámara gástrica (Simon, 2009a).

La isoforma de tripsina más abundante (según el número de copias de RNAm) en *P. argus* es la única que incrementa su expresión en respuesta a la ingestión de alimento (Capítulo 6). Aunque se sabe que los elementos que regulan la transcripción se encuentran fundamentalmente en las regiones (Cis) 5´ (Wray *et al*., 2003), en otros estudios se ha encontrado que los genes que muestran niveles altos de expresión presentan indicios de evolución adaptativa en las regiones 3´ UTR y adyacentes (Holloway *et al*., 2007). Todos los cDNA de tripsina encontrados en *P. argus* presentan regiones 5´ UTR cortas y casi idénticas, mientas que las regiones 3´ UTR de *PaTry1* y *PaTry3* difieren en 5 sustituciones nucleotídicas de la región 3´ UTR de *PaTry2*, la cual es la isoforma con menores niveles de expresión (Capítulo 5). No obstante, será necesario el estudio de las secuencias génicas de estas enzimas, y regiones adyacentes del genoma (sobre todo hacia el extremo 5´), para determinar los elementos estructurales implicados en la regulación de la expresión.

Muchos son los factores de transcripción y moléculas relacionadas (co-activadores, co-represores, remodeladores de la cromatina, acetilasas de histonas, de-acetilasas, quinasas, metilasas, etc.) que intervienen en la regulación de la transcripción en eucariontes (ver revisión de Brivanlou y Darnell, 2002). De igual manera, las regiones del genoma que interactúan con estos factores y están involucradas en la regulación de la transcripción han sido objeto de estudio por muchos autores, fundamentalmente en los organismos modelo. Por ejemplo, se han encontrado 155 048 sitios de unión para factores de transcripción y 19 937 sitios de unión para de-acetilasas de histonas en el genoma de *Drosophila* (Nègre *et al*., 2001).

La región adyacente al extremo 5´ de genes de tripsina en algunos insectos posee secuencias "TATA box" similares a los promotores de la actina en *Drosophila* (Xiong y Jacobs-Lorena, 1995; Mazumdar-Leighton y Broadway, 2001) y secuencias AAAGAAA y AAAATAAAA, que son similares a los sitios de unión de factores de transcripción en *Drosophila*, aunque no se ha demostrado la relevancia de estas secuencias en la regulación de la expresión de las tripsinas (Mazumdar-Leighton y Broadway, 2001). También en insectos, en las regiones adyacentes al extremo 5´ de los genes de tripsina se han identificado otros elementos que parecen también estar involucrados en la regulación de la trascripción. Entre estos elementos está un sitio iniciador 5´ACAGT conservado en artrópodos [la secuencia (TAG)CA(GT)T está presente en el 25% de los 112 sitios promotores de artrópodos estudiados por Cherbas y Cherbas, 1993] y dos secuencias conservadas 5´GGATTAA y 5´TGTTTCCT que aparecen a distancias equivalentes del sitio de inicio de la trascripción en los genes de tripsina de varios insectos (Xiong y Jacobs-Lorena, 1995). En el copépodo *Lepeophtheirus salmonis* también se han encontrado regiones promotoras asociadas a varios de los genes de tripsinas (Kvamme *et al*., 2005) pero en general, los estudios de este tipo son escasos en crustáceos. La diferencias tan marcadas en los niveles de expresión entre las isoformas de tripsina en *P. argus* es un escenario ideal para estudiar las bases moleculares de la expresión de estas enzimas digestivas.

Además, aunque se acepta que existe correlación entre los altos niveles de expresión y la baja tasa de evolución de las proteínas (Drummond *et al*., 2005), en nuestro trabajo

(Capítulo 6) se encontraron algunos sitios sometidos a selección positiva en la isoforma de mayor expresión génica. Otros trabajos han mostrado evidencias de evolución adaptativa reciente en las regiones codificadoras de genes con valores altos de expresión (Holloway *et al*., 2007). Estudios futuros encaminados a determinar la correspondencia de los cDNA de tripsina y las enzimas caracterizadas bioquímicamente en este trabajo, aportarían evidencias sobre las fuerzas (neutrales o adaptativas) que pudieran estar influyendo en la evolución de estas enzimas.

La gran variación fenotípica (referida en este caso a la actividad) de las tripsinas de *P. argus* tiene sus bases en la presencia de diferentes isoformas (Capítulo 2, 3, 4) y en las diferencias catalíticas (Capítulo 7) y de expresión entre las mismas (Capítulo 5 y 6). Además, la acción conjunta de factores intrínsecos, como los estadios de la muda y de desarrollo (Capítulo 3), y extrínsecos como la alimentación (Perera *et al*., 2005; Capítulo 6), que afectan la expresión y secreción de tripsinas en la especie, determinan aún mayores variaciones en la actividad tripsina total tanto en la glándula digestiva como en el jugo gástrico. Por tanto, las características del alimento se deben manejar de manera especie-específica para aprovechar el mayor potencial de la especie e incrementar la eficiencia de la digestión proteica. En este sentido, se detectaron (Capítulo 4) algunas características de los piensos para langostas espinosas, que no están en concordancia con las capacidades digestivas de estos crustáceos (exceso de proteínas de origen vegetal, baja solubilidad de proteínas de origen animal, digestión incompleta de la mayoría de las fracciones proteicas que componen las harinas más utilizadas). Se deben, por tanto, realizar estudios encaminados a mejorar la solubilidad de las proteínas de la dieta en la cámara gástrica de *P. argus* y su digestibilidad, así como determinar el nivel de inclusión de harina de soja que no impida el progreso de los diferentes procesos digestivos.

Los resultados de la presente Tesis Doctoral contribuyen a incrementar la comprensión de las capacidades digestivas, valor fisiológico del polimorfismo en las enzimas digestivas, y factores involucrados en la regulación de estas enzimas en la langosta *P. argus* (Fig. 1). Sin embargo, la cuestión más interesante que surge de esta Tesis Doctoral es: ¿cómo la presencia de isoformas y las diferencias en expresión y eficiencia catalítica entre las isoformas, se integran a los niveles superiores de organización produciendo *in vivo*, si lo hacen, diferencias en el crecimiento?

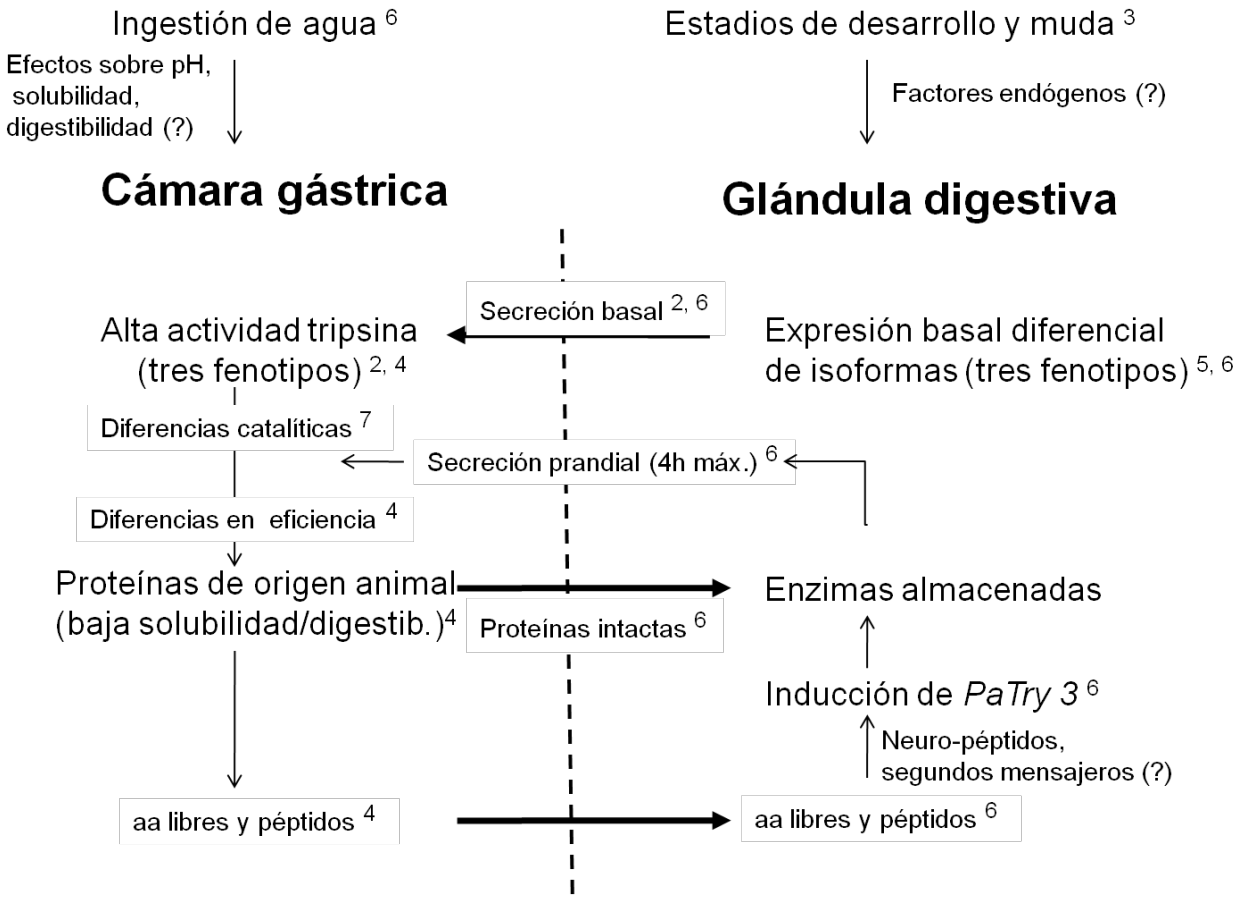

Fig. 1. Factores extrínsecos e intrínsecos involucrados en la regulación de las enzimas tipo tripsina y la digestión de proteínas en la langosta *P. argus*. Los números indicados como superíndices se refieren a los capítulos de la presente Tesis Doctoral donde se demuestran o abordan determinados aspectos de los procesos señalados.

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Capítulo 9

Conclusiones

1. La langosta *Panulirus argus* presenta, en concordancia con sus hábitos carnívoros y oportunistas, una amplia batería de enzimas digestivas (tripsinas, quimotripsinas, amilasas, esterasas y lipasas), cuya actividad y variaciones responden tanto a sus requerimientos durante el ciclo de vida como durante la muda. Las características más distintivas de las enzimas digestivas de la especie son el polimorfismo y la alta actividad tripsina presente.

2. La digestión de proteínas en la langosta *P. argus* comienza en la cámara gástrica, donde se localizan altas actividades tripsina y quimotripsina. Sin embargo, la digestión de dietas formuladas está limitada por la baja solubilidad de las proteínas contenidas en las harinas de pescado, lo cual limita la acción de las proteasas digestivas sobre sus sustratos. Por este motivo, se debe mejorar la solubilidad de las proteínas en estas dietas para poder aprovechar las potencialidades digestivas que la especie presenta en la cámara gástrica.

3. La langosta *P. argus* posee al menos cinco isoformas de tripsina, con secuencias primarias y estructuras tridimensionales similares, de las cuales las tres isoformas principales manifiestan diferencias en su movilidad electroforética, características cinéticas y eficiencia digestiva: i) la isoforma PaT1 presenta la menor movilidad electroforética, es la única que está presente en todos los individuos, tiene una alta eficiencia catalítica (K_{cat}/K_m) y es la más eficiente digiriendo sustratos proteicos nativos; ii) la isoforma PaT2 posee la movilidad electroforética mayor, una alta eficiencia catalítica y ostenta una eficiencia media digiriendo proteínas nativas; y iii) la isoforma PaT4 muestra movilidad electroforética media, la menor eficiencia catalítica y la menor capacidad para digerir sustratos proteicos nativos.

4. La variación en los ejemplares de la langosta *P. argus*, respecto a su capacidad de digerir las proteínas de la dieta, está determinada por la combinación de isoformas de tripsina presentes y se fundamenta en las características cinéticas y de actividad de cada una de ellas. De este modo, se definen tres fenotipos: i) fenotipo A, que presenta las tres isoformas principales de la tripsina (PaT1, PaT2 y PaT4) y es el más eficiente en los procesos digestivos; ii) fenotipo B, que se caracteriza por la presencia de las isoformas PaT1 y PaT4, resultando el menos eficiente; y iii) fenotipo C, que posee tanto las isoformas PaT1 como PaT2 y una eficiencia media.

5. La secreción y expresión de tripsinas en la langosta *P. argus* son procesos regulados cronológicamente por las características del alimento y los productos de la digestión. De este modo, las proteínas del alimento recién ingerido estimulan la secreción de tripsinas a la cámara gástrica, mientras que los productos finales de la digestión proteica (fundamentalmente aminoácidos libres) promueven la transcripción diferencial de isoformas de tripsina en la glándula digestiva, probablemente para reponer las enzimas secretadas. Estos procesos se suceden normalmente cuando la fuente de proteína en la dieta es de origen animal (pescado, calamar), pero están inhibidos cuando existe un exceso de proteínas de origen vegetal (soja).

6. En la langosta *P. argus* la digestibilidad de la harina de calamar es baja. Sin embargo, durante la digestión de las fracciones proteicas de bajo peso molecular que componen esta harina se producen cantidades significativas de aminoácidos libres que estimulan la expresión de la isoforma de tripsina más abundante en esta especie. Por este motivo, dicha fuente de proteína puede usarse en las dietas formuladas para incrementar las capacidades digestivas de *P. argus*.

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