CENTRO DE INVESTIGACIÓN EN ALIMENTACIÓN Y DESARROLLO, A. C.

PURIFICACIÓN Y CARACTERIZACIÓN BIOQUÍMICA Y CINÉTICA DE PROTEASAS DE VÍSCERAS DE PEZ DIABLO (*Pterygoplichthys disjunctivus*)

POR

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TESIS COMO REQUISITO PARCIAL PARA OBTENER EL GRADO DE

DOCTORADO EN CIENCIAS

HERMOSILLO, SONORA DICIEMBRE DE 2011

CARTA DE APROBACIÓN

Los miembros del comité designado para revisar la tesis de la M. en C. Ana Gloria Villalba Villalba, la han encontrado satisfactoria y recomiendan sea aceptada como requisito parcial para obtener el grado de Doctorado en Ciencias, dentro del Programa de Doctorado en Ciencias del Centro de Investigación en Alimentación y Desarrollo A. C.

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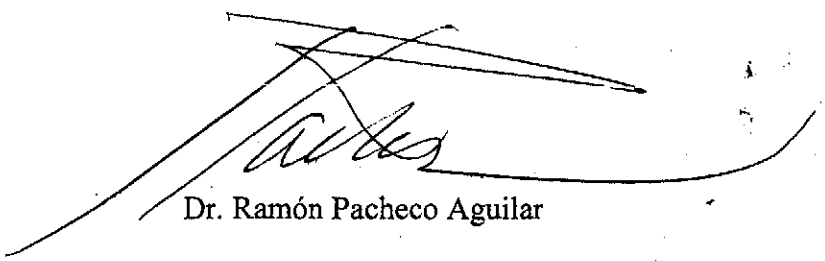

Dr. Ramón Pacheco Aguilar

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C.I.A.D., A.C.

AGRADECIMIENTOS

Al Consejo Nacional de Ciencia y Tecnología (CONACYT) por la beca otorgada durante el programa de Doctorado.

Al Centro de Investigación en Alimentación y Desarrollo, A.C., (CIAD) por haberme aceptado en su programa de Doctorado.

Especialmente a mi director de tesis, Dr. Ramón Pacheco Aguilar, por todos sus consejos y enseñanzas a lo largo de todo este tiempo, pero sobre todo gracias por confiar en mí en todo momento.

Al comité de tesis, al Dr. Juan Carlos Ramírez Suárez por todo su empeño y esfuerzo dedicado en el mejoramiento de los manuscritos. Al Dr. Fco. Javier Castillo y Dr. Enrique Márquez Ríos, por estar siempre atentos y al pendiente de mis avances y mostrar siempre su mejor disposición de ayuda en el momento que se los solicité. Y finalmente a la Dra. Elisa M. Valenzuela Soto, por todas sus enseñanzas, consejos, ayuda, asesorías, y por estar siempre disponible cuando la necesité.

Al personal de la biblioteca, cómputo y mantenimiento por brindarme su servicio amable, oportuno y de calidad.

A mis compañeros de generación Laura, Hugo, Erika, Marisela y Enrique.

Al equipo del Laboratorio de Bioquímica y Calidad de Productos Pesqueros, a las maestras María Elena Lugo, Gisela Carvallo y Guillermina García por su disposición de apoyo en todo momento, pero especialmente gracias por su amistad. A mis compañeros de Laboratorio, Lucina, Hugo, Carlos, Mariel, Aristeo, Josué, Aarón, Marco, Gabriel, Hilda por las experiencias y tiempo compartido, también a mis compañeros Elba J. y Wilfrido, por haberse convertido en mis amigos.

DEDICATORIA

A mi familia

A mis padres Laura Alicia y Juan Ángel por brindarme siempre su amor y apoyo incondicional.

A mi hermano Joel y mi sobrina Andrea Melisa por ser parte de mi vida.

A Edgard por estar a mi lado y apoyarme siempre. Gracias por todo.

A mis mascotas Luna, Gordis y Rambo por recibirme siempre con tanta alegría al llegar a casa.

Los quiero.

CONTENIDO

SINOPSIS

Pez diablo y su situación en México

Los peces plecos o armados de la familia Loricariidae, son nativos de Sudamérica (Nico et al., 2009) y se han establecido fuera de su área de distribución en varias regiones del mundo, entre ellas México, detectándose por primera vez en 1995 en el río Mezcala. En México se acuñó el nombre de peces diablo (*Pterygoplichthys spp*) para estas especies. Durante los últimos años, estos peces se han expandido rápidamente y es común encontrarlos en varias de las cuencas hidrológicas más grandes del país. Este fenómeno se ha caracterizado por una alta tasa de dispersión y una significativa proliferación en sus poblaciones. Diversas particularidades de su morfología, fisiología y comportamiento acentúan su potencial invasivo, como una reproducción precoz y una alta tasa reproductiva. Además el desarrollo de escamas con fuertes espinas y placas óseas, en gran medida esto explica la carencia de depredadores, en su hábitat nativo, son depredados por cocodrilos, nutrias y peces grandes. Su crecimiento es rápido, la mayoría de las especies son pequeñas o medianas aunque algunas alcanzan tallas de 50 – 70 cm y alrededor de 3 Kg de peso. Son extremadamente adaptables a diversos ambientes, algunos son tolerantes a la salinidad moderada, y su gran estómago vascularizado funciona como pulmón, permitiéndole soportar hipoxia durante días, entre otras características que favorecen su potencial invasivo (CONABIO, 2007). Estos peces desplazan a otras especies, algunas de ellas endémicas, ya sea por la ingesta incidental de sus huevos y la competencia por alimento (algas y detritus). Al desplazarse en grandes cardúmenes, dañan la vegetación nativa, la cual es utilizada como fuente de alimento, sitio de anidación y refugio de las especies endémicas. Además, al anidar cavan galerías de alrededor de 1.5 m, desplazando grandes cantidades de sedimento, perturbando la estabilidad de las riveras, aumentando la erosión e incrementando la turbidez, lo que ocasiona que se afecte la calidad del agua. Los ambientes acuáticos en particular han demostrado ser extremadamente sensibles a las especies invasoras; aproximadamente 40% de las extinciones de especies que habitan estos ambientes han estado relacionadas con la depredación, el parasitismo o la competencia de especies invasoras (Pimentel et al. 2001). Se estima que la tasa de extinción en los ambientes acuáticos, particularmente en los dulceacuícolas, es cinco veces mayor que en los ambientes terrestres (Ricciardi y Rasmussen 1999).

Por otro lado, estos peces han devastado a la pesquería de tilapia y carpa en la presa Adolfo López Mateos (El Infiernillo), la cual llegó a registrar producciones de 20, 000 toneladas anuales. Esta actividad fue una alternativa económica durante años para 119 comunidades de los alrededores de la presa, sin embargo, un manejo inadecuado del recurso y la presencia del pez diablo condujeron al descenso de esta pesquería. En los últimos años, entre el 70 y 80 % de la captura de tilapia y carpa (8,000 ton) ha sido sustituida por pez diablo. Sin embargo, estos no tienen valor económico ni son aceptados como alternativa alimenticia, es decir, es un recurso no aprovechado. La evaluación de costos asociados a los daños causados por la introducción de los peces loricáridos a la presa Infiernillo, en el límite entre Michoacán y Guerrero, ha llevado a pérdidas económicas estimadas de 13 millones de dólares (Stabridis-Arana et al. 2009). Ha tenido además, un costo social importante al dejar desempleados a miembros de 46,000 familias. El tema de las invasiones biológicas incluye una dimensión ecológica, socioeconómica e incluso ética. Las acciones de control o erradicación con frecuencia resultan costosas, por lo que, la generación de experiencias en el manejo y control, la cooperación y el intercambio de información constituyen las máximas fortalezas para solucionar estos problemas ecológicos (Schüttler y Karez, 2008).

De ahí, que surge la necesidad de buscar alternativas de uso para este recurso, en este trabajo se estudiaron las principales características bioquímicas y catalíticas de las proteasas de las vísceras del pez diablo y para poder así establecer un uso biotecnológico. En la actualidad las proteasas constituyen el grupo más importante de enzimas utilizadas en la industria a nivel mundial, en 2010 se vendieron alrededor de 2.2 billones de dólares, esperando un incremento en la demanda de estas enzimas en los próximos años (Market Research Reports and Technical Publications, 2011). Sin embargo la mayoría de las proteasas en el mercado son de origen bovino, porcino o microbiano. Se ha visto que las proteasas de organismos acuáticos presentan mejores características bioquímicas y catalíticas que sus homólogas de otros organismos (Haard, 1998 y Simpson, 2000). Las principales proteasas presentes en las vísceras de los peces son las ácidas como la pepsina, aislada del estómago y las alcalinas como la tripsina y quimotripsina aisladas de los intestinos (Simpson, 2000). Los peces loricáridos, como *P. disjunctivus*, presentan un estómago modificado, el cual realiza funciones de órgano respiratorio, éste no se involucra en el proceso de digestión; usualmente está lleno de aire y es alcalino (German y Bittong, 2009). Por lo tanto no secreta pepsina y la hidrólisis de las proteínas que consume es realizada por las proteasas

alcalinas. La mayoría de las especies de peces presentan un 5% de vísceras, respecto a su peso corporal total, pero el pez diablo tiene un 10%, es decir pueden ser una fuente importante de estas enzimas. Así el objetivo del presente estudio fue estudiar las principales características bioquímicas y catalíticas de las proteasas de las vísceras del pez diablo y para poder así establecer un uso biotecnológico.

Proteasas de peces

Como ya se mencionó previamente, las proteasas utilizadas actualmente en la industria son obtenidas del tracto digestivo de bovinos y porcinos, así como de microorganismos. Sin embargo en los últimos años ha crecido el interés por el estudio de proteasas de las vísceras de los peces, al ser consideradas una buena alternativa para la industria por sus características; entre las que destacan: baja temperatura óptima de actividad, baja termoestabilidad, alto pH óptimo de actividad con respecto a sus similares de mamíferos y baja energía de activación (Simpson, 2000). Además muestran alta actividad en un amplio rango de pH y a concentraciones relativamente bajas (Haard, 1998). Estas características les confieren potencial de uso en una amplia variedad de industrias, como los detergentes, alimentos, agroquímicos, farmacéuticos, etc. (Gupta et al., 2002).

Varias enzimas proteolíticas han sido aisladas a partir de las vísceras de diferentes especies de peces y otros organismos. Siendo las más importantes la tripsina (EC 3.4.21.4) y quimotripsina (EC 3.4.21.1) (Yang et al., 2009), pertenecientes al grupo de las serina-proteasas.

Tripsina

La tripsina posee una secuencia de aminoácidos en su sitio activo característica del grupo de las serina-proteasas, consiste en His, Asp y Ser. Muestra especificidad para romper el enlace peptídico por el lado carboxilo donde se encuentran los aminoácidos con cadenas laterales voluminosas y con carga positiva como lisina y arginina. Esta enzima ha sido aislada y caracterizada de especies de peces tanto de agua salada como de agua dulce, así como también de aguas frías hasta templadas (Khaled et al., 2011).

Incluyendo entre las especies de agua dulce y aguas de templadas a tropicales están las carpas como *Catla catla, Labeo rohita* y *Hypophthalmichthys molitrix,* en este trabajo se observó un

rango de pH de 9 – 11, en el cual la tripsina mostró su máxima actividad y pesos moleculares de 26.7, 21.6 y 32.5, respectivamente para cada una de estas especies (Kumar et al., 2007). El pez mandarin es uno de los peces de agua dulce más cultivado en China, a partir de este se obtuvieron las vísceras para aislar y purificar la tripsina, con el objetivo de proveer información acerca de las principales características bioquímicas de esta enzima. Se encontró una tripsina catiónica (A) y otra aniónica (B) de 21 y 21.5 kDa, respectivamente; ambas tripsinas mostraron máxima actividad a un pH de 8.5 y a 35 – 40 °C y tuvieron más del 45% de su actividad máxima a 20 °C (Lu et al., 2008). En otro estudio realizado recientemente por Klomklao et al. (2011), purificaron tripsina a partir de las vísceras de un híbrido de pez gato (*Clarias macrocephalus* x *Clarias gariepinus*), la enzima mostró un peso molecular aproximado de 24 kDa y un pH y temperatura óptimos de 8.0 y 60 °C. Además mostró alta estabilidad en condiciones hipersalinas, ya que con 30% de NaCl conservó alrededor de 55% de actividad.

Tripsina también se ha logrado purificar de las vísceras de bogue (*Boops boops*), un pez marino, de la familia Sparidae, al cual se le puede encontrar en el Atlántico y Mar Mediterráneo, se determinó un peso molecular estimado de 23 kDa para esta tripsina. Un pH óptimo de 9.0 y temperatura óptima de 55 °C, siendo estable en un rango de pH de 6.0 – 11.0 y a una temperatura por debajo de 40 °C, en cuanto a las constantes cinéticas, se obtuvo una *Km* de 0.13 mM y una k*cat*/*Km* de 12 s^{-1} mM^{-1} (Barkia et al., 2010). Khaled et al., 2011 purificaron y caracterizaron tripsina de sardina (*Sardinella aurita)*, encontrando para esta enzima un peso molecular aproximado de 28.8 kDa y un pH óptimo de 9 y altamente estable en un rango de pH de 8.0 -10.0. Además la enzima tuvo la capacidad de retener más del 60% de actividad después de su incubación durante 1 h a 45 °C. Para esta enzima se encontró una *Km* de 0.125 mM y una eficiencia catalítica (k*cat*/*Km*) de 17.92 s^{-1} mM^{-1}; mientras que para la tripsina de origen bovino se ha encontrado una *Km* de 0.65 - 0.92 mM y una eficiencia catalítica de 3.1 s^{-1} mM^{-1}, es decir las constantes cinéticas de la tripsina de *Sardinella aurita* fueron mejores.

Ktari et al., (2012), purificaron y caracterizaron tripsina con potencial de uso en los detergentes, a partir de vísceras de una especie de robalo (*Salaria basilisca*). El peso molecular de esta tripsina fue de 27 kDa y fue altamente activa a temperaturas de 30 a 50 °C, con un óptimo de 60 °C. A 50 °C, la enzima retuvo alrededor de 65% actividad después de 1 h de incubación. El pH óptimo fue de 8.0, y mostró alta estabilidad en el rango de pH de 7.0 – 11.0; además fue altamente estable a la presencia de surfactantes no iónicos como Tween 80 y Triton X-100, ya que

retuvo 86 y 100 % de actividad, respectivamente después de 30 min de incubación. La mojarra plateada (*Diapterus rhombeus*) es otro pez marino, del cual se ha purificado tripsina, su peso molecular es de 26.5 kDa. Esta enzima fue muy estable en rango de pH de 7.5 – 11.0 con un pico máximo de actividad a pH 8.0 y la temperatura óptima de 55 °C; en los ensayos de termoestabilidad se observó una caída importante de la actividad a temperaturas cercanas a los 60 °C. Y se encontraron valores de *Km* y kcat/*Km* de 0.27 mM y 3.48 s^{-1} mM^{-1}, respectivamente (Silva et al., 2011).

Fuchise et al., (2009) purificaron y caracterizaron tripsina de peces de zonas de aguas frías, *Gadus macrocephalus* y *Eleginus gracilis*, para ambos peces la enzima mostró un peso molecular de 24 kDa, un pH óptimo de 8.0 y termo estabilidad más baja que la tripsina pancreática de mamíferos. El pez ballesta (*Balistes capriscus*) también es de aguas frías y a partir de sus vísceras se purificó y caracterizó la tripsina, encontrando una temperatura de 40 °C como óptima. La enzima fue altamente estable a temperaturas por debajo de 40 °C, pero se inactivó a temperaturas más altas, observando 78.7% y 8.5% de actividad después de 1h de incubación a 50 y 60 °C, respectivamente. Además la enzima fue muy estable en un rango de pH muy amplio de 7.0 – 12.0 (Jelloulli et al., 2009).

En general las tripsinas aisladas de peces son similares a sus homólogas de otras organismos en características como el peso molecular, que varía entre 22 y 28 kDa aproximadamente. Así como en la susceptibilidad a inhibidores, todas son inhibidas por fenil-metil-sulfonil fluoruro (PMSF), inhibidor de tripsina de soya (SBTI), benzamidina y toluen-lisil-clorometil cetona (TLCK). Pero, difieren en cuanto a los efectos que les provocan el pH y la temperatura, las tripsinas de peces son más estables a pH alcalinos en comparación a sus homólogas de mamíferos, por otro lado éstas últimas son más termoestables.

Quimotripsina

Entre las proteasas de peces, la tripsina ha recibido mucho interés y está bien documentada (El Hadj et al., 2009), sin embargo la información disponible de quimotripsina es menor. Tiene capacidad de hidrolizar el enlace peptídico por el lado carboxilo de aminoácidos hidrofóbicos como fenilalanina, tirosina, triptófano y leucina. Las quimotripsinas de peces tienen pesos moleculares entre 22-30 kDa y muestran pH óptimo de actividad entre 7.5 y 9.0 y 45 y 55 °C como temperatura óptima (Heu et al., 1995). Esta enzima ha sido aislada y caracterizada de varias

especies de peces como carpa común (*Cyprinus carpio*) (Cohen, et al., 1981), bacalao (*Gadus morhua*) (Raae y Walter, 1989), trucha arcoíris (*Oncorhynchus mykiss*) (Kristjansson y Nielsen, 1992) y anchoveta (*Engralius japonica*) (Hue, et al., 1995). Sin embargo, los trabajos más recientes sobre esta enzima aislada a partir de las vísceras de peces son los realizados por Castillo-Yáñez et al., (2006), en el cual la quimotripsina se logró purificar y caracterizar de la sardina Monterey (*Sardinops sagax caerulea*). El peso molecular estimado fue de 26 kDa y el punto isoeléctrico de 5.0, y mostró máxima actividad a pH 8.0 y a 50 °C, además fue muy estable en el rango de pH de 7.5 – 10.0 y a temperaturas menores de 45 °C. Además se estimaron las constantes cinéticas, se encontró una *Km* de 0.074 mM y una eficiencia catalítica (k*cat*/*Km*) de 251 s^{-1} mM^{-1}, mientras que para quimotripsina bovina se han estimado valores de Km de 0.008 mM y eficiencia catalítica de 0.0011 251 s^{-1} mM^{-1}, es decir la quimotripsina de sardina Monterey es varias veces más eficiente en su catálisis comparada con la quimotripsina de bovino.

También se ha caracterizado la quimotripsina de una especie de carpa (*Carassius auratus*), en este estudio de encontraron dos isoformas (quimotripsina A y B) con un peso molecular aproximado de 28 y 27 kDa, respectivamente. Las temperaturas óptimas fueron de 40 y 50 °C y los pH´s óptimos de 7.5 y 8.0 para quimotripsina A y B. Ambas enzimas fueron estables en un rango de pH de 6.0 – 11.0 a temperaturas menores a 45 °C. La *Km* estimada para cada una de las enzimas fue de 0.0014 y 0.0005 mM, y la eficiencia catalítica fue de 0.0019 y 0.0068 s^{-1} mM^{-1} (Yang et al., 2009). En otro trabajo se aisló y purificó la quimotripsina de *Lateolabrax japonicus*, el peso molecular estimado fue de 27.5 kDa y un punto isoeléctrico de 8.0. Se observó un pico máximo de actividad en un pH de 8.0 y a 45 °C. En el rango de pH de 7.0 – 11.0 la enzima fue muy estable, mostrando más del 80% de actividad, la estabilidad térmica disminuyó drásticamente a temperaturas ≥ 50 °C. Se encontró una *Km* de 0.0007 mM y una k*cat*/*Km* de 0.0026 s^{-1} mM^{-1} (Jiang et al., 2010).

El Hadj et al., (2010) por su parte purificaron y caracterizaron a la quimotripsina obtenida de las vísceras de lobina rayada (*Lithognathus mormyrus*). Encontrando un peso molecular estimado de 27 kDa para esta enzima, con un pH óptimo entre 10.0 – 11.0, además fue extremadamente estable en un rango de pH de 6.0 a 12.0. La temperatura óptima fue de 50 °C y la enzima mostró alta actividad a bajas temperaturas comparada con otras quimotripsinas, pero fue muy lábil a temperaturas ≥ 40 °C.

Estos estudios revelan que las quimotripsinas de peces tienen pesos moleculares similares a quimotripsinas de bovinos o porcinos. Sin embargo, presentan diferencias importantes, incluyendo una eficiencia catalítica mayor, baja termoestabilidad y la composición de aminoácidos de su estructura primaria (Racicot y Hultin, 1987; Cohen et al., 1981). Además en estos trabajos se concluye que tanto las tripsinas como las quimotripsinas de peces, presentan características que sugieren su potencial aplicación en procesos en los cuales se requieran bajas temperaturas y alta actividad enzimática (El Hadj et al., 2010; Jiang et al., 2010; Silva, et al., 2011).

Por todo lo anterior se concluyó que la recuperación de enzimas digestivas de vísceras, puede ser una alternativa viable para el aprovechamiento de la población del pez diablo (*Plecostomus sp*), y con ello reducir los problemas de sustitución e invasión de hábitats que actualmente está generando. Por lo que en éste trabajo se planteó que era necesario, investigar en detalle las características moleculares y catalíticas de las enzimas digestivas más relevantes de este pez, lo cual permitiría lograr un mejor aprovechamiento de las mismas. Debido a que un conocimiento a fondo de las características de las enzimas de una determinada fuente, es el primer paso en el camino de su recuperación y posible uso en la industria.

Hipótesis

Las proteasas de las vísceras de pez diablo (*Pterygoplichthys disjunctivus*), presentan características bioquímicas y cinéticas que permitirán su utilización como herramientas biotecnológicas.

Objetivo General

Generar información básica acerca de las características bioquímicas y cinéticas de las proteasas de las vísceras de pez diablo (*P. disjunctivus*).

Desarrollo del Trabajo Experimental

Se dividió en 3 etapas principales:

1. Caracterización bioquímica de las proteasas presentes en el extracto crudo obtenido a partir de las vísceras de pez diablo (*P. disjunctivus*).
2. Purificación y caracterización bioquímica y cinética de quimotripsina de las vísceras de pez diablo (*P. disjunctivus*).
3. Purificación y caracterización bioquímica y cinética de tripsina de las vísceras de pez diablo (*P. disjunctivus*).

A continuación se describen en capítulos los resultados obtenidos, ordenados de acuerdo a las etapas mencionadas previamente.

CAPÍTULO I. Caracterización bioquímica de las proteasas presentes en el extracto crudo obtenido a partir de las vísceras de pez diablo (*P. disjunctivus*).

En este capítulo se muestran los resultados del primer artículo publicado derivado del trabajo de tesis, el cual se titula "Partial characterization of alkaline proteases from viscera of vermiculated sailfin catfish (*Pterygoplichthys disjunctivus*,Weber, 1991)". En este estudio se caracterizó el extracto enzimático crudo obtenido de las vísceras del pez diablo. Con la finalidad de conocer que proteasas predominaban en dicho extracto enzimático, así como el comportamiento de éstas al someterlas a diferentes condiciones de pH, temperatura, salinidad, etc. Se encontró la presencia de proteasas alcalinas, en su mayoría del grupo de la serina-proteasa, en base a los ensayos de inhibición con el inhibidor fenil-metil-sulfonil fluoruro (PMSF) específico para este grupo de enzimas. El pH óptimo para estas enzimas fue de 9.0 y 50 °C la temperatura óptima. En los zymogramas se detectaron proteínas en un rango de peso molecular entre 14 – 116 kDa, todas con actividad tipo proteasas, pero predominaron las de 25 – 30 kDa, pesos moleculares reportados para tripsina y quimotripsina. Tanto la tripsina como la quimotripsina mostraron alta estabilidad en un rango de pH de 6.0 – 11.0 y de temperaturas de 30 – 50 °C, después de 1 h de incubación. Además de gran estabilidad a condiciones hipersalinas, incluso con 30% de NaCl ambas enzimas se encontró alrededor de 65% de actividad. Estos resultados constituyen

información valiosa para futuros estudios encaminados a la posible utilización del extracto enzimático crudo de vísceras de pez diablo en procesos biotecnológicos.

CAPÍTULO II. Purificación y caracterización bioquímica y cinética de quimotripsina de las vísceras de pez diablo (*P. disjunctivus*).

Después de realizar la caracterización parcial de las principales proteasas presentes en el extracto crudo, se procedió a su purificación. En este capítulo se describe el segundo artículo de investigación original con los resultados de la purificación y caracterización de quimotripsina de las vísceras del pez diablo titulado, "Purification and characterization of chymotrypsin from viscera of vermiculated sailfin catfish, *Pterygoplichthys disjunctivus*, Weber, 1991*"* y fue enviado a la revista Fish Physiology and Biochemistry. La estrategia experimental empleada para lograr purificar a la quimotripsina, incluyó fraccionación con sulfato de amonio al 30% y 70% de saturación, seguido por cromatografía de filtración en gel (Sephadex G-75), de afinidad (benzamidina) y finalmente intercambio iónico (DEAE-Sepharosa y SP-Sepharosa). La quimotripsina mostró una sola banda en el zymograma y el peso molecular estimado por SDS-PAGE fue de 29 kDa. El análisis de isoelecctroenfoque sugiere que la enzima es de naturaleza aniónica con un punto isoeléctrico de alrededor de 3.9, mostrando un pico máximo de actividad a pH 9.0 y a 50 °C. Quimotripsina fue altamente estable en un rango de pH de 5.5 – 11.0 y a temperaturas menores de 45°C. La enzima fue altamente inhibida por PMSF y tosil fenilalanina clorometil cetona (TPCK) en un 99% y 94%, respectivamente. Se encontró una K*m* de 7.2 µM y una eficiencia catalítica de 2.67 s^{-1} µM $^{-1}$, este último valor fue 2.43 veces mayor que el de quimotripsina de bovino.

CAPÍTULO III. Purificación y caracterización bioquímica y cinética de tripsina de las vísceras de pez diablo (*P. disjunctivus*).

En este capítulo se muestran los resultados del artículo titulado "Trypsin from viscera of vermiculated sailfin catfish, *Pterygoplichthys disjunctivus*, Weber, 1991, purification and characterization", el cual fue preparado para enviarlo a la revista Food Chemistry. La tripsina fue

purificada de las vísceras del pez diablo por fraccionación con sulfato de amonio en 30% y 70% de saturación, cromatografía de filtración en gel (Sephadex G-75), de afinidad (benzamidina) esta etapa fue crucial para lograr la separación de tripsina de quimotripsina. Y finalmente se utilizó cromatografía de intercambio iónico (DEAE-Sepharosa) para tener a la tripsina pura. El peso molecular para enzima fue de aproximadamente 27.5 kDa y mostró una sola banda en el zymograma. El pH óptimo fue de 9.5 y la temperatura óptima 40 °C, la enzima fue estable en el rango de pH de 5.5 – 11.0, manteniendo más del 80% de actividad. En cuanto a la termoestabilidad fue muy estable a temperaturas menores de 45 °C, disminuyendo drásticamente la actividad a temperaturas desde los 50 °C, se considera una enzima de baja termoestabilidad. Los valores de K*m* y kcat/K*m* fueron 0.13 mM y 11.24 s^{-1} mM^{-1}, la eficiencia catalítica de la tripsina de pes diablo fue 3.6 veces mayor que la de tripsina de bovino.

Conclusiones y recomendaciones

Integrando los resultados obtenidos al desarrollar las etapas experimentales fue posible contrastar la hipótesis planteada.

Se establecieron las condiciones para la obtención de un extracto enzimático crudo a partir de las vísceras de pez diablo, con actividad de proteasas alcalinas, así como para la purificación de tripsina y quimotripsina presentes en dicho extracto.

Las vísceras de pez diablo pueden ser consideradas una buena fuente de proteasas alcalinas. Con base en sus características bioquímicas y cinéticas, estas enzimas tienen potencial de uso en procesos industriales que involucren desde pH´s ligeramente ácidos hasta alcalinos, bajas temperaturas o condiciones hipersalinas.

Se recomienda desarrollar estrategias para la implementación y aplicación de las enzimas del presente estudio, inicialmente a nivel laboratorio, utilizando un modelo de algún proceso industrial. Además realizar estudios de comparación de eficiencia de las enzimas purificadas con las enzimas comerciales utilizas actualmente por la industria.

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CAPÍTULO I

Partial characterization of alkaline proteases from viscera of vermiculated sailfin catfish

(*Pterygoplichthys disjunctivus*,Weber, 1991).

Publicado en Fisheries Science (2011), 77:697-705

CAPÍTULO II

Purification and characterization of chymotrypsin from viscera of vermiculated sailfin catfish, *Pterygoplichthys disjunctivus*, Weber, 1991.

Enviado a Fish Physiology and Biochemistry

Purification and characterization of chymotrypsin from viscera of vermiculated sailfin catfish, *Pterygoplichthys disjunctivus*, Weber, 1991.

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Enviado a la Revista Fish Physiology and Biochemistry

Abstract

Pterygoplichthys disjunctivus viscera chymotrypsin was purified by fractionation with ammonium sulfate, gel filtration, affinity and exchange (DEAE-and SP-Sepharose) chromatography. Chymotrypsin molecular weight was approximately 29 kDa according to SDS-PAGE, shown a single band in zymogram. Electrofocusing study suggested being an anionic enzyme (pI ≈3.9), exhibiting maximal activity at pH 9 and 50 °C, using Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SAAPNA) as substrate. Enzyme was effectively inhibited by phenyl methyl sulfonyl fluoride (PMSF) (99%), and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (94%). Enzyme activity was slightly affected by metal ions (Fe^{2+} >Hg^{2+} >Mn^{2+} > K^{1+} >Mg^{2+} > Li^{1+} > Cu^{2+}). Chymotrypsin activity decreased continuously as NaCl concentration increased (from 0 to 30%). *Km* and k_{cat} values were 7.2 µM and 19.24 s^{-1}, respectively.

Keywords: chymotrypsin, enzyme purification, *Pterygoplichthys disjunctivus*, viscera.

Introduction

The search for proteases from different sources has increased in the last years. Special attention has been given on those obtained from fish viscera, which enzymes have shown an advantage over commercial proteases due to their diverse enzymatic characteristics that differ from homologous proteases obtained from warm blooded animals (Shahidi et al. 1995). Although fish proteases are basically similar to their mammalian counterparts, differences in structural and functional properties have been reported (Fong et al. 1998). Fish proteases have shown higher catalytic activities over a wide range of pH and temperature conditions (Shahidi and Kamil 2001) at relatively low concentrations (Haard 1998). Industrial applications of serine proteinases in detergent, food, pharmaceutical, leather and silk industries have also been studied (Klomklao et al. 2005).

One of the most important fish and aquatic invertebrate viscera digestive enzyme is chymotrypsin, EC 3.4.21.1. This enzyme has a much broader specificity than trypsin, cleaving peptide bonds involving bulky side chains and non polar amino acids such as phenylalanine, tyrosine, tryptophan and leucine (Simpson 2000), as well as synthetic substrates such as benzoyl-L-tyrosine-ethyl-ester (BTEE) and succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (SAAPNA) (Hue et al. 1995). Chymotrypsin have been isolated and characterized from several fish species, including freshwater fish such as common carp (*Cyprinus carpio*) (Cohen et al. 1981), grass carp (*Ctenopharyngodon idellus*) (Fong et al. 1998), rainbow trout (*Oncorhynchus mykiss*) (Kristjansson and Nielsen 1992), crucian carp (*Carassius auratus*) (Yang et al. 2009), as well as marine fish such as dogfish *(Squalus acanthias)* (Racicot and Hultin 1987), Atlantic cod (*Gadus morhua*) (Asgeirsson and Bjarnason 1991), anchovy (*Engralius japonica*) (Heu et al. 1995), monterrey sardine (*Sardinops sagax caeruleus*) (Castillo-Yáñez et al. 2006),

and recently from Japanese sea bass (*Lateolabrax japonicus*) (Jiang et al. 2010) and striped seabream (*Lithognathus mormyrus*) (El Hadj et al. 2010).

Vermiculated sailfin catfish (*Pterygoplichthys disjunctivus*), classified within the locaridae family, is native from the Amazonia Basin in Brazil and Bolivia (Gibbs et al. 2008); however, it has successfully invaded several inland waters around the world. It was introduced in México either as an ornamental fish or as a fish tank cleaner; however, somehow species made it through the wild, invading most of the inland waters south, central and parts of the northwest of Mexico. Interestingly, its viscera correspond to approximately 10% of the total body weight (Ramirez-Suarez JC, pers. Comm., 2008) *versus* 5% of most fish species. Although at the present time is considered a waste in the region where is caught, it has the potential to become an important fishing resource due to the easiness to proliferate in water bodies around the world; thus, studies about its possible use must be conducted. Previous work at our laboratory has shown a partial characterization of alkaline proteases from viscera of vermiculated sailfin catfish (*Pterygoplichthys disjunctivus*, Weber, 1991) (Villalba-Villalba et al. 2011). Hence, the aim of this study was to purify and characterize chymotrypsin from vermiculated sailfin catfish (*Pterygoplichthys disjunctivus*) intestine, thus generating basic information about this by-product.

Materials and methods

Fish samples

Vermiculated sailfin catfish specimens were obtained from the Adolfo López Mateos dam also commonly known as “El Infiernillo”, located at the boundary of the mexican states of Michoacán and Guerrero (18°52’-18°15’ North and 101°54’-102°55’ West). Samples were cryogenically

frozen *in situ* with liquid N_2, placed between layers of CO_2 and transported by airplane, to the CIAD Seafood Products Quality and Biochemistry Laboratory located in Hermosillo, Sonora, México.

Preparation of crude enzyme extract

At the laboratory, thawed vermiculated sailfin catfish specimens were dissected, their intestines (containing juice and food) removed and immediately frozen, keeping them at -80°C until further analysis. Intestines (100g) were homogenized at 20,000 × g with 200 mL of 50 mM Tris-HCl buffer (pH 8.0), with 10 mM $CaCl_2$ and 0.5 M NaCl for 2 min and centrifuged at 18,000 × g for 30 min at 2-4°C. Then, supernatant (enzyme extract) was frozen and kept at –80°C until further analysis (Heu et al. 1995; Whitaker 1994).

Enzyme purification

Crude extract was fractionated with ammonium sulfate at 30% and 70% saturation. Each time sample was centrifuged at 20,000 × *g* for 20 min at 4°C Precipitate was dissolved in 20 mL of 50 mM Tris-HCl buffer (pH 8.0) with 10 mM $CaCl_2$ (buffer A), loaded into a 1.6 cm x 120 cm G-75 Sephadex gel filtration chromatography column (Amersham Pharmacia Biotech), eluted at 0.3 mL/min and equilibrated with buffer A, collecting 5 mL fractions. Then, fractions with chymotrypsin activity were pooled and loaded into a 1.1 cm x 15 cm Benzamidine-Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech) equilibrated with same buffer. Unabsorbed fractions with chymotrypsin activity were collected for further analysis as described below. Then,

fractions were dialyzed for 24 h at 4°C using repeated changes of 20 mM Tris-HCl (pH 7.5) buffer. Dialyzed fractions were then loaded into a 1.6 x 10 cm DEAE-Sepharose Fast Flow column (Amersham Pharmacia Biotech) and equilibrated with 20 mM Tris-HCl buffer (pH 7.5) (buffer B). Unabsorbed protein was washed with same buffer, and the column was eluted with a linear gradient of NaCl from 0 to 0.5 M. Fractions revealing chymotrypsin activity were pooled and loaded into a 2.6 cm x 10 cm SP-Sepharose column (Amersham Pharmacia Biotech) pre-equilibrated with buffer B. Then, the column was washed with the same buffer, and eluted with a linear gradient of NaCl from 0 to 0.5 M in the equilibrating buffer. Active fractions eluted were pooled for further analysis.

Protein concentration determination

Protein concentration in all samples was determined according to Bradford (1976), using bovine serum albumin as standard.

Polyacrilamide gel electrophoresis (SDS-PAGE) and gelatin zymography

SDS-PAGE was carried according to the method of Laemmli (1970) using 4 and 14% stacking and separating gels, respectively. For protein band analysis, a volume of enzyme extract was mixed with two volumes of sample buffer (125mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue), heated at 95°C for 4 min, cooled immediately, and loaded into the gel. After electrophoresis, gels were stained with 0.25% Coomasie brilliant blue R-250 (CBB) in 40% methanol, 10% acetic acid or silver staining

depending of purification process (protein concentration). Bovine serum albumin (66 kDa), ovoalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α-lactoalbumin (14.2 kDa) were used as molecular weight markers. Zymogram was performed according to the procedure of Laemmli (1970) except that samples were not heated and no reducing agents were added. After electrophoresis, the gel was soaked for 30 min in 1.25% casein in 50 mM Tris-HCl at pH 8.0 at 4°C. Then, gel was immersed in same solution at 37°C for 60 min and then soaked in trichloroacetic acid for 30 min to stop the reaction, washed in distilled water, fixed and stained with 0.05% Coomasie Blue solution, and finally destained with 40% methanol and 10% acetic acid.

Electrofocusing

Isoelectric point of isolated enzyme was evaluated by analytical electrofocusing in thin-layer polyacrylamide flat gel (LKB ampholyne PAG plate) containing ampholynes in the pH ranges of 3.5-9.5. An isoelectric focusing calibration kit (Amersham Pharmacia Biotech), containing 11 proteins with known isoelectric points, was used as a reference. Proteins were stained with silver staining as described by Castillo-Yáñez et al. (2006).

Amidase activity

Chymotrypsin amidase activity was evaluated according to Erlanger et al. (1961) using 0.1 mM Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SAAPNA) as substrate. Thus, appropriate diluted enzyme

solutions (10 µL) was added to 990 µL of substrate solution at 25ºC and production of *p*-nitroaniline was measured by monitoring its increment in absorbance at 410 nm every 30 s for 10 min. One unit of the enzyme was defined as the activity which produced 1 µM *p*-nitroaniline released/min, using 8800 M^{-1} cm^{-1} as the extinction coefficient of *p*-nitroaniline at 410 nm.

Optimum pH and temperature

The effect of pH on chymotrypsin activity was evaluated using a universal buffer from pH 4 to 12 at 25°C for 15 min (Stauffer 1989). In order to study the effect of temperature over enzyme activity, extract was incubated at 25, 30, 40, 50, 60, 70 and 80°C for 15 min in 50 mM Tris-HCl buffer under optimal pH.

pH and thermal stability

The effect of pH and temperature on enzyme stability was evaluated by measuring the residual activity after incubation at various pH's (from 4 to 12) for 60 min at 25°C using universal buffers (Stauffer 1989). Enzyme solution temperature stability was evaluated by incubation at various temperatures (30, 40, 50, 60, 70 and 80°C) for 60 min.

Effect of inhibitors

In order to elucidate the effect of inhibitors on chymotrypsin activity, a series of inhibitors, at different concentrations, were evaluated. Phenylmethylsulfonyl fluoride (PMSF) (100mM in 2-

propanol), Nα-p-Tosyl-L-lysine chloromethyl ketone (TLCK) (10mM in dimethyl sulfoxide, DMSO), benzamidine (10mM in DMSO), soybean trypsin inhibitor (1g/L in distilled water), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (5mM in methanol), ethylenediamine tetra-acetic acid (EDTA) (10mM in distilled water) and pepstatin A (1mM in DMSO) were used for the analysis. Mixture of 50 µL inhibitor solution and 50 µL enzyme extract was incubated for 60 min at 25°C; then 950 µL of substrate solution (0.1 mM SAAPNA in 50 mM Tris·HCl buffer, pH 8.0) was added and residual activity was measured. Control tests were performed in absence of inhibitors.

Effect of metal ions

Samples of the purified enzyme (30 µL) were preincubated with various metal ions (KCl, LiCl, $CaCl_2$, $MnCl_2$, $MgCl_2$, $CuSO_4$, $FeSO_4$ and $HgSO_4$ at 5 mM) (70 µL) for 60 min at 25 ºC; then 900 µL of substrate solution (0.1 mM SAAPNA in 50 mM Tris·HCl buffer, pH 8.0) was added and residual activity was measured. Control tests were performed in absence of metal ions.

Effect of NaCl on enzyme activity

Chymotrypsin activity was assayed in the presence of NaCl at varying final concentrations (0, 5, 10, 15, 20, 25 and 30%, w/v). Residual activity was determined at 25 ºC at pH 8 using SAAPNA as a substrate. The activity of the enzyme in the absence of NaCl was taken as a control.

Kinetic parameters

The Michaelis-Menten constant (K_m) and catalysis constant (k_{cat}) were evaluated. The initial velocity of the enzymatic reaction was evaluated at 25°C by varying SAAPNA substrate concentration (0.01, 0.05, 0.1, 0.2, 0.4, 0.8 mM). K_m and V_{max} wer calculated from Lineweaver-Burk plots (Lineweaver and Burk 1934). The value of the turnover number or k_{cat} was calculated from the following equation $k_{cat} = V_{max}/[E]$, where [E], is the active enzyme concentration. (Heu et al., 1995; Copeland, 2000; El Hadj Ali et al., 2010).

Results and discussion

Purification of chymotrypsin

Table 1 summarizes the procedure used to purify chymotrypsin from *P. disjunctivus* viscera and the results obtained during each step. Chymotrypsin was purified 112.74 fold, achieving a 14.6% yield. Protein elution and fractions containing chymotrypsin activity resulted from gel filtration chromatography were pooled and applied to affinity chromatography (Benzamidine-Sepharose); unabsorbed fractions with chymotrypsin activity were collected and subjected to DEAE-Sepharose exchange chromatography. At this point, column was washed with buffer B (see section Enzyme purification), eluting bound proteins with a linear gradient of NaCl (from 0 to 0.4M) in the same buffer. Then, adsorbed fractions with chymotrypsin activity were applied to SP-Sepharose, thus obtaining the purified anionic chymotrypsin (Fig. 1) (called “chymotrypsin” from now on). Other anionic chymotrypsins have also been purified from different fish species, such as Monterey sardine (*Sardinops sagax caerulea*) viscera (Castillo-Yañez et al., 2006) and crucian carp (*Carassius auratus*) hepatopancreas (Yang et al., 2009).

SDS-PAGE and zymography

Purified chymotrypsin showed a single band on SDS-PAGE (Fig. 2a) and zymography (Fig. 2b) suggesting their high purity and showing only one dominant isoform of chymotrypsin in *P. disjunctivus* viscera. The purified enzyme showed a molecular weight of 29 kDa, similar to various fish chymotrypsins such as crucian carp anionic chymotrypsin (27 kDa) (Yang et al. 2009), rainbow trout (28.2 and 28.8 kDa) (Kristjansson and Nielsen 1992), grass carp (28 and 27 kDa) (Fong et al. 1998), Monterrey sardine (26 kDa) (Castillo-Yañez et al. 2006) and close to mammalian chymotrypsins (22-30 kDa).

β-mercaptoethanol (reducing agent) treatment of chymotrypsin from *P. disjunctivus* led to a single protein band (Figure 2a), in accordance to chymotrypsin from other fish species (Raae and Walther 1989; Ásgeirsson and Bjarnason 1991; Kristjansson and Nielsen 1992; Castillo-Yañez et al. 2006; Yang et al. 2009). However, Bender and Killheffer (1973) showed that bovine chymotrypsin split into various polypeptide chains (A, B, C) under reducing conditions, suggesting a different evolution of these enzymes.

Electrofocusing

This analysis suggested that *P. disjunctivus* chymotrypsin has a pI of about 3.9 (Fig. 3), supporting the assumption that this enzyme is anionic, having a high acid to basic amino acid ratio. Similar results were found by Kristjansson and Nielsen (1992), Castillo-Yañez et al. (2006) and Yang et al. (2009) for other fish chymotrypsins.

Optimum pH and pH stability

The optimum pH for *P. disjunctivus* chymotrypsin activity was 9.0 (Fig. 4), although high activity was observed throughout the pH range of 5.0 to 10.0, showing activities of 88 (pH 7.5), 94 (pH 8.0), 95 (pH 8.5), 96 (pH 9.5) and 71% (pH 10.0). This optimum pH falls in the range for alkaline fish digestive proteases. A considerable loss of activity, although still remaining, was observed at pH 4.5 and 4.0, with 56 and 48% of activity, respectively.

On the other hand, chymotrypsin showed a remarkable stability, remaining high over a broad pH range (from 5.5 to 11.0, maintaining more than 89% of activity); however, the enzyme was unstable at lower pH (4.5 and 4.0). Similar results were found by Jiang et al. (2010), Yang et al. (2009), Kristjansson and Nielsen (1992) for chymotrypsins from Japanese sea bass, crucian carp and rainbow trout, respectively.

The stability of enzymes at a particular pH may be related to its net charge at that pH; a reduced stability corresponds with a net charge change that occurs below the enzyme´s pI, thus affecting their tertiary structure (Castillo-Yañez et al. 2006).

Since *P. disjunctivus* chymotrypsin showed a high activity and stability at high alkaline pH conditions, it makes this enzyme a potential candidate for applications for certain food processing operations that requires high alkaline conditions or for its applications in detergents.

Optimum temperature and thermal stability

The optimum temperature for *P. disjunctivus* chymotrypsin activity under experimental conditions was 50°C (Fig. 5). The same result was obtained for anionic chymotrypsin from crucian carp (Yang et al. 2009) and Monterrey sardine (Castillo-Yañez et al. 2006), and similar to anchovy chymotrypsin (45°C) (Heu et al. 1995). The difference in optimal temperatures might be related to the fish inhabiting environmental area, and also to the enzymes nature (anionic *versus* cationic).

The thermal stability profile (Fig. 6) showed that *P. disjunctivus* chymotrypsin was highly stable at temperatures below 45°C, decreasing its activity sharply at temperatures above 50°C. Thermostability profile shown by this enzyme is similar to other fish chymotrypsins (Yang et al. 2009, Castillo-Yañez et al. 2006; Heu et al. 1995; Kristjansson and Nielsen 1992), showing stabilities around 30°C and being labile at 55 °C. This thermostability could be related to a high proportion of charged residues and fewer polar hydrogen-bond forming residues as suggested by Leth-Larsen et al. (1996).

This low thermostability can be beneficial for industrial applications, as these enzymes can be easily inactivated using less heat treatment (Jiang et al. 2010).

Effect of inhibitors and metal ions

The effect of several proteinase inhibitors on *P. disjunctivus* chymotrypsin activity is summarized in Table 2. As observed, the pattern of inhibition presented by these inhibitors was characteristic of other chymotrypsins, supporting the finding that the isolated enzyme is indeed, a chymotrypsin. Enzyme was almost completely inhibited by PMSF, a serine-protease inhibitor and by TPCK, a specific chymotrypsin inhibitor. Soybean trypsin inhibitor affected more than 80% of

chymotrypsin activity, and enzyme was barely affected by pepstatin A, an aspartic proteases inhibitor. Similar results were observed in crucian carp, Monterrey sardine, Atlantic cod, anchovy, and rainbow trout (Yang et al. 2009; Castillo-Yañez et al. 2006; Asgeirsson and Bjarnason 1991; Heu et al. 1995; Kristjansson and Nielsen 1992).

The effect of metal ions over enzyme activity is shown in Table 2. It can be observed that Ca^{2+} ion did not show an effect over the proteolytic activity; however, the enzyme was slightly inactivated by Mn^{2+}, K^{1+}, Mg^{2+}, Li^{1+} and Cu^{2+} ions with 96, 94, 93, 92 and 80% residual activity, respectively. On the other hand, Hg^{2+} ion greatly affected chymotrypsin activity, showing only 44% residual activity. Similar results were observed in a trypsin from *Sardinella aurita* (Khaled et al. 2011). It has been reported that Hg^{2+} ions bind to -SH groups on the enzymes, thus inhibiting their action (Klee 1988).

Effect of NaCl on enzyme activity

The effect of NaCl on *P. disjunctivus* chymotrypsin showed a continuous decreased in its activity with increasing NaCl concentration. However, it remained very active even at high salt concentrations, showing 95, 91, 85, 74, 68 and 63% residual activity at 5, 10, 15, 20, 25 and 30% of NaCl, respectively. Loss of enzyme activity is due to the denaturation of chymotrypsin caused by the “salting out” effect.

Kinetic studies

Kinetic constants, K_m and k_{cat} of purified *P. disjunctivus* chymotrypsin, hydrolyzing Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SAAPNA) at 25°C, were determined using a Lineaweaver-Burk plot. The values of K_m and k_{cat} presented by the purified chymotrypsin were 7.2 µM and 19.24 s^{-1}, respectively (Table 3). K_m was similar to that reported for grass carp chymotrypsin II (Fong et al. 1998) but lower than those from *Lithognatus mormyrus* (El Hadj et al. 2010), *Gadus morhua* (Leth-Larsen et al. 1996), *Oncorhynchus mykiss* (Kristjansson and Nielsen 1992), *Sardinops sagax caerulea* (Castillo-Yañez et al. 2006) and *Engraulis japonica* (Heu et al. 1995). This result suggests that *P. disjunctivus* chymotrypsin has higher SAAPNA affinity than the ones mentioned. k_{cat} value was similar for chymotrypsins from *Gadus morhua* (Leth-Larsen et al. 1996), *Sardinops sagax caerulea* (Castillo-Yañez et al. 2006) and *Engraulis japonica* (Heu et al. 1995) (Table 3). The catalytic efficiency (k_{cat}/K_m) value of *P. disjunctivus* chymotrypsin (2.67 $s^{-1}\mu M^{-1}$) compared with bovine chymotrypsin (0.7 $s^{-1}\mu M^{-1}$) revealed a higher catalytic efficiency (3.8 folds at 25°C).

Conclusions

Based on the results of the present study, it can be concluded that the enzyme isolated and purified from viscera of *P. disjunctivus* was chymotrypsin, which shows a high activity at pH 5.0-10.0, 15-40°C and 30% salt concentration. Results suggest the enzyme possess a potential application where low processing temperatures are needed. Further, it may find applications in laundry detergents or food industries in which high salt concentration is used such as in fish sauce production.

Acknowledgements

This study was supported by the Fondo Mixto CONACYT-Gobierno del Estado de Michoacán under the project “Desarrollo Tecnológico para el Aprovechamiento e Industrialización del Pez Diablo en la Región del Bajo Balsas en Michoacán, FOMIX # 37147.

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Fig. 6. Thermal stability of purified chymotrypsin from viscera of *Pterygoplichthys disjunctivus.*

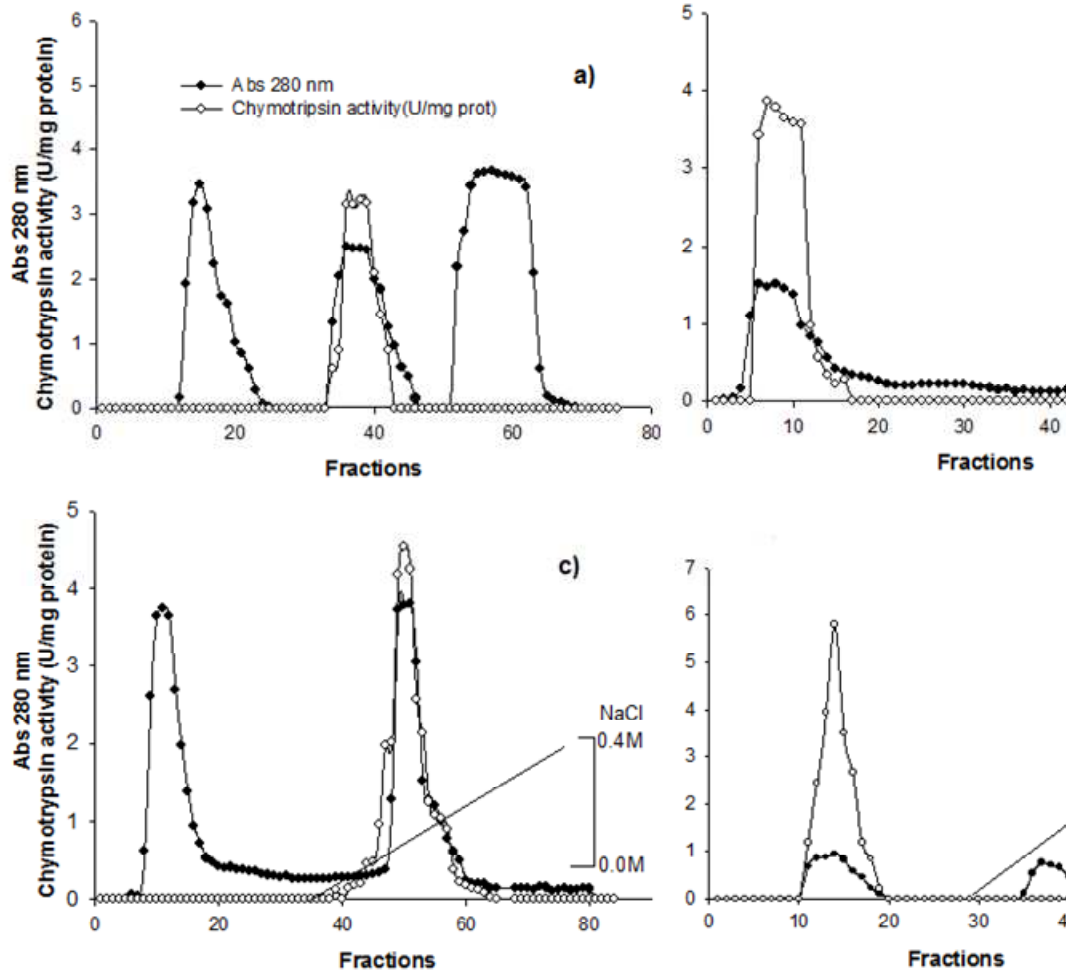

Fig. 1. Chromatographic purification of chymotrypsin from viscera of *Pterygoplichthys disjunctivus.* (a) G-75 Sephadex gel filtration chromatography. (b) Affinity chromatography. (c) DEAE-Sepharose chromatography. (d) SP-Sepharose chromatography . Absorbance 280 nm (●); chymotrypsin activity, Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA) (o).

Paint, Windows 2007

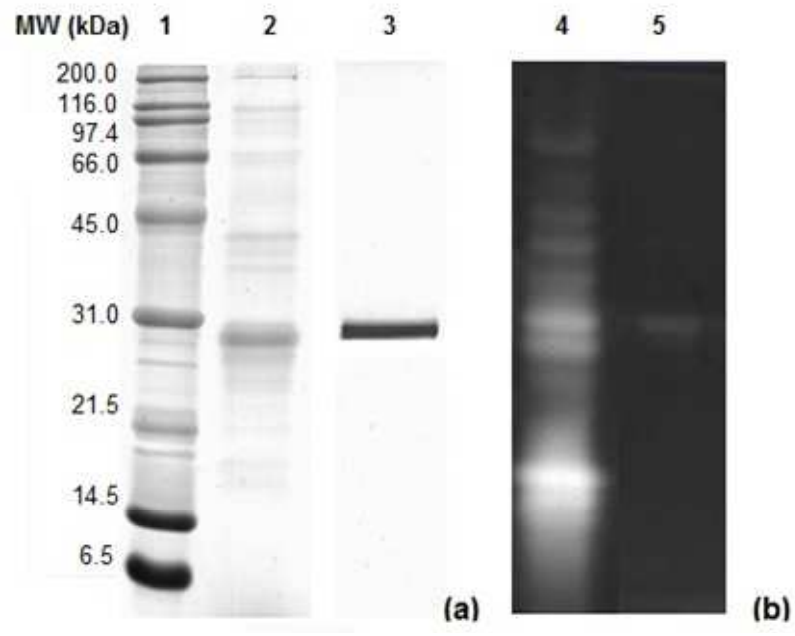

Fig. 2. SDS-PAGE (a) and gelatine zymography (b) of purified chymotrypsin from viscera of *Pterygoplichthys disjunctivus.* Lane 1: molecular weighs markers; lane 2: crude extract; lane 3: SP-Sepharose fraction; lane 4: crude extract; lane 5: SP-Sepharose fraction.

Paint, Windows 2007

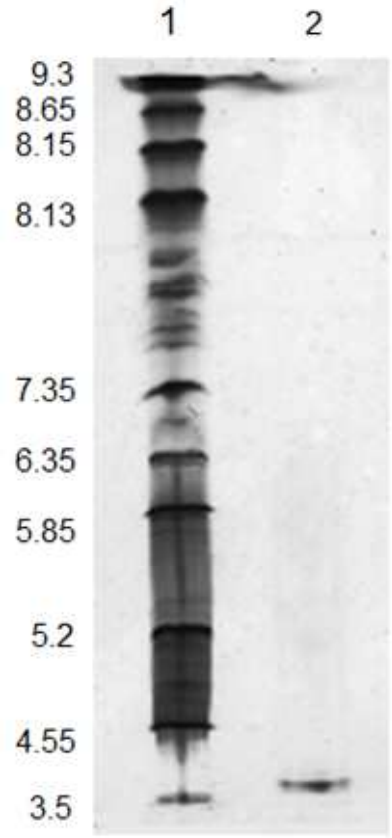

Fig. 3 Isoelectrofocusing of chymotrypsin from viscera of *Pterygoplichthys disjunctivus*. Lane 1: protein markers; lane 2: SP-Sepharose fraction.

Paint, Windows 2007

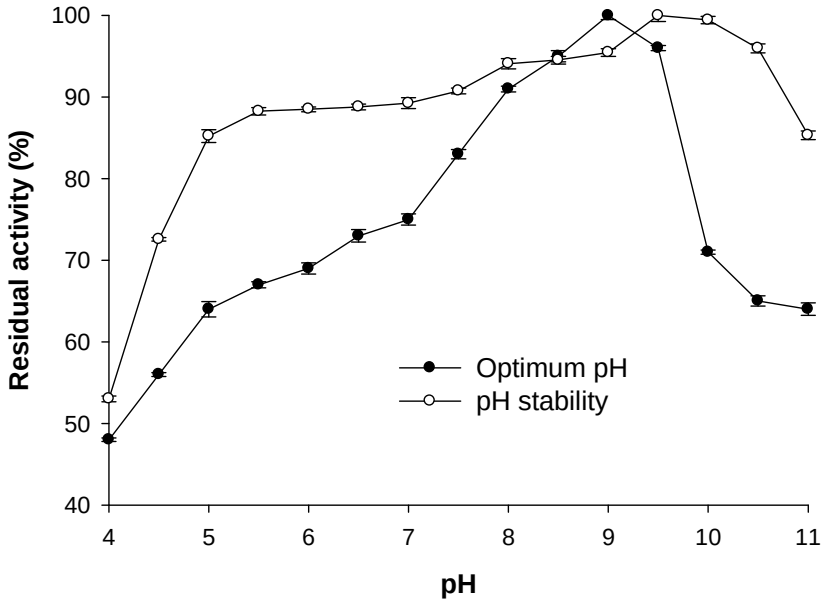

Fig. 4. Optimum pH and pH stability of purified chymotrypsin from viscera of *Pterygoplichthys disjunctivus*.

SigmaPlot 11.0

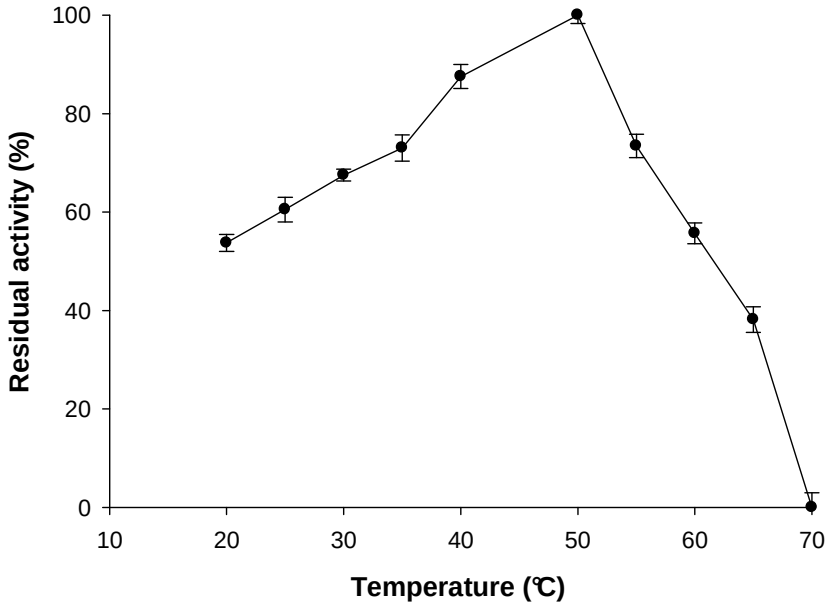

Fig. 5. Optimum temperature of purified chymotrypsin from viscera of *Pterygoplichthys disjunctivus.*

SigmaPlot 11.0

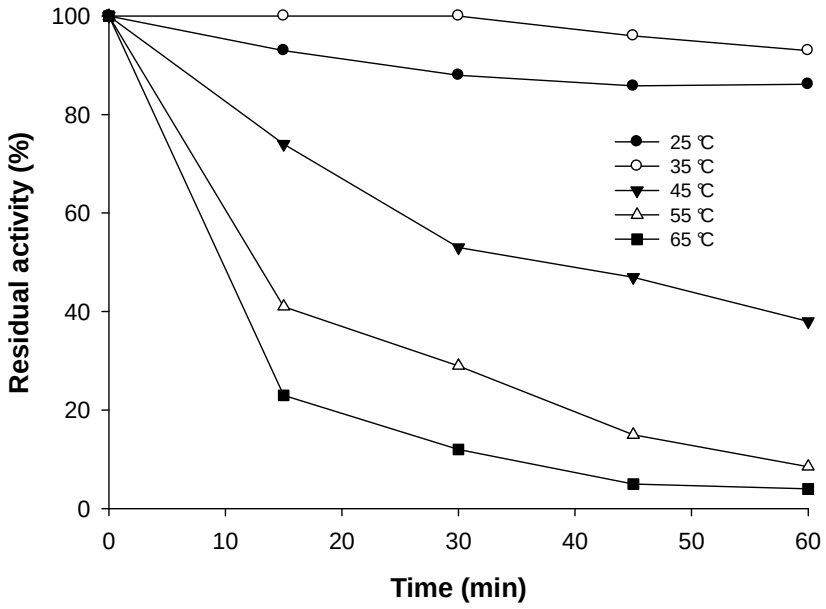

Fig. 6. Thermal stability of purified chymotrypsin from viscera of *Pterygoplichthys disjunctivus.*

SigmaPlot 11.0

Purificacion steps	Total protein (mg)	Total activity (U)	Sp. Act. (U/mg)	Purity (Folds)	Yield (%)
Crude extract	5187.12	3008.53	0.58	1	100
Ammonium sulphate	653.46	2430.87	3.72	6.41	80.80
Sephadex G-75	115.57	1367.23	11.83	20.40	56.24
Benzamidine	17.05	477.64	28.01	48.29	34.93
DEAE-Sepharose	2.42	112.15	46.31	79.84	23.48
SP-Sepharose	0.25	16.37	65.39	112.74	14.60

Table 1. A summary of purification of chymotrypsin from viscera of *Pterygoplichthys disjunctivus.*

Excel, Windows 2007

Inhibitor/Metal ion	Concentration	Residual activity (%)
Control (No inhibitor/Ion)		100
TPCK	5 mM	5.8
PMSF	5 mM	0.7
Soybean trypsin inhibitor	1 g/L	14.3
TLCK	10 mM	88.4
Benzamidine	10 mM	76.5
Pepstatin A	0.1 mM	94.6
EDTA	2 mM	80.7
$CaCl_2$	5 mM	100
$MnCl_2$	5 mM	96
KCl	5 mM	94
$MgCl_2$	5 mM	93
LiCl	5 mM	92
$CuSO_4$	5 mM	80
$FeSO_4$	5 mM	73
$HgSO_4$	5 mM	44

Table 2. Effect of protease inhibitors and metal ions on the activity of chymotrypsin from *Pterygoplichthys disjunctivus.*

Excel, Windows 2007

Enzyme	Source	Km (μM)	K_{cat} (s^{-1})	K_{cat}/Km (s^{-1} μM^{-1})	Temp	Ref
Chymotrypsin	*P. disjunctivus*	7.2	19.24	2.67	25℃	This study
Chymotrypsin	*L. mormyrus*	30.70	14.35	0.47		El Hadj et al., 2010
Chymotrypsin I	Grass carp	42	ND	ND		Fong et al., 1998
Chymotrypsin II		11	ND	ND		Fong et al., 1998
Chymotrypsin A	*L. japonicus*	0.7	1.8	2.6		Jiang et al., 2010
Chymotrypsin B		1.2	1.3	1.1		Jiang et al., 2010
Chymotrypsin	*O. mykiss*	35	2.2	0.0628		Kristjasson and Nielsen, 1992
Chymotrypsin I	*G. morhua*	12	22.7	1.89		Leth-Larsen et al., 1996
Chymotrypsin II		30.8	25	0.81		Leth-Larsen et al., 1996
Chymotrypsin	Bovine	1.3	0.9	0.7		Jiang et al., 2010
Chymotrypsin	*S. sagax caerulea*	74	18.6	0.251	30℃	Castillo-Yáñez et al., 2006
Chymotrypsin	*E. japonica*	89	14.7	0.165		Heu et al., 1995
Chymotrypsin A	*C. auratus*	1.4	2.7	1.9	37℃	Yang, et al., 2009
Chymotrypsin B		0.5	3.4	6.8		Yang, et al., 2009
Chymotrypsin	Bovine	0.8	0.9	1.1		Yang, et al., 2009

Substrate: SAAPNA. ND: No Data

Table 3. Comparison of kinetics parameters from *Pterygoplichthys disjunctivus* chymotrypsin with other chymotrypsins.

Excel, Windows 2007.

CAPÍTULO III

Trypsin from viscera of vermiculated sailfin catfish, *Pterygoplichthys disjunctivus*, Weber, 1991, purification and characterization.

Preparado para enviar a Food Chemistry

Trypsin from viscera of vermiculated sailfin catfish, *Pterygoplichthys disjunctivus*, Weber, 1991, purification and characterization.

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Preparado para enviar a la Revista Food Chemistry

Abstract

Pterygoplichthys disjunctivus viscera trypsin was purified by fractionation with ammonium sulfate, gel filtration, affinity chromatography and ionic exchange chromatography (DEAE-Sepharose). Trypsin molecular weight was approximately 27.5 kDa according to SDS-PAGE, also shown a single band in zymogram. It exhibited maximal activity at pH 9.5 and 40 ºC using N-benzoyl-DL-arginine p-nitroanilide (BAPNA) as substrate. Enzyme was effectively inhibited by phenyl methyl sulfonyl fluoride (PMSF) (100%), N-α-p-Tosyl-L-lysine chloromethyl ketone (TLCK) (85.4%), benzamidine (80.2%), and soybean trypsin inhibitor (75.6%) and partially inhibited by N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (10.3%), ethylendiamine tetra-acetic acid (EDTA) (8.7%) and pepstatin A (1.2%). Enzyme activity was slightly affected by metal ions (Fe^{2+} >Hg^{2+} >Mn^{2+} > K^{1+} >Mg^{2+} > Li^{1+} > Cu^{2+}). Trypsin activity decreased continuously as NaCl concentration increased (0-30%). K*m* and k*cat* values were 0.13 mM and 1.46 s^{-1}, respectively.

Keywords: trypsin, enzyme purification, *Pterygoplichthys disjunctivus*, viscera.

1. Introduction

The search for proteases from different sources has increased in the last years and about 50% of the total enzyme sale in industries consists of proteases. To obtain a variety of proteases, particularly those with unique properties, new sources of proteolytic enzymes have been studied, including proteases from fish, especially fish viscera (Souza, Amaral, Santo, Carvalho & Bezerra, 2007). Although fish proteases are basically similar to their mammalian counterparts, differences in structural and functional properties have been reported (Fong, Chan & Lau, 1998). Fish proteases have shown higher catalytic activities over a wide range of pH and temperature conditions (Shahidi & Kamil 2001) at relatively low concentrations (Haard 1998). Industrial applications of serine proteinases in detergent, food, pharmaceutical, leather and silk industries have also been studied (Klomklao et al. 2005). Therefore, studies describing enzymes isolated from these animals represent the first step to evaluate their potential for technological application. In fact, experiments at laboratory conditions are essential for future production at industrial scale (Silva et al, 2011).

In the digestive tract of fish, one of the main peptidases is trypsin (EC 3.4.21.4) known as the serine endoprotease that hydrolyse peptide bonds at the carboxylic end of the amino acid residues arginine (R) and lysine (K), trypsins plays major roles in biological process including digestion, activation of zymogens chymotrypsin and other enzymes of (Klomklao, Kishimura, Nonami, & Benjakul, 2009).

Trypsin have been isolated and characterized from several fish species, spotted goatfish (*Pseudupeneus maculatus*) (Souza, Amaral, Espíritu Santo, Carvalho Jr., Bezerra, 2007), brownstripe red snapper (*Lutjanus vitta*) (Khantaphant & Benjakul, 2010), hybrid catfish (*Clarias macrocephalus* x *Clarias gariepinus*) (Klomklao, Benjakul, Kishimura & Chaijan, 2011), sardinelle (*Sardinella aurita*) (Khaled et al., 2011), silver mojarra (*Diapterus rhombeus*) (Silva et al., 2011), zebra blenny (*Salaria basilisca*) (Ktari et al., 2012).

Vermiculated sailfin catfish (*Pterygoplichthys disjunctivus*), classified within the locaridae family, is native from the Amazonia Basin in Brazil and Bolivia (Gibbs, Shields, Lock, Talmadge & Farrell, 2008); however, it has successfully invaded several inland waters around the world. It was introduced in México either as an ornamental fish or as a fish tank cleaner; somehow species made it through the wild, invading most of the inland waters south, central and parts of the northwest of Mexico. Interestingly, its viscera correspond to approximately 10% of the total body weight (Ramirez-Suarez JC, pers. Comm.) *versus* 5% of most fish species. Although is considered a waste by the fisherman in Mexico, it has the potential to become an important fishing resource due to the easiness to proliferate in water bodies around the world; thus, studies about its possible use must be conducted. Previous work at our laboratory has shown a partial characterization of alkaline proteases from viscera of vermiculated sailfin catfish (*Pterygoplichthys disjunctivus*, Weber, 1991) (Villalba-Villalba et al. 2011). Hence, the aim of the present study was to purify and characterize trypsin from vermiculated sailfin catfish (*Pterygoplichthys disjunctivus*) intestine, thus generating basic information about this by-product.

2. Materials and methods

2.1. Fish samples

Vermiculated sailfin catfish specimens were obtained from the Adolfo López Mateos dam also commonly known as "El Infiernillo", located at the boundary of the mexican states of Michoacán and Guerrero (18°52'-18°15' North and 101°54'-102°55' West). Samples were cryogenically frozen *in situ* with liquid N_2, placed between layers of CO_2 and transported by airplane, to the CIAD Seafood Products Quality and Biochemistry Laboratory located in Hermosillo, Sonora, México.

2.2. Preparation of crude enzyme extract

At the laboratory, thawed vermiculated sailfin catfish specimens were dissected, their intestines (containing juice and food) removed and immediately frozen, keeping them at -80 °C until further analysis. Intestines (100 g) were homogenized at 20,000*g* with 200 mL of 50 mM Tris-HCl buffer (pH 7.5), with 10 mM $CaCl_2$ and 0.5 M NaCl for 2 min and centrifuged at 18,000*g* for 30 min at 2-4 °C. Then, supernatant (enzyme extract) was frozen and kept at –80 °C until further analysis (Heu, Kim & Pyeun, 1995; Whitaker, 1994).

2.3. Enzyme purification

Crude extract was fractionated with ammonium sulfate at 30% and 70% saturation. Each time sample was centrifuged at 20,000*g* for 20 min at 4 °C Precipitate was dissolved in 20 mL of 50 mM Tris-HCl buffer (pH 7.5) with 10 mM $CaCl_2$ (buffer A), loaded into a 1.6 cm x 120 cm G-75 Sephadex gel filtration chromatography column (Amersham Pharmacia Biotech), eluted at 0.3 mL/min and equilibrated with buffer A, collecting 5 mL fractions. Then, fractions with trypsin activity were pooled and loaded into a 1.1 cm x 15 cm Benzamidine-Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech) equilibrated with same buffer. Retained fractions were eluted by changing the mobile phase pH from 7.5 to 3.0, using a 50 mM Gly-HCl, pH 3.0 buffer. Fractions eluted were combined and dialyzed against 6 L 20 mM Tris-HCl, pH 7.5 (buffer B) (Cohen, Gertler & Birk, 1981, Simpson & Haard, 1984, Castillo-Yáñez et al., 2005). Dialyzed fractions with trypsin activity were then loaded into a 1.6 x 10 cm DEAE-Sepharose Fast Flow column (Amersham Pharmacia Biotech) equilibrated with buffer B. Unabsorbed protein was washed with same buffer, and the column was eluted with a linear gradient of NaCl from 0 to 0.4 M. Fractions revealing trypsin activity were pooled for further analysis.

2.4. Protein concentration determination

Protein concentration in all samples was determined according to Bradford (1976), using bovine serum albumin as standard.

2.5. Polyacrilamide gel electrophoresis (SDS-PAGE) and gelatin zymography

SDS-PAGE was carried according to the method of Laemmli (1970) using 4 and 14% stacking and separating gels, respectively. For protein band analysis, a volume of enzyme extract was mixed with two volumes of sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue), heated at 95°C for 4 min, cooled immediately, and loaded into the gel. After electrophoresis, gels were stained with 0.25% Coomasie brilliant blue R-250 (CBB) in 40% methanol, 10% acetic acid or silver staining depending of purification process (protein concentration). Bovine serum albumin (66 kDa), ovoalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α-lactoalbumin (14.2 kDa) were used as molecular weight markers. Zymogram was performed according to the procedure of Laemmli (1970) except that samples were not heated and no reducing agents were added. After electrophoresis, the gel was soaked for 30 min in 1.25% casein in 50 mM Tris-HCl at pH 8.0 at 4 ºC. Then, gel was immersed in same solution at 37 ºC for 60 min and then soaked in trichloroacetic acid for 30 min to stop the reaction, washed in distilled water, fixed and stained with 0.05% Coomasie Blue solution finally destained with 40% methanol and 10% acetic acid.

2.6. Specific activity

Trypsin amidase activity was evaluated according to Erlanger, Kokowski & Cohen, 1961 using 10 µL of enzyme solution was combined with 990 µL N-benzoyl-DL-arginine p-nitroanilide (BAPNA) 1 mM dissolved in 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$ buffer at 25 °C. Production of *p*-nitroaniline was measured by monitoring the increment in Abs_{410nm} every 30 sec for 10 min. BAPNA hydrolysis units (U) were calculated with the following equation: $U = A_{(410)}/min \times 1000 \times 1 / 8{,}800 \times mg$ enzyme, where 8,800 is the *p*-nitroaniline molar extinction coefficient. Esterase activity was evaluated according to Hummel (1959) using *N*-*α*-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) 1 mM as substrate. Briefly, 10 µL of enzyme solution was mixed with 990 µL of TAME dissolved in 50 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$ buffer at 25 °C. Production of *p*-tosyl-arginine was measured by monitoring the increment in Abs_{247nm} every 30 sec for 10 min. TAME units (U) were calculated with the following equation: $U = A_{(247)}/min \times 1000 \times 1 / 540 \times mg$ enzyme, where 540 is *p*-tosyl-arginine molar extinction coefficient.

2.7. Optimum pH and temperature

The effect of pH on trypsin activity was evaluated using a universal buffer from pH 4 to 12 at 25 °C for 15 min (Stauffer 1989). In order to study the effect of temperature over enzyme activity, extract was incubated at 25, 30, 40, 50, 60, 70 and 80 °C for 15 min in 50 mM Tris-HCl buffer under optimal pH.

2.8. pH and thermal stability

The effect of pH and temperature on enzyme stability was evaluated by measuring the residual activity after incubation at various pH's (from 4 to 12) for 60 min at 25 °C using universal buffers (Stauffer 1989). Enzyme solution temperature stability was evaluated by incubation at various temperatures (30, 40, 50, 60, 70 and 80 °C) for 60 min.

2.9. Effect of inhibitors

In order to elucidate the effect of inhibitors on trypsin activity, a series of inhibitors, at different concentrations, were evaluated. Phenylmethylsulfonyl fluoride (PMSF) (100mM in 2-propanol), Nα-p-Tosyl-L-lysine chloromethyl ketone (TLCK) (10mM in dimethyl sulfoxide, DMSO), benzamidine (10mM in DMSO), soybean trypsin inhibitor (1 g/L in distilled water), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (5mM in methanol), ethylenediamine tetra-acetic acid (EDTA) (10mM in distilled water) and pepstatin A (1mM in DMSO). Mixture of 50 µL inhibitor solution and 50 µL enzyme extract was incubated for 60 min at 25 ºC; then 950 µL of substrate solution (1 mM BAPNA in 50 mM Tris-HCl buffer, pH 8.0) was added and residual activity was measured. Control tests were performed in absence of inhibitors.

2.10. Effect of metal ions

Samples of the purified enzyme (30 µL) were preincubated with various metal ions (KCl, LiCl, $CaCl_2$, $MnCl_2$, $MgCl_2$, $CuSO_4$, $FeSO_4$ and $HgSO_4$ at 5 mM) (70 µL). Mixture was incubated for 60 min at 25 ºC; then 900 µL of substrate solution (1 mM BAPNA in 50 mM Tris-HCl buffer, pH 8.0) was added and residual activity was measured. Control tests were performed in absence of metal ions.

2.11. Effect of NaCl on enzyme activity

Trypsin activity was assayed in the presence of NaCl at varying final concentrations (0, 5, 10, 15, 20, 25 and 30%, w/v). Residual activity was determined at 25 ºC at pH 8 using TAME 1 mM as a substrate. The activity of the enzyme in the absence of NaCl was taken as a control.

2.12. Kinetic parameters

The Michaelis-Menten constant (K_m) and catalysis constant (k_{cat}) were evaluated. The initial velocity of the enzymatic reaction was evaluated at 25 °C by varying BAPNA substrate concentration (0.01, 0.05, 0.1, 0.25, 0.5, 0.75 and 1.0 mM). K_m and V_{max} were calculated from Lineweaver-Burk plots. The value of the turnover number or k_{cat} was calculated from the following equation $k_{cat} = V_{max}/[E]$, where [E], is the active enzyme concentration (Heu, Kim, & Pyeun, 1995; Copeland, 2000, El Hadj, N., Hmidet, N., Zouari-Fakhfakh, N., Khaled, H., & Nasri, M., 2010).

3. Results and discussion

3.1. Trypsin purification

Results on purification of trypsin extracted from viscera from *P. disjunctivus* viscera are summarized in Table 1; trypsin was purified 25.86 fold, achieving a 2.40 % yield. Gel filtration chromatography (G-75 Sephadex) separated the high molecular weight protein group with low trypsin specific activity (BAPNA), from the low molecular weight, high trypsin-like specific activity protein group. Fractions with high activity, between 36 and 41 were combined for affinity chromatography (Benzamidine-Sepharose). As shown in Fig. 1a, in affinity chromatography protein was detected in fractions 4 to 13, but without trypsin specific activity. During elution based on pH change, fractions 48 – 51 showed high trypsin specific activity were combined for of ionic exchange chromatography (DEAE-Sepharose). In ionic exchange chromatography fractions 1-75 corresponding to the “wash” with equilibration buffer, scarce protein with no enzymatic activity was detected in fractions 5 to 13. A linear gradient of NaCl was applied to fractions 75 and on, with most protein eluted from fractions 82 to 88, all showing trypsin activity (Fig.1b).

Electrophoresis showed that fractions 83, 84 and 85 generated only one band, with proteolytic activity revealed by substrate-gel electrophoresis (Fig. 2). Based on its specific activity, and on the single band on the protein gel and zymogram, this fraction was considered to contain pure trypsin.

The adsorbed trypsin in DEAE-Sepharose was regarded as an anionic enzyme; trypsins from fish intestine have high rations of acidic to basic aminoacids. The anionic nature of the trypsin like enzyme might have originated from the aminoacids composition. This is quite different from mammalian trypsins which have basic isoelectric points (Heu, Kim & Pyeun, 1995). Anionic trypsins are common in other fish such capelin (*Mallotus villosus*), salmon (*Oncorhynchus keta*), sardine (*Sardinops melanostica*), anchovy (*Engralius encrasicholus*), sardine (*Sardinops sagax caurulea*) (Murakami & Noda, 1981; Martínez, Olsen & Serra, 1988; Sekizaki, Itoh, Murakami, Toyota & Tanizawa, 2000; Castillo-Yáñez et al., 2005).

3.2. SDS-PAGE and zymography

Purified trypsin showed a single band on SDS-PAGE (Fig. 2a) and zymography (Fig. 2b) suggesting their high purity. Generally, trypsins have reported to have molecular masses between 20 and 30 kDa (Gendry & Launay, 1992). Trypsin from *P. disjunctivus* showed a molecular weight of 27.5 kDa, similar to various fish trypsins such as rainbow trout (25.7 kDa) (Kristjansson, 1991), common carp (28 kDa) (Cao et al., 2000), sardine (*Sardinops sagax caerulea*) (25 kDa) (Castillo-Yáñez et al., 2005), Atlantic bonito (*Sarda sarda*) (29 kDa) (Klomklao et al., 2007), walleye polock (24 kDa) (Kishimura, Klomklao, Benjakul & Chun, 2008), Grey triggerfish (*Balistes capriscus*) (23.2 kDa) (Jellouli et al., 2009), sardinelle (*Sardinella aurita*) (28.8 kDa) (Khaled et al., 2011), zebra blenny (*Salaria basilisca*) (27 kDa) (Ktari et al., 2012). The differences of molecular mass in trypsins may due to genetic variation among species, but the possibility that these differences are caused by autolytic degradation should not be excluded (Lu et al., 2008)

3.3. Optimum pH and pH stability

The optimum pH for *P. disjunctivus* trypsin activity was 9.5 (Fig. 3). This optimum pH falls in the range (8.0-10.0) for alkaline fish digestive proteases like sardine (*Sardinops sagax caerulea*) (pH 8.0) (Castillo-Yáñez et al., 2005), mandarin fish (*Siniperca chuatsi*) (pH 8.5) (Lu et al., 2008), silver mojarra (*Diapterus rhombeus*) (pH 8.5), (Silva et al., 2011), hybrid catfish (*Clarias macrocephalus* x *Clarias gariepinus*) (pH 8.0) (Klomklao et al., 2011). A considerable loss of activity, although still remaining, was observed at pH 5.0 and 4.5, with 48.27 and 44.83 % of activity, respectively. Trypsins generally belong to the alkaline proteinase group (Simpson & Haad, 1984), under acid conditions the charge distribution and conformation were changed and enzyme could not bind to substrate properly (Benjakul & Morrisey, 1997)

Trypsin stability at different pH remained high over a broad pH range from 5.5 to 11.0, maintaining more than 80% of activity; however the enzyme was unstable at lower pH (4.5 and 4.0). Similar results were found by Klomklao et al. 2006, Kishimura et al., 2008, Lu et al., 2008, Jellouli et al., 2009 for Tongol Tuna (*Thunnus tonggol*) , walleye pollock (*Theragra chalcogramma*), mandarin fish (*Siniperca chuatsi)*, and Grey triggerfish (*Balistes capriscus*)*,* respectively.

The stability of enzymes at a particular pH may be related to its net charge at that pH; a reduced stability corresponds with a net charge change that occurs below the enzyme´s pI, thus affecting their tertiary structure (Castillo-Yañez et al. 2006).

Since *P. disjunctivus* trypsin showed a high activity and stability at high alkaline pH conditions, it makes this enzyme a potential candidate for applications for certain food processing operations that requires high alkaline conditions or for its applications in detergents.

3.4. Optimum temperature and thermal stability

The optimum temperature for *P. disjunctivus* trypsin activity under experimental conditions was 40 °C (Fig. 4). The same result was obtained for trypsin from Grey triggerfish (*Balistes capriscus*) (Jellouli et al. 2009) and mandarin fish (*Siniperca chuatsi*) (Lu et al., 2008). The optimum temperature was similar for trypsins from other fish species such a cod (*Gadus ogac*) (Simpson & Haard, 1984) and anchovy (*Engraulis encrasicholus*) (Martínez, Olsen & Serra, 1988), which have optimum activity at temperatures between 40 and 45 °C. However, is lower than trypsin from silver mojarra (*Diapterus rhombeus*) (55 °C) (Silva et al., 2011), hybrid catfish (*Clarias macrocephalus* x *Clarias gariepinus*) (60 °C) (Klomklao et al., 2011) and zebra blenny (*Salaria basilisca*) (70 °C) (Ktari et al., 2012). The difference in optimal temperatures might be related to the fish inhabiting environmental.

The thermal stability profile (Fig. 5) showed that *P. disjunctivus* trypsin was highly stable at temperatures below 45 °C, decreasing its activity sharply at higher temperatures. The purified enzyme retaining almost 100% and about 85% of activity after 60 min of incubation at 25 °C and 35 °C, respectively. At 45 °C, trypsin retained more than 50% of activity after 15 min of incubation, but only 15% after 60 min of incubation. The enzyme was inactivated at 65 °C after 15 min of incubation. Thermostability profile shown by this enzyme is similar to other fish trypsins, like sardine (*Sardinops sagax caerulea*) (Castillo-Yañez et al. 2005), bogue (*Boops boops*) (Barkia et al., 2010) and zebra blenny (*Salaria basilisca*) (Ktari et al., 2012). This low thermostability could be related to a high proportion of charged residues and fewer polar hydrogen-bond forming residues as suggested by Leth-Larsen et al. (1996).

High activity of trypsin from *P. disjunctivus* at low temperatures may be interesting for many biotechnological and food processing applications; for other hand this low thermostability can be beneficial as these enzymes can be easier inactivated using less heat treatment in industrial applications (Jiang et al. 2010).

3.5. Effect of inhibitors and metal ions

Proteases can be classified by their sensibility to various inhibitors (North, 1982). The effect of several proteinase inhibitors on *P. disjunctivus* trypsin activity is summarized in Table 2. As observed, the pattern of inhibition presented by these inhibitors was characteristic of other trypsins, supporting the trypsin nature of this enzyme. It was 100 % inhibited by PMSF, a serine-protease inhibitor. Trypsin activity was strongly inhibited by TLCK (85.4%) and benzamidine (80.2%), both specifics inhibitors for trypsin. But, trypsin activity was not affected by EDTA, metallo protease inactivator or and pepstatin A, an aspartic proteases inhibitor. These results confirm that the single band detected by SDS-PAGE and zymogram corresponds to trypsin. Similar results were observed in Monterrey sardine (Castillo-Yañez et al. 2005), Pacific cod (*Gadus macrocephalus*) (Fuchise et al., 2009) and brownstripe red snapper (*Lutjanus vitta*) (Khantaphant and Benjakul, 2010).

The effect of metal ions over enzyme activity is shown in Table 2. It can be observed that Ca^{2+} ion did not show an effect over the proteolytic activity; however, the enzyme was slightly inactivated by Mn^{2+}, K^{1+}, Mg^{2+}, Li^{1+} and Cu^{2+} ions by 91, 89, 85, 84 and 71%, respectively. On the other hand, Hg^{2+} ion greatly affected trypsin activity, showing only 31% residual activity. Similar results were observed in a trypsin from *Sardinella aurita* (Khaled et al. 2011) and bogue (*Boops boops*) (Barkia et al., 2010). It has been reported that Hg^{2+} ions bind to -SH groups on the enzymes, inhibiting their action (Klee 1988).

3.6. Effect of NaCl on enzyme activity

The effect of NaCl on the activity of *P. disjunctivus* trypsin showed a continuous decreased in its activity was observed with increasing NaCl concentration. Loss of enzyme activity is due to the denaturation of chymotrypsin caused by the "salting out" effect. Similar results were observed in trypsin from bogue (Barkia et al., 2010). NaCl at higher concentration possibly competed with the enzyme in water binding, resulting in a stronger protein-protein interaction, which was possibly associated with precipitation (Klomklao et al., 2007). However, it remains very active even at high salt concentrations, showing 90, 85, 73, 68, 55 and 48% residual activity at 5, 10, 15, 20, 25 and 30% of NaCl, respectively. Therefore, tryspin from *P. disjunctivus* may have potencial may be used to accelerate hydrolysis of proteins under hypersaline condition in fermented products like as fish sauce.

3.7. Kinetic studies

Kinetic constants, K_m and k_{cat} of purified *P. disjunctivus* trypsin, hydrolyzing BAPNA at 25 °C, were determined using a Lineaweaver-Burk plot. The values of K_m and k_{cat} presented by the purified trypsin were 0.13 mM and 59.92 s^{-1}, respectively (Table 3). K_m was similar to that reported for sardinelle (*Sardinella aurita*) (0.125 Mm) (Khaled et al. 2011) but lower than those from anchovy (*Engraulis encrasicholus*) (Martínez et al., 1988), bigeye snapper (*P. macracanthus*) (Hau & Benjakul 2006), and brownstripe red snapper (*Lutjanus vita*) (Khantaphant & Benjakul, 2010).

This result suggests that *P. disjunctivus* trypsin has higher BAPNA affinity. k_{cat} value was similar for trypsins from anchoveta (*E. japonica*) (Heu et al., 1995), Monterey sardine (*Sardinops sagax caerulea*)

(Castillo-Yáñez et al., 2005), sardinelle (*Sardinella aurita*) (Khaled et al., 2011) and anchoveta (*E. encrasicholus*) (Martínez et al. 1988). (Table 3). The catalytic efficiency (k_{cat}/K_m) value of *P. disjunctivus* trypsin (11.24 $s^{-1}mM^{-1}$) compared with bovine trypsin (3.1 $s^{-1}mM^{-1}$) revealed a higher catalytic efficiency (3.63 folds at 25 °C).

4. Conclusions

Based on SDS-PAGE and the different activity analyses such as specific substrate used for measuring trypsin activity (BAPNA), and inhibitors susceptibly, it can be concluded that the enzyme isolated and purified from viscera of *P. disjunctivus* was trypsin with a high activity at pH 5.5-11.0, 25-40°C and 30% salt concentration. Therefore, viscera of vermiculated sailfin catfish (*P. disjunctivus)* may be an important source of trypsin, could be biotechnological tool where low processing temperatures or high salt concentration are needed.

Acknowledgements

This study was supported by the Fondo Mixto CONACYT-Gobierno del Estado de Michoacán under the project “Desarrollo Tecnológico para el Aprovechamiento e Industrialización del Pez Diablo en la Región del Bajo Balsas en Michoacán, FOMIX # 37147.

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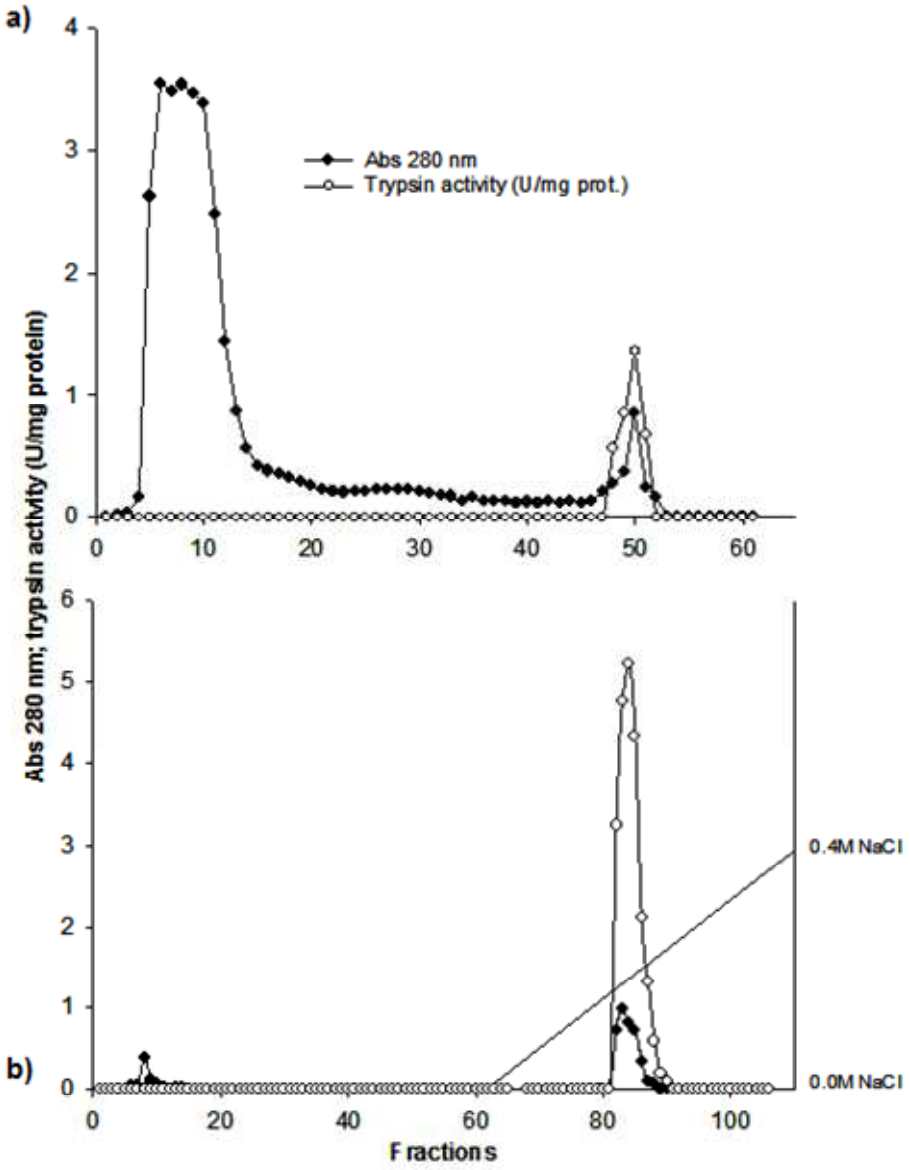

Fig. 1. Chromatographic purification of chymotrypsin from viscera of *Pterygoplichthys disjunctivus.* (b) Affinity chromatography. (b) DEAE-Sepharose chromatography. Absorbance 280 nm (●); trypsin activity (BAPNA) (o).

Paint, Windows 2007

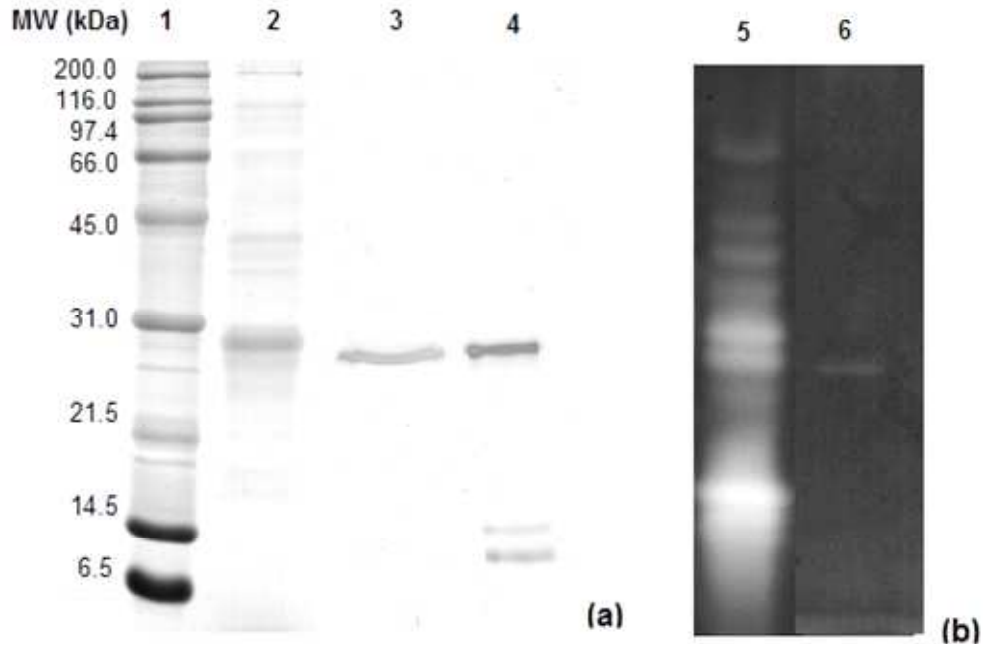

Fig. 2. SDS-PAGE (a) and gelatine zymography (b) of purified trypsin from viscera of *Pterygoplichthys disjunctivus.* Lane 1: molecular weighs markers; lane 2: crude extract; lane 3: DEAE-Sepharose fraction; lane 4: bovine trypsin; lane 5:crude extract; lane 6: DEAE-Sepharose fraction.

Paint, Windows 2007

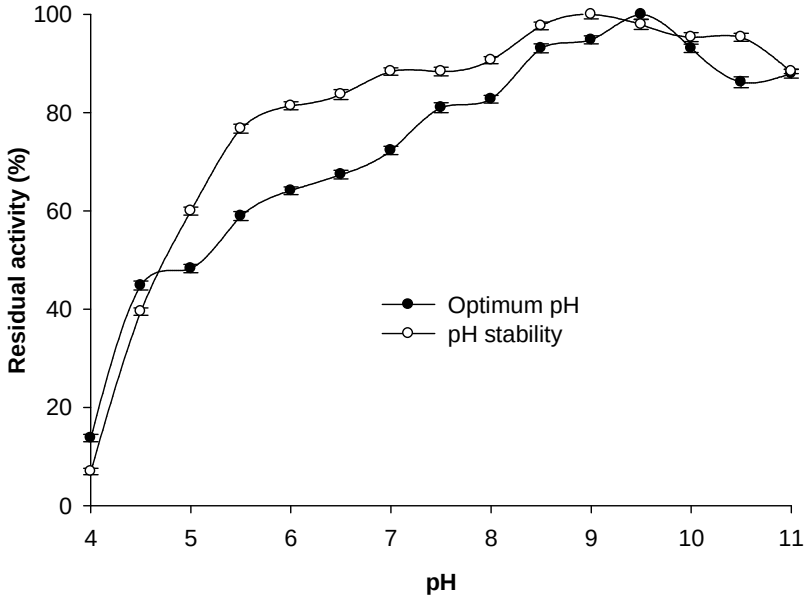

Fig. 4. Optimum pH and pH stability of purified trypsin from viscera of *Pterygoplichthys disjunctivus*.

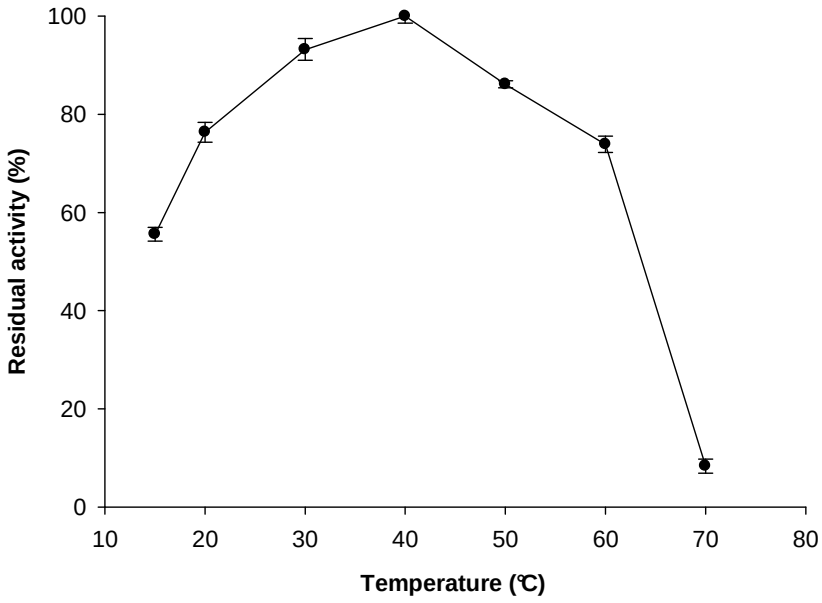

Fig. 5. Optimum temperature of purified trypsin from viscera of *Pterygoplichthys disjunctivus.*

SigmaPlot 11.0

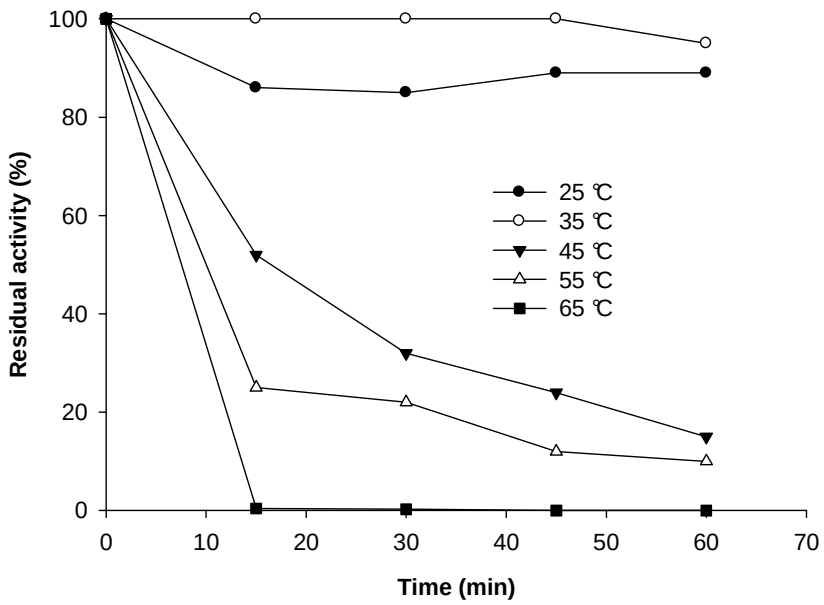

Fig. 6. Thermal stability of purified trypsin from viscera of *Pterygoplichthys disjunctivus.*

SigmaPlot 11.0

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	5187.12	3008.53	0.58	1	100
Ammonium sulfhate	1253.46	2155.95	1.72	2.96	71.66
Sephadex G-75	215.30	604.99	2.81	4.84	28.06
Affinity	13.34	102.05	7.85	13.53	16.86
DEAE-Sepharose	0.16	2.45	15.34	25.86	2.40

Table 1. A summary of purification of trypsin from viscera of *Pterygoplichthys disjunctivus.*

Excel, Windows 2007

Inhibitor/Ion	Concentration	Residual activity (%)
Control (No inhibitor/Ion)		100
PMSF	5 mM	0
TLCK	10 mM	14.6
Benzamidine	10 mM	19.8
Soybean trypsin inhibitor	1 g/L	24.4
TPCK	5 mM	89.7
EDTA	2 mM	91.3
Pepstatin A	0.1 mM	98.8
$CaCl_2$	5 mM	100
$MnCl_2$	5 mM	91
KCl	5 mM	89
$MgCl_2$	5 mM	85
LiCl	5 mM	84
$CuSO_4$	5 mM	72
$FeSO_4$	5 mM	64
$HgSO_4$	5 mM	31

Table 2. Effect of protease inhibitors and metal ions on the activity of chymotrypsin from *Pterygoplichthys disjunctivus.*

Excel, Windows 2007

Trypsin	Km (mM)	K_{cat} (s^{-1})	K_{cat}/Km (s^{-1} mM^{-1})	Temp (℃)	Reference
P. disjunctivus	0.13	1.46	11.24	25	This study
S. aurita A	0.13	2.24	17.92	25	Khaled et al., 2011
B. capriscus	0.07	2.76	40.6	25	Jellouli et al., 2009
S. officinalis	0.06	2.32	36.3	25	Balti et al., 2009
O. keta	0.029	2.29	79	25	Sekizaki et al. 2000
E. encrasicholus	0.66	3.2	4.84	25	Martínez et al., 1988
G. morhua	0.077	4	51.9	25	Asgeirsson et al., 1989
S. sagax caerulea	0.051	2.12	41	25	Castillo-Yañez et al., 2005
L. vitta	0.507	4.71	9.27	30	Khantaphant & Benjakul, 2010
E. japonica	0.049	1.55	31	45	Heu et al., 1995
P. macracanthus	0.312	1.06	3.5	55	Hau & Benjakul, 2006
Bovine	0.65 - 0.92	2	3.1	25	Asgeirsson et al., 1989

*Sustrato: Nα-benzoyl-DL-arginine-p-nitroanilide (BAPNA).

Table 3. Comparison of kinetics parameters from *Pterygoplichthys disjunctivus* chymotrypsin with other chymotrypsins.

Excel, Windows 2007.

ANEXOS

Imagen 1. Pez diablo (*Pterygoplichtys disjunctivus*). Foto por Laboratorio de Bioquímica y Calidad de Productos Pesqueros, CIAD, A.C.

Imagen 2. Vísceras de pez diablo (*Pterygoplichtys disjunctivus*). Foto por Laboratorio de Bioquímica y Calidad de Productos Pesqueros, CIAD, A.C.