UNIVERSIDAD DE SONORA

DIVISIÓN DE CIENCIAS BIOLÓGICAS Y DE LA SALUD

DEPARTAMENTO DE INVESTIGACIÓN Y POSGRADO EN ALIMENTOS

Programa de Posgrado en Ciencias y Tecnología de Alimentos

"El saber de mis hijos hará mi grandeza"

"Colágeno del Músculo de Calamar Gigante (*Dosidicus gigas*): Relación entre estructura, actividad de lisil oxidasa y comportamiento térmico"

TESIS

Como requisito parcial para obtener el grado de:

DOCTOR EN CIENCIAS DE LOS ALIMENTOS

Presenta:

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Hermosillo, Sonora

Noviembre de 2017

Universidad de Sonora

Repositorio Institucional UNISON

APROBACIÓN

Colágeno del Músculo de Calamar Gigante (*Dosidicus gigas*): Relación entre estructura, actividad de lisil oxidasa y comportamiento térmico

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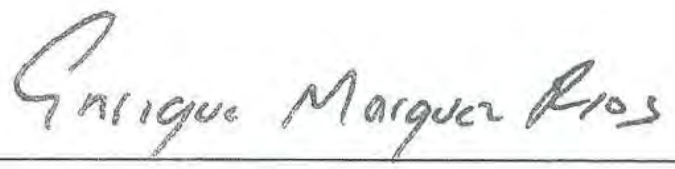

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AGRADECIMIENTOS

Al **Consejo Nacional de Ciencia y Tecnología** (CONACYT), por la ayuda económica asignada atreves del proyecto 180214 y la beca otorgada para la realización de mis estudios de doctorado.

A la **Universidad de Sonora**, por mi formación profesional y al **Departamento de Investigación y Posgrado en Alimentos** (DIPA), por darme la oportunidad de crecer académicamente y formarme como doctor en ciencias de los alimentos.

A mi comité de tesis, la **Dra. Ofelia Rouzand**, la **Dra. Hisila Santacruz,** la **Dra. Elisa Valenzuela y** al **Dr. Enrique Márquez,** por sus consejos y valiosos comentarios que enriquecieron mi trabajo y mi formación como doctor.

A mi directora de tesis, la **Dra. Marina Esquerra** por su paciencia, sus consejos, su calidad como persona, la cual siempre vio por mí, más allá de un estudiante brindándome siempre su ayuda en momentos difíciles.

Al **Dr. Wilfredo Torres**, por el apoyo y ayuda recibida para la realización de mis estudios.

A **Dra. Gabriela Ramos**, por apoyarme siempre y ser parte de mi formación profesional.

A **Javier Valdez**, por toda la ayuda brindada durante todo este tiempo.

A **Manuel Carretas, Sergio Arce, Emilio Vázquez, Odilia Higuera**, por su amistad y apoyo durante todo este tiempo.

A mis tíos **Patria Martínez, Lino Ávila**, a mi primo **Rosario Gómez** y a toda mi familia por apoyarme siempre.

A mi novia **Evangelina Padilla** por acompañarme, apoyarme, creer en mí y motivarme para mejorar siempre

DEDICATORIAS

*Dedico este trabajo a mis padres **Ramona Sainz** y **José Sarabia**, las personas más grandes en mi vida.*

*A mi abuela **Elena Sainz** y mis abuelos **Amparo Meza** y **Manuel Sarabia.***

*A mis hermanos **Ilse Sarabia** y **Andre-I Sarabia.***

Héctor Manuel

RESUMEN

El calamar gigante (*Dosidicus gigas*) se sigue considerando como una de las especies marinas de mayor importancia para la industria pesquera en varias partes del mundo, esto derivado de las características y propiedades funcionales de su músculo. Sin embargo, el filete del calamar presenta la desventaja qué durante su procesamiento y conservación, la textura de sus principales regiones anatómica que se consumen, tienden a presentar cierta dureza, incluso, después de la cocción poseen una consistencia gomosa y difícil de masticar. Mientras que la estabilidad térmica del músculo del calamar se ha atribuido a la presencia de un colágeno térmicamente estable. Y se ha establecido que las características estructurales del colágeno pueden variar dependiendo del nivel de entrecruzamiento, el cual se ha vinculado principalmente a la concentración de la piridinolina, cuya formación ocurre mediante la oxidación de residuos de lisina e hidroxilisina, catalizada por la enzima lisil oxidasa (LOX). La LOX además puede inducir la formación de otras moléculas como la hidroxi-merodesmosina, que puede afectar las propiedades del colágeno.

En este estudio, se compararon las características químico estructurales, térmicas y nanoestructurales de dos fracciones del colágeno presente en el manto, aleta y tentáculos. Se extrajeron del tejido conectivo del músculo de cada región anatómica, y a la fracción soluble en pepsina a la que se le denominó (FSP) y a la fracción sin solubilizar se le denominó insoluble (FIC). Las dos fracciones fueron posteriormente separadas a través de una columna de intercambio catiónico. El objetivo del trabajo fue establecer la interdependencia entre la actividad de la LOX, la concentración de piridinolina (Pyr), la estructura secundaria y nanoestructura del colágeno de cada región anatómica, con la firmeza de su tejido.

El músculo de la aleta presentó la mayor actividad de LOX, además, se detectó que en sus fracciones (FSP y FIC), la temperatura (T_{max}) y la entalpía (ΔH) de transición endotérmica, medidas por Calorimetría de Barrido Diferencial, fueron más bajas, que en el de las otras regiones anatómicas. Al comparar la resistencia al corte del tejido se detectó el siguiente orden: tentáculo>aletas>manto. La mayor firmeza detectada en tentáculos se puede explicar por el mayor contenido de FIC, de iminoácidos, el

mayor grado de hidroxilación de la prolina y lisina y la mayor concentración de piridinolina en su colágeno. Además, al comparar los densitogramas obtenidos por SDS-PAGE de las fracciones entre cada región anatómica, se observó que tanto FSP como FIC presentaron la mayor intensidad de la banda asociada a componentes entrecruzantes β del colágeno. El orden de la intensidad de ésta banda, fue tentáculo>aleta>manto. También, los análisis espectrofotométricos de Infrarrojo Transformada de Fourier (FTIR), de Resonancia Magnética Nuclear de Protón (RMN^1H) y de RAMAN, así como, las observaciones a nivel nanoestrucutural por Miscroscopía de Absorción de Fuerza Atómica (AFM), indicaron mayor orden molecular del colágeno presente en los tentáculos que en manto y aletas. Se detectó que había una correlación significativa entre la actividad de la lisil oxidasa (LOX) con T_{max} y el grado de hidroxilación de la prolina y lisina. En cuanto a la firmeza, ésta mostró correlación positiva con el grado de ordenamiento del colágeno medido por FTIR y el contenido de Pyr. Si bien no se detectaron correlaciones altas entre Pyr con la T_{max}, la detección de un pico asociado a los protones de la hidroximerodesmosina por RMN^1H, sugiere que además de la Pyr existen otras moléculas que afectan la estabilidad térmica del colágeno. Estos resultados proveen bases teóricas para que en un futuro se lleven estudios enfocados al desarrollo de nuevos productos a base de calamar.

ABSTRACT

Jumbo squid (*Dosidicus gigas*) is considered an important fishery resource in many countries' fishing industries due to its muscle characteristics and functional properties. However, fresh squid has a sticky consistency, and it is not easy to chew. A close relationship has been found between muscle firmness and collagen content in seafood. Pyridinoline (Pyr) is one of the major intermolecular crosslinks in collagen related to heat stability of squid collagen. The Pyr molecule is formed from the oxidation of lysine and hydroxylysine by the enzyme lysyl oxidase (LOX). LOX activity also formed another molecule, hydroxymerodesmosin, which can affect the properties of collagen.

The chemical structure, thermal denaturation and nanostructure of two collagen fractions [pepsin soluble (PSC) and insoluble (ISC)], obtained from a cation-exchange separation of the mantle, fins and tentacles of giant squid, were comparatively studied. The main idea of this work was to provide an in-depth understanding of the interdependence between LOX, pyridinoline (Pyr) content, secondary chemical structure and nanostructure of squid collagen with squid tissue firmness.

Fins showed the highest LOX activity and its collagen fractions (PSC and ISC) presented the lowest temperature and enthalpy of transition by Differential Scanning Calorimetry than the mantle and tentacles. The tentacle required more shear force than mantle and fins muscle. The tentacle firmness may be explained by the higher ISC concentration, imino amino acid content, proline and lysine hydroxylation degrees and Pyr content of its collagen. Moreover, a comparison among the regions studied, in both collagen fractions, the order of the intense intramolecular crosslinked components α band chain, from highest to lowest was tentacles> fins>mantle. In addition, the spectroscopy studies of Fourier transform infrared analysis (FTIR), nuclear magnetic resonance of proton (^{1}H NMR) and, Raman and atomic force microscopy (AFM), implied that the collagen in the tentacles, was more intermolecularly ordered than the mantle and fins. A relationship was detected among LOX activity with T_{max} and proline and lysine hydroxylation degrees. Firmness values showed a relationship with the intermolecularly ordered measured by FTIR and Pyr

content. Although, low relationship was detected among Pyr content with T_{max}, detection of hydroximerodesmosin band by ^{1}H NMR, might suggest that Pyr itself contributed partially to the collagen thermal stability. These results may provide a theoretical basis for further investigation into the development of new squid muscle products.

CONTENIDO

INTRODUCCIÓN

El colágeno es una de las proteínas más abundantes en los mamíferos, llega a constituir hasta una tercera parte del contenido proteico de un animal, su función es dar soporte y mantener la unión entre las células de los distintos tejidos en vertebrados (Gelse *et al.*, 2003). Los tejidos que requieren soportar fuerzas mecánicas, como la piel, el tendón, el hueso, el músculo, son ricos en colágenos fibrilares y colágenos asociados a fibras, por lo que el tipo de colágeno condiciona las propiedades físicas y mecánicas del tejido. Existen al menos 27 tipos de colágeno, sin embargo, en la mayoría de los organismos el tipo I es el más abundante (Muyonga *et al.*, 2004). En músculo su concentración está muy relacionada con la firmeza, por lo que sus características se vuelven cruciales para comprender la textura del músculo de varios organismos, incluyendo los marinos, como el calamar gigante (Eyre, 1987; Ando *et al.*, 2001; Badii y Howell, 2003).

El calamar gigante representa un importante recurso económico en varios países como Chile, Perú, México, China y Japón (FAO, 2016). Actualmente, existe una fuerte demanda de calamar en los mercados asiáticos, europeos y un creciente mercado en Estados Unidos. La captura de este organismo a nivel mundial representa más de 130 mil toneladas anules las cuales son para consumo humano (FAO, 2016). Esta especie generalmente es comercializada seca, fresca o cocida, sin embargo, su aceptación para el consumo está ligada con la textura del músculo, la cual es más firme que otros organismos acuáticos (Ando *et al.*, 1999). Lo anterior es trascendente desde el punto de vista comercial, ya que los atributos texturales son importantes para el consumo o el procesamiento mecánico de los filetes por la industria de alimentos de pescados y mariscos (Bjørnevik *et al.*, 2003).

El músculo de calamar ha sido ampliamente estudiado por poseer un colágeno con características inusuales para organismos marinos, ya que posee una estabilidad térmica comparable con la de organismos terrestres (Valencia-Pérez *et al.*, 2008). Esto puede deberse a su composición de aminoácidos, al orden molecular y al grado de entrecruzamiento (Ando *et al.*, 2001), ya que el colágeno de calamar se

caracteriza por contener mayor concentración de hidroxiprolina (Hyp) que cualquier otro organismo marino (Gómez-Guillen *et al.*, 2002). La concentración de Hyp es importante para la estabilidad térmica ya que estabiliza las cadenas α mediante puentes de hidrogeno. Además, se sabe que la rigidez de la molécula, es el resultado de la acumulación del entrecruzamiento covalente entre las fibras de colágeno. Este entrecruzamiento se debe a la actividad enzimática de la lisil oxidasa (LOX), quien propicia la formación de moléculas de piridinolina (Pyr) mediante la oxidación de residuos de lisina e hidroxilisina (Morales *et al.*, 2000; Ando *et al.*, 2001). Así, se ha indicado, que un alto contenido de piridinolina tiene un efecto sobre la textura del músculo y con la solubilidad del colágeno (Eyre, 1987; Badii y Howell, 2003; Ando *et al.*, 2006).

Por otro lado, cada región anatómica del calamar posee una función diferente (O´Dor, 1988), que está definida por la concentración, la solubilidad y la estabilidad del colágeno (Torres-Arreola *et al.*, 2008). Esto hace suponer que el colágeno presente en cada región poseerá diferente orden molecular, vinculado principalmente a la concentración de Pyr. Sin embargo, hay que hacer notar que la LOX además de inducir la formación de Pyr, también puede promover la formación de otras moléculas entrecruzantes en las proteínas del tejido conectivo, como la hidroximerosdesmosina (Avery y Bailey, 2008) la cual, aunque se considera un agente entrecruzante minoritario ha sido identificado, sobre todo, en el colágeno maduro de organismos terrestres (Tanzer *et al.*, 1973; Masuda *et al.*, 1976; Voet *et al.*, 2002).

Los estudios de comparación de las características estructurales, propiedades térmicas y el grado de entrecruzamiento del colágeno, entre las diferentes regiones anatómicas del calamar gigante son muy escasos. Más aún, no se ha estudiado la relación entre la actividad de LOX con la concentración de colágeno soluble en pepsina y colágeno insoluble al momento de la captura del organismo. De acuerdo a la literatura, la fracción insoluble se presupone que presenta mayor grado de entrecruzamiento, y a su vez, que tiene mayor efecto sobre la firmeza del músculo después de haber sido sometido a un tratamiento térmico. Si bien existe bastante información sobre el colágeno presente en el calamar gigante, son pocos los trabajos

que han abordado estudios de la relación existente entre la Pyr y la actividad de la LOX, con las propiedades fisicoquímicas del colágeno y con la firmeza del músculo de este organismo.

Por lo antes mencionado, el objetivo del trabajo fue establecer la interdependencia existente entre la actividad de la LOX, la concentración de Pyr, la estructura secundaria y nanoestrucutra del colágeno presente en las tres principales regiones anatómicas que se comercializan del calamar gigante con la firmeza de su tejido. La información generada en este estudio servirá de base teórica para el desarrollo de estudios tendientes al desarrollo de nuevos procesos tecnológicos y a un mejor aprovechamiento del calamar gigante.

REVISIÓN BIBLIOGRÁFICA

Importancia del Calamar Gigante en la Industria Pesquera

El calamar gigante, a pesar de las bajas producciones que se han presentado a nivel mundial en los últimos años, sigue siendo considerado un recurso económico importante, esto derivado de la alta demanda a nivel comercial que el mismo posee (FAO, 2017). Lo atractivo del músculo de este organismo desde el punto de vista industrial es su coloración y las características inusuales de su textura, por lo que puede ser usado como base para la obtención de una amplia gama de productos, tales como los concentrados proteicos (Gómez-Guillen *et al.*, 2003; Sánchez-Alonso *et al.*, 2007; Gálvez-Rongel *et al.*, 2013) o bien enlatarse y ofrecerse como producto tipo abulón (CONAPESCA, 2013).

Las principales regiones que se comercializan son el manto, las aletas y los tentáculos, principalmente como productos frescos congelados, pero en diferentes presentaciones, como el caso de las anillas o aros tratadas contra la acidez, o los tentáculos en forma de flores (Luna-Raya *et al.*, 2009).

Características del Músculo de Calamar Gigante

La distribución histológica y las propiedades bioquímicas de las proteínas en el músculo de los organismos acuáticos son de suma importancia para proporcionar información fundamental sobre sus funciones en el desarrollo de la textura de los tejidos comestibles. El colágeno y las fibras musculares son componentes proteicos en los tejidos de los animales multicelulares que cumplen funciones mecánicas y fisiológicamente importantes para la textura del músculo. Las fibras de colágeno se distribuyen principalmente en el tejido conectivo del músculo (epimisio, perimisio y endomisio) rodeando las fibras del tejido muscular (Bremner y Hallett, 1985) y la degradación de estas proteínas está relacionada con la pérdida de los parámetros texturales en pescados y mariscos (Ayala *et al.*, 2010).

En el caso particular del calamar gigante fresco, este posee una consistencia pegajosa por lo que no es fácil de masticar y esta consistencia está relacionada con la distribución única de sus fibras musculares (Figura 1) (Otwell y Hamann, 1979a;

Otwell y Hamann 1979b; Otwell y Giddins, 1980). Las fibras musculares del calamar están repartidas en múltiples capas reforzadas y endurecidas por el colágeno del tejido conectivo, el cual es de 3 a 5 veces más abundante que en los peces, cuyas fibras poseen la décima parte de la densidad de las fibras presentes en el músculo del calamar (Mizuta *et al*., 2000). Además, el músculo del calamar se encuentra intercalado entre dos túnicas de tejido conectivo, una interna y otra externa, las cuales representan cerca del 5% del grosor total, siendo su principal componente el colágeno.

Asimismo, se ha observado que después de un proceso de cocción, el músculo del calamar se contrae aumentando la resistencia al corte y cuando es sometido a un proceso de secado el tejido puede ser cortado transversalmente, pero no longitudinalmente. Estas propiedades únicas exhibidas por el calamar, son atribuidas a la estructura y composición bioquímica del colágeno (Maza *et al*., 2003).

Características del Colágeno de Organismos Marinos

La concentración de tejido conectivo de organismos marinos es menor que en terrestres. En peces el colágeno juega un papel muy importante para el mantenimiento de la estructura del músculo, ya que proporciona la fuerza tensil y está estrechamente relacionado con la capacidad natatoria (Sato *et al*., 1989).

La concentración de colágeno en los organismos marinos puede variar del 1 al 12% del total de proteínas y entre un 0.2 y 2.2 % del peso en músculo (Sikorski y Borderias, 1994). En el caso particular del músculo de calamar gigante, este puede poseer una concentración de colágeno hasta un 18.33% (Torres-Arreola *et al*., 2008), la cual puede variar dependiendo de la función fisiológica que posea cada región anatómica (Montero y Gómez-Guillen, 2000; Morales *et al*., 2000). Otro factor que influye en el contenido de colágeno es la edad y el sexo del organismo sujeto de estudio (Morales *et al*., 2000).

En cuanto a las características químico estructurales del colágeno de los cefalópodos, éstas son similares al colágeno de mamíferos (Thuy *et al*., 2014). A la fecha se han identificado dos tipos de colágeno en varias especies de calamar, considerándose como el más abundante el colágeno tipo I.

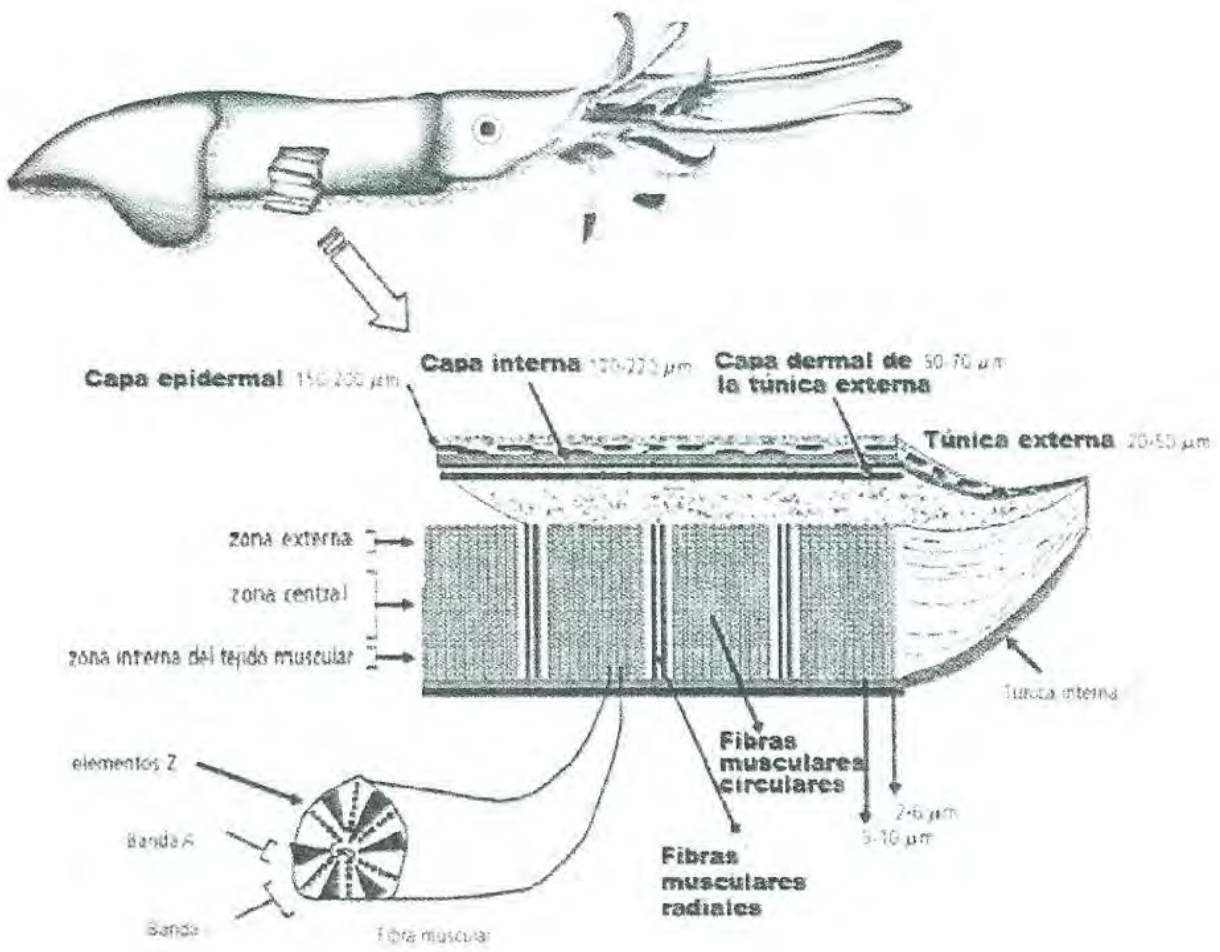

Figura 1. Representación de la estructura del tejido muscular en manto de calamar. Túnica externa e interna del manto. Hacia dentro se encuentra la capa epidermal, la capa interna y la capa dermal. Entre la túnica externa e interna se ubican las fibras musculares circulares, las fibras musculares radiales, estas dos regiones se dividen en tres zonas, externa, central y una zona interna del tejido muscular. La fibra muscular con los elementos Z, la banda A y banda I.

Fuente: Mizuta *et al.* (2000).

El segundo tipo de colágeno, el cual se ha reportado que está presente en bajas concentraciones, es el colágeno tipo V (Morales *et al.*, 2000; Kittiphattanabawon *et al.*, 2015).

Para entender las diferencias entre las características de textura del calamar con las otras especies, es necesario purificar y caracterizar completamente la proteína de colágeno, lo cual no se ha realizado en esta especie. Después de la obtención mediante solubilización con pepsina, es importante examinar las características de colágeno en su forma de fibra, para comprender al desarrollo de la textura y las propiedades físicas de la carne. La digestión con pepsina altera la estructura de las cadenas de colágeno debido a que elimina la región telopetidica y los entrecruzamientos. Sin embargo, el músculo del calamar posee fibras con alto grado de entrecruzamiento incluso después de la digestión enzimática.

Estructura del Colágeno

El colágeno está formado por tres subunidades en forma de triple hélice llamadas cadenas α. Las tres cadenas están enrolladas con giro dextrógiro, mientras que cada cadena α posee giro levógiro. Las cadenas α pueden variar en tamaño y composición, dando lugar a diferentes tipos de colágeno (Lehninger *et al.*, 2001). Existen al menos 27 tipos (I-XXVII) de los cuales solo el tipo I y V se han reportado en músculo, piel, escamas y espinas de organismos acuáticos (Montero *et al.*, 1990), a diferencia del tipo III, el cual es abundante en mamíferos (Mizuta *et al.*, 2002).

La resistencia de los tejidos a estirarse es debida a la estructura en triple hélice del colágeno (Figura 2). El plegamiento de la triple hélice del colágeno se debe a la abundancia inusual de glicina, prolina e hidroxiprolina. Estos aminoácidos forman la peculiar repetición de Gly-X-Y, donde X e Y generalmente están ocupados por prolina (Pro) e Hidroxiprolina (Hyp) y en menor proporción Lisina (Lys) (Lehninger *et al.*, 2001). La cadena lateral de glicina con un átomo de H, es la única que puede caber en el centro de una hélice de tres cadenas. Los puentes de hidrógeno, establecidos entre los grupos amino (N-H) de un residuo de glicina, con un grupo carbonilo (C=O) del enlace peptídico en la cadena adyacente, ayudan a mantener juntas las tres cadenas del polipéptido.

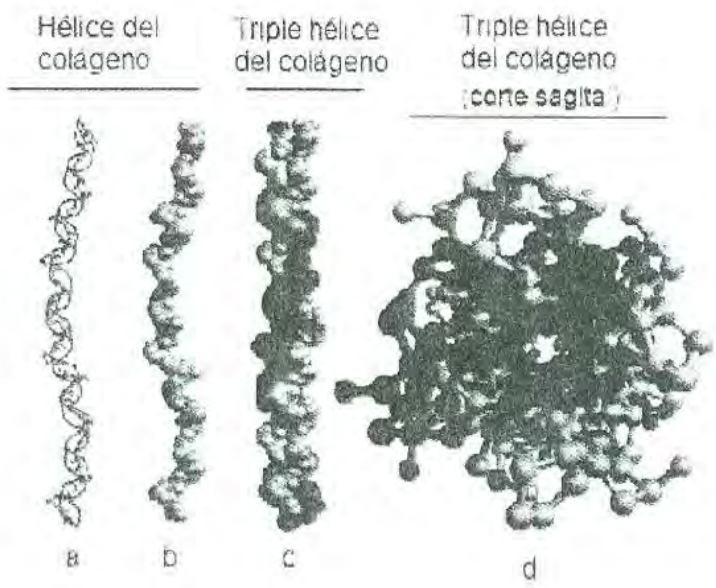

Figura 2. Estructura del colágeno. (a) Cadena α de colágeno con estructura helicoidal levógira; (b) modelo de esferas de la misma cadena α; (c) tres hélices α enrolladas entre sí de forma dextrógira; (d) la superhélice de tres cadenas del colágeno vista desde un extremo, en representación de bolas y varillas.
Fuente: Lehninger, 2001.

El ángulo fijo del enlace C-N de la peptidil-prolina o un enlace peptidil-hidroxiprolina, permite a cada cadena plegarse en una geometría tal que forman una triple hélice, es decir tres cadenas polipeptídicas giran en conjunto, formando así la molécula de tropocolágeno (Li *et al.*, 2005). Cada molécula de tropocolágeno posee 300 nm de longitud y pueden formar fibrillas con un diámetro entre 50-200 nm mediante interacciones laterales, donde la unidad es una molécula de triple cadena helicoidal. Este tipo de acomodo espacial induce que el colágeno presente un alto grado de polimorfismo, formando una gran variedad de diferentes estructuras (Lin, 2002).

Otra característica estructural de las moléculas de colágeno es que poseen segmentos muy cortos llamados telopéptidos, los cuáles no forman parte de la estructura helicoidal, pero son importantes en la formación de fibrillas de colágeno. Estos segmentos contienen hidroxilisina, un aminoácido inusual, que participa en la formación de enlaces covalentes, induciendo el entrecruzamiento de la molécula (Asghar y Henrickson, 1982). Este entrecruzamiento se presenta tanto intra- como inter-molecularmente e involucra principalmente a la lisina e hidroxilisina y los residuos de aldehído derivados de ellos, y se localiza principalmente en las regiones telopeptídicas (Ricard-Blum y Ruggiero, 1996).

El grado de entrecruzamiento es altamente variable y depende del tipo de colágeno, tejido, especies animales, edad, entre otros. Las alteraciones en el grado de entrecruzamiento intra- e inter-molecular son derivados de la desaminación oxidativa de los grupos ε-NH_2 de la lisina e hidroxilisina (Badii y Howell, 2003; Asghar y Henrickson, 1982). Debido a que el entrecruzamiento estabiliza de lado a lado las moléculas de colágeno, se generan fibras más fuertes que repercuten en la consistencia del tejido muscular, así como en la solubilidad y estabilidad térmica del colágeno (Badii y Howell, 2003; Asghar y Henrickson, 1982).

Estabilidad Térmica del Colágeno en Organismos Marinos

La estabilidad térmica del colágeno se atribuye a los aminoácidos que estabilizan su triple hélice, principalmente por el contenido de prolina e hidroxiprolina (Nomura *et al.*, 2000). En particular la hidroxiprolina juega un papel preponderante en la estabilización de la triple hélice del colágeno, ya que tiene la capacidad de formar

puentes de hidrógeno a través de su grupo -OH (Burjanadze, 1979, 1982; Ledward, 1986). Se ha detectado que colágenos con baja concentración de prolina e hidroxiprolina tienden a desnaturalizarse a temperaturas menores, que aquellos que los contienen en concentraciones mayores.

Al comparar el colágeno de los organismos marinos con el de los terrestres, este generalmente posee menor concentración de hidroxiprolina (Fennema, 2002) y menor grado de entrecruzamiento. (Badii y Howell, 2003; Sikorski y Borderias, 1994). Sin embargo, hay que hacer notar que debido a la diversidad de organismos marinos y al hábitat donde se pueden desarrollar éstos, tanto el contenido de hidroxiprolina, como el grado de entrecruzamiento del colágeno varían gradualmente (Sato *et al.*, 1989). Así se detectó que los colágenos presentes en la piel del tiburón azul (*Prionace glauca*) y en las espinas de bacalao (*Gadusmorua*) poseían una menor temperatura de desnaturalización (T_d) comparado con el colágeno presente en la piel del cerdo, atribuido su menor contenido de prolina e hidroxiprolina detectado en la molécula (Nomura *et al.*, 2000; Badii y Howell, 2003). Mientras que el colágeno extraído de las escamas de carpa (*Cyprinuscarpio*) y siluro gigante (*Pangasianodon gigas*), presentaron alto porcentaje de prolina e hidroxiprolina (~21%) y una T_d aproximada de 35 °C, cercana al rango de la de piel de cerdo (Rigby, 1968; Pati *et al.*, 2010). Lo cual se atribuyó a la amplia variación de temperatura del hábitat en el cual éstos últimos se desarrollan, la cual es de 5°C en invierno y 45°C en verano.

De los estudios llevados a cabo en cefalópodos, se tiene que el colágeno presente en el pulpo (*Octopus vulgaris*) presentan T_d consideradas bajas, entre 25-27°C (Mizuta *et al.*, 2003). Sin embargo, en calamar las T_d son más altas (>50%) (Valencia-Pérez *et al.*, 2008; Uriarte-Montoya *et al.*, 2010; Arías-Moscoso *et al.*, 2011). Al analizar el contenido de hidroxiprolina en el colágeno extraído de diferentes regiones anatómicas del calamar gigante, se ha detectado una alta concentración de prolina (Torres-Arreola *et al.*, 2008; Uriarte-Montoya *et al.*, 2010; Arías-Moscoso *et al.*, 2011) comparado con lo reportado para otras especies marinas (Gómez-Guillén *et al.*, 2002). Además, se ha reportado que el colágeno presente en el calamar

gigante posee un alto grado de hidroxilación de la lisina (Gómez-Guillén *et al.*, 2002), la cual influye en el grado de entrecruzamiento de la proteína.

Entrecruzamiento de las Fibras de Colágeno

El entrecruzamiento es un importante mecanismo de modificación de las proteínas que ocurre intra- e intermolecularmente entre las fibras de colágeno. Estos cambios pueden presentarse durante el crecimiento y desarrollo del animal, o bien durante el manejo post-captura del producto (Badii y Howell, 2003; Ando *et al.*, 2001). Una vez sintetizadas y fuera del espacio intracelular, las fibras de tropocolageno son ensambladas mediante interacciones cabeza cola. Estas interacciones no covalentes, que se establecen inicialmente entre las moléculas de colágeno que formaran la fibrilla, no proporcionan la suficiente fuerza mecánica para mantener la estructura. Para aumentar la fuerza tensil y estabilidad mecánica, se realiza un proceso adicional de estabilización mediante la formación de enlaces covalentes. Los residuos que participan en la formación de estos enlaces estabilizadores son la Lys e Hyl localizadas en la región telopéptidica o en regiones de la triple hélice relajadas (tripletes que contienen poca prolina). Para ello los residuos son oxidados por la acción de la lisil oxidasa, promoviendo la condensación para la formación de enlaces covalentes entre las fibras de colágeno.

La oxidación de la lisina e hidroxilisina por la actividad de la enzima da como resultado distintas moléculas tales como la piridinolina, desoxi-piridinolina, hidroxi-ferodesmosina, 5-hidroxi-lisino-norleucina, glucosil-galactosil-hidroxi-lisino-norleucina, responsables del entrecruzamiento covalente de las fibras de colágeno (Masuda *et al.*, 1976, Borel, 1991, Leroy *et al.*, 2014). La condensación entre los residuos de Lys e His entre las cadenas laterales, llevan a la formación de la formación de histidinodihidrohidroxi-merodesmosina (Voet *et al.*, 2002).

La alta estabilidad térmica del músculo del calamar se ha atribuido principalmente a la presencia de la piridinolina (Ando *et al.*, 2001). La presencia de dicha molécula puede afectar la solubilidad del colágeno. En tejido conectivo extraído a partir del manto de varias especies de calamares (*Loligoforbensil, Loligopealei, Sepia pharaonis* y *Sepia officinalis*) se ha detectado que la solubilidad disminuye hasta un

70% después de un tratamiento de 30 min en agua hirviendo. Así mismo, se ha sugerido que existe una mayor concentración de la fracción insoluble de colágeno, la cual se presupone que es la que afecta mayormente la estabilidad térmica del colágeno en el músculo del calamar (Ando *et al.*, 2001). El colágeno posee otras estructuras entrecruzantes generadas por la actividad de la LOX, como la hidroximerodesmosina, la cual también se ha vinculado con la estabilidad de la molécula (Tanzer *et al.*, 1973; Masuda *et al.*, 1976; Voet *et al.*, 2002), aunque la mayoría de los estudios se han centrado en la piridinolina.

Formación de Piridinolina

El mecanismo de formación de la piridinolina (Figura 3) ha sido ampliamente estudiado en mamíferos, existiendo poca información en organismos marinos (Eyre *et al.*, 2005). La piridinolina se produce a partir de la oxidación de los aminoácidos lisina e hidroxilisina por la actividad de la lisil oxidasa, la cual cataliza la desaminación oxidativa de la lisina e hidroxilisina, convirtiéndolas en los correspondientes aldehídos (Smith-Mungo y Kagan, 1998; Kagan y Li, 2003). Posteriormente se producen reacciones químicas de condensación, sin la participación de otras enzimas.

La piridinolina se forma por medio de dos rutas. La ruta de alisina y la ruta de la hidroxialisina. En la primera, un residuo de lisina (Lys) dentro del telopéptido se convierte en el aldehído alisina por la acción de LOX, mientras que, en la segunda un residuo de hidroxilisina dentro del telopéptido se convierte en el aldehído hidroxialisina. En ambas rutas, el aldehído reacciona posteriormente con un residuo de Lys, Hyl y/o histidina (His) dentro de la triple hélice para formar enlaces cruzados di-, tri- o tetra-funcionales (Reiser *et al.*, 1992, Robins, 1983). Se ha propuesto que la formación de la piridinolina se puede deber a que se forman dos residuos oxidados (cetoiminas) los cuáles inducen el cierre del anillo mediante una reacción de oxidación (Eyre y Oguchi, 1980) o bien a través de una reacción entre cetoimina con una aldehído-hidroxilisina libre presente en un segundo telopéptido en la misma molécula de colágeno (Robins y Duncan, 1983). En ambos casos las cetoiminas desaparecen de los tejidos maduros y las piridinolinas se convierten esencialmente en los únicos residuos de entrecruzamiento (Wu y Erye, 1984).

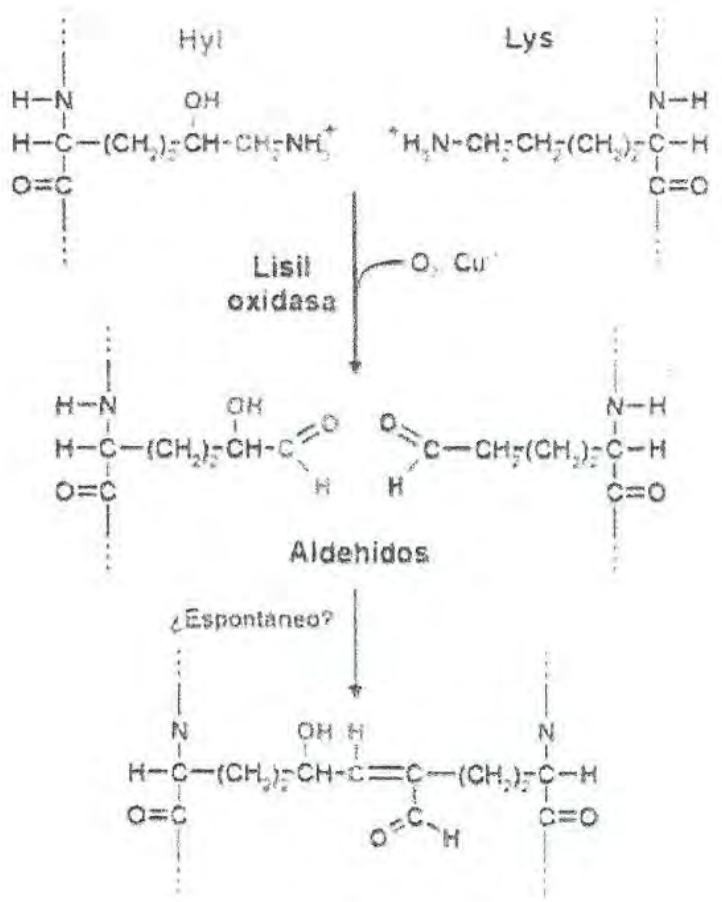

Figura 3. Oxidación de los residuos de Lys e Hyl por la lisil oxidasa para la formación del entrecruzamiento covalente.

LOX en Organismos Marinos

Los primeros reportes acerca de la presencia de la enzima en organismos marinos fueron realizados en el pez cebra y en salmón, enfocándose en la secuenciación del gen que codifica para la enzima (Consuegra y Johnston, 2006; Reynaud *et al.*, 2008). Sin embargo, los reportes acerca de su actividad en el músculo de organismos marinos son relativamente recientes. Uno de estos reportes se realizó en el músculo del calamar gigante (Torres-Arreola *et al.*, 2011). Posteriormente, se detectó que había una correlación positiva entre la actividad de la enzima, la concentración de piridinolina y el aumento de la textura que presentaba el manto del calamar gigante durante el almacenamiento en hielo (Ramírez-Guerra *et al.*, 2015).

Debido a que, además del manto del calamar a nivel internacional se comercializan tentáculos y aletas, y que la piridinolina no es la única estructura que puede afectar la estabilidad de la molécula de colágeno (Miles *et al.*, 2005), se considera que es importante realizar más estudios que profundicen y permitan establecer la relación existente entre la actividad de la LOX, la concentración piridinolina, la presencia de otra molécula entrecruzante en el colágeno, la estabilidad térmica de la molécula y la firmeza del músculo.

BIBLIOGRAFÍA

Ando, M., Tsukamasa, Y., Makinodan, Y. y Miyoshi, M. (1999). Muscle firmness and structure of raw and cooked arrow squid mantle as affected by freshness. *Journal of Food Science*, *64*(4), 659-662.

Ando, M., Makino, M., Tsukamasa, Y., Makinodan,Y. y Miyoshi, M. (2001). Interdependence between heat stability and pyridinoline contents of squid mantle collagen. *Journal of Food Science. 66*(2), 265-269.

Ando, M., Nakagishi, Y., Yoshida, K., Nakao, M., Nakawawa, T., Makinodan, Y., Tsukamasa, Y. y Kawasaki, K. (2006). Pyridinoline concentration in muscular and skin collagen of fish and relationship between collagen solubility and pyridinoline concentration in fish muscular collagen. *Fisheries Science 72*(5), 1104-1108.

Asghar, A. y Henrickson, R. L. (1982). Chemical, biochemical, functional, and nutritional characteristics of collagen in food systems. *Advances in Food Research*, *28*, 231-372.

Ayala, M. D., Abdel, I., Santaella, M., Martínez, C., Periago, M. J., Gil, F., Blanco, A. y Albors, O. L. (2010). Muscle tissue structural changes and texture development in sea bream, *Sparus aurata* L., during post-mortem storage. *LWT-Food Science and Technology*, *43*(3), 465-475.

Badii, F. y Howell, N. (2003). Elucidation of the effect of formaldehyde and lipids on frozen stored cod collagen by FT-raman spectroscopy and differential scanning calorimetry. *Journal of Agriculture and Food Chemistry, 51*(5), 1440-1446.

Bjørnevik, M., Karlsen, Ø. Johnston, I. A., y Kiessling, A. (2003). Effect of sustained exercise on white muscle structure and flesh quality in farmed cod (*Gadus morhua L.*). *Aquaculture Research, 34*(1), 55-64.

Borel J. P. (1991). Les collagènes. *l'Eurobiologiste* 25(195): 247-271

Bremner, H. A. y Hallett, I. C. (1985). Muscle fiber–connective tissue junctions in the fish blue grenadier (*Macruronus novaezelandiae*). *Journal of Food Science, 50*(4), 975–980.

Burjanadze, T. V. (1979). Hydroxyproline content and location in relation to collagen thermal stability. *Biopolymers*, *18*(4), 931-938.

Burjanadze, T. V. (1982). Evidence for the role of 4-hydroxyproline localized in the third position of the triplet (Gly-X-Y) in adaptational changes of thermostability of a collagen molecule and collagen fibrils. *Biopolymers*, *21*(8), 1489-1501.

CONAPESCA (2013). Estadísticas de Producción Pesquera. Comisión Nacional de Acuacultura y Pesca. www.conapesca.gob.mx.

Consuegra. S. y Johnston, I. (2006). Polymorphism of the lysyl oxidase in relation to muscle collagen crosslink concentration in Atlantic salmon. *Aquaculture Research, 37*(12), 1699-1702.

Eyre, D. (1987). Collagen cross-linking amino acids. *Methods in Enzymology*, *144*, 115-139.

Eyre, D. y Wu, J. (2005). Collagen cross-links, En: *Collagen. Topics in Current Chemistry,* vol. 247. Brinckmann J., Notbohm H., Müller P.K. (eds). Springer, Berlin, Heidelberg.

Eyre, D. R. y Oguchi, H. (1980). The hydroxypyridinium crosslinks of skeletal collagens: their measurement, properties and a proposed pathway of formation. *Biochemical and Bbiophysical Research Communications*, *92*(2), 403-410.

FAO (2017). Food and Agriculture Organization of the United Nations, Globefish-Analysis and Information on World Fish Trade. (Market reports 2017). http://www.fao.org/in-action/globefish/market-reports/cephalopods/en/.

Fennema, O. (2002). *Química de los Alimentos*. 2da ed. Acribia, España.

Gálvez-Rongel, A., Ezquerra-Brauer, J. M., Ocano-Higuera, V. M., Ramirez-Wong, B., Torres-Arreola, W., Rouzaud-Sandez, O. y Marquez-Rios, E. (2014). Methods to obtain protein concentrates from jumbo squid (*Dosidicus gigas*) and evaluation of their functionality. *Food Science and Technology International, 20*(2), 109-117.

Gómez-Guillén, M. C., Martínez-Alvarez, O. y Montero, P. (2003). Functional and thermal gelation properties of squid mantle proteins affected by chilled and frozen storage. *Journal of Food Science*, *68*(6), 1962-1967

Gómez-Guillén, M. C., Turnay, J., Fernández-Dıaz, M. D., Ulmo, N., Lizarbe, M. A., y Montero, P. (2002). Structural and physical properties of gelatin extracted from different marine species: a comparative study. *Food Hydrocolloids, 16*(1), 25-34.

Kagan H. y Li, W. (2003). Lysyl oxidase: Properties, specificity, and biological roles inside and outside of the cell. *Journal of Cell Biochemestry 88*(4), 660–672.

Kittiphattanabawon, P., Nalinanon, S., Benjakul, S. y Kishimura, H. (2015). Characteristics of pepsin-solubilised collagen from the skin of splendid squid (*Loligo formosana*). *Journal of Chemistry, Article ID 482354,* 8 páginas.

Ledward, D. A. (1986). Gelation of gelatin. En: *Functional Properties of Food Macromolecules*, pp. 171-201. Hill, S.E., Ledward, D.A. y Mitchell, J.R. A Chapman & Hall Food Sciecne Book, Gaithersburg, Maryland.

Lehninger, A. L., Nelson, D. L., Cox, M. M. (2001). Principios de Bioquímica. 3ra ed. Omega, Barcelona, España.

Leroy, M., Labbé, J. F., Ouellet, M., Jean, J., Lefèvre, T., Laroche, G., Auger, M. y Pouliot, R. (2014). A comparative study between human skin substitutes and normal human skin using Raman microspectroscopy. *Acta Biomaterialia*, *10*(6), 2703-2711.

Li, X., Bickerdike, R., Nickell, D., Campell, P., Dingwall, A. y Johnston I. A. (2005). Hydrylysyl pyridinoline cross-link concentration affects the textural properties of fresh and smoked Atlantic salmon (*Salmo salar L.*) flesh. *Journal of Agricultural and Food Chemistry*, *53*(17), 510–515.

Lin, A. C. y Goh, M. C. (2002). Investigating the ultrastructure of fibrous long spacing collagen by parallel atomic force and transmission electron microscopy. *Proteins: Structure, Function, and Bioinformatics*, *49*(3), 378-384.

Luna-Raya, M., Aragón-Noriega, E. A. y Salinas-Zavala, C. A. (2009). Análisis del consumo de calamar gigante en el noroeste de México. *Región y Sociedad*, *21*(46), 145-157.

Masuda, M., Karube, S., Hayashi, Y., Shindo, H. y Igarashi, M. (1976). Direct measurement of collagen crosslinks with automatic amino acid analyzer-identification of peaks due to crosslinks. *FEBS letters*, *63*(2), 245-249.

Maza, S., Salas, A., Rosales, M. y Castro, R. (2003). Efectos de un proceso de lixiviación ácida salina sobre la calidad del surimi de *Dosidicus gigas* "pota". *Boletín de Investigación del Instituto Tecnológico Pesquero del Perú*, *5*, 81-88.

Mizuta, S., Mutoh, M., Sugihara, K. y Yoshinaka, R. (2000). Immunochemical and immunohistochemical identification of a minor collagen in raw muscles of decapod mollusks. *Journal of Food Science*, *65*(4), 570-574.

Mizuta, S., Isobe, S. y Yoshinaka, R. (2002). Existence of two molecular species of collagen in the muscle layer of the ascidian (*Halocynthiaroretzi*). *Food Chemistry 79*(1), 9–13.

Mizuta, S., Tanaka, T. y Yoshinaka, R. (2003). Comparison of collagen types of arm and mantle muscles of the common octopus (*Octopus vulgaris*). *Food Chemistry, 81*(4), 527-532.

Montero, P., Gómez-Guillen, M. y Borderías, A.J. (1999). Functional characterization of muscle and skin collagenous material from hake (*Marluccius merluccius* L.). *Food Chemistry, 65*(1), 55-59.

Montero, P. y Gómez-Guillén, M. (2000). Extracting conditions for megrim (*Lepidorhombusboscii*) skin collagen affect functional properties of the resulting gelatin. *Journal of Food Science, 65*(3), 434-438.

Morales, J., Montero, P. y Moral, A. (2000). Isolation and partial characterization of two types of muscle collagen in some cephalopods. *Journal of Agricultural and Food Chemistry*, *48*(6), 2142-2148.

Muyonga, J. H., Cole, C. G. B., & Duodu, K. G. (2004). Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Latesniloticus*). *Food Chemistry, 85*(1), 81-89.

Nomura, Y., Toki, S., Ishii, y Shirai, K. (2000). The physicochemical property of shark type I collagen gel and membrane. *Journal of Agricultural and Food Chemistry 48* (6), 2028- 2032.

O'Dor, R. (1988). Limitations on locomotor performance in squid. *Journal of Applied Physiology, 64* (1), 1 128-134.

Otwell, W. S. y Hamann, D. D. (1979a) Textural characterization of squid (*Loligo pealei LESUER*): scanning electron microscopy of cooked mantle. *Journal of Food Science 44*(6), 1629–1635, 1643.

Otwell, W. S. y Hamann, D. D. (1979b). Textural characterizati, on of squid *(Loligo pealei L.)*: instrumental and panel evaluations. *Journal of Food Science*, *44*(6), 1636-1643.

Otwell, W. S., y Giddins, G. G. (1980). Scanning electron microscopy of squid, *Loligo pealei*: raw, cooked and frozen mantle. *Marine Fisheries Review 42*(7-8), 67–73.

Pati, F., Adhikari, B. y Dhar, S. (2010). Isolation and characterization of fish scale collagen of higher thermal stability. *Bioresource Technology, 101*(10), 3737–3742.

Ramírez-Guerra, H. E., Fimbres-Romero, M. D. J., Tapia-Vazquez, A. E., Ezquerra-Brauer, J. M., Márquez-Ríos, E., Suarez-Jimenez, G. M. y Torres-Arreola, W. (2015). Relationship between lysyl oxidase activity, pyridinoline content and muscle texture during ice storage of jumbo squid (*Dosidicus gigas*). *International Journal of Food Science & Technology, 50*(12), 2700-2706.

Reiser, K., McCormick, R. J., y Rucker, R. B. (1992). Enzymatic and nonenzymatic cross-linking of collagen and elastin. *The FASEB Journal*, *6*(7), 2439-2449.,

Reynaud, C., Baas, D., Gleyzal, C., Le G. y Sommer, P. (2008). Morpholino knockdown of lysyl oxidase impairs zebrafishdevelopment, and reflects some aspects of copper metabolism disorders. *Matrix Biology, 27*(6), 547–560.

Ricard-Blum, S. y Ruggiero, F. (2005). The collagen superfamily: from the extracelular matrix to the cell membrane. *Pathology Biology (Paris)*, 53(7), 430-442.

Rigby, B.J. (1968). Amino-acid composition and thermal stability of the skin collagen of the Antarctic ice-fish. *Nature, 219*(1), 166-167.

Robins, S. P. (1983). Cross-linking of collagen. Isolation, structural characterization and glycosylation of pyridinoline. *Biochemical Journal*, *215*(1), 167-173.

Robins, S. P. y Duncan, A. (1983). Cross-linking of collagen. Location of pyridinoline in bovine articular cartilage at two sites of the molecule. *Biochemical Journal*, *215*(1), 175-182.

Sato, K., Yoshinaka, R., Sato, M. y Tomita, J. (1989). Biochemical characterization of collagen in myocommata and endomysium fractions of carp and spotted mackerel muscle. *Journal of Food Science*, *54*(6), 1511-1514.

Sikorski, Z. y Borderias, J. (1994). Collagen in the muscles and skins of marine animals. In: Sea Food Protein (edited by Z.E. Sikorski, B.S.Pan&F.Shahidi). Pp. 58–70. Chapman and Hall, New York.

Smith-Mungo, L. y Kagan, H. (1998). Lysyl oxidase: Properties, regulation and multiple fuctions in biology. *Matrix Biology*, *16*(7), 387-398.

Sánchez-Alonso, I., Careche, M. y Borderías, A. J. (2007). Method for producing a functional protein concentrate from giant squid (*Dosidicus gigas*) muscle. *Food Chemistry*, *100*(1), 48-54.

Tanzer, M.L. (1973). Croos-linking of collagen. *Science, 180*(4086), 561-566.

Thuy, L.T.M., Okazaki, E. y Osako, K. (2014) Isolation and characterization of acid-soluble collagen from the scales of marine fishes from Japan and Vietnam. Food Chemistry *149*, 264–270.

Torres-Arreola, W., Pacheco-Aguilar R., Sotelo-Mundo R., Rouzaud-Sández O. y Ezquerra-Brauer J.M. (2008). Caracterización parcial de colágeno soluble en pepsina (CSP) extraído a partir del manto, aleta y tentáculos de calamar gigante (*Dosidicus gigas). Ciencia y Tecnología Alimentaria, 6*(2), 101-116

Torres-Arreola, W., Ezquerra-Brauer, M., Figueroa-Soto, C., Valenzuela-Soto, E., Garcia-Sanchez, G., Marquez-Rios, E. y Pacheco-Aguilar M. (2011). Lysyl Oxidase from jumbo squid (*Dosidicus gigas*) muscle: detection and partial Purification. *International Journal of Food Science and Technology*, *46*(8), 1711–1715.

Uriarte-Montoya, M. H., Santacruz-Ortega, H., Cinco-Moroyoqui, F. J., Rouzaud-Sández, O., Plascencia-Jatomea, M. y Ezquerra-Brauer, J.M. (2011). Giant squid skin gelatin: chemical composition and biophysical characterization. *Food Research International 44*(10), 3243–3249.

Valencia-Pérez, A.Z., García-Morales, M.H., Cárdenas-López, J.L., Herrera-Urbina, J.R., Rouzaud-Sández, O. y Ezquerra-Brauer, J.M. (2008). Effect of thermal process on connective tissue from jumbo squid (*Dosidicus gigas*) mantle. *Food Chemistry, 107*(4), 1371-1378.

Voet, D., Voet, J. G. y Pratt, C. W. (2006). Fundamentals of Biochemistry: Life at the Molecular Level. Pp. 142. 2nd. Edn. Wiley & Son., United States.

Wu, J. J. y Eyre, D. R. (1984). Identification of hydroxypyridinium crosslinking sites in type II collagen of bovine articular cartilage. *Biochemistry*, *23*(8), 1850-1857

HIPÓTESIS

La composición, estructura y grado de entrecruzamiento del colágeno de las regiones anatómicas, manto, aleta y tentáculos del calamar gigante, depende de la actividad de la lisil oxidasa, que cuanto mayor sea ésta, mayor es su estabilidad térmica y la firmeza del músculo que lo contiene.

OBJETIVO GENERAL

Purificar y caracterizar las fracciones de colágeno soluble en pepsina e insolubles de aleta, manto y tentáculos del calamar gigante, y relacionar su estructura química con la actividad de la lisil oxidasa, el comportamiento térmico de la molécula y la firmeza que presente el músculo de cada región.

Objetivos Específicos

1. Extraer y purificar la fracción de colágeno soluble en pepsina e insoluble del músculo de manto, tentáculos y aletas de calamar gigante.
2. Caracterizar las fracciones purificadas del colágeno presente en manto, tentáculo y aletas de calamar gigante, mediante análisis químicos y biofísicos.
3. Determinar y comparar la concentración de piridinolina en el tejido conectivo del músculo del manto, tentáculo y aletas de calamar gigante.
4. Correlacionar la concentración de piridinolina en el tejido conectivo, con la actividad de la lisil oxidasa y la resistencia al corte que presente el músculo de manto, tentáculos y aleta.

DESARROLLO DEL TRABAJO DE INVESTIGACIÓN

El trabajo se divide en tres capítulos, en los cuales se describen los artículos publicados y enviados a revistas internacionales de considerable factor de impacto, indizadas en el Journal Citation Report (JCR) del Instituto de Información Científica en la base de datos Thomson-Reuters.

Descripción del Capítulo I

En este capítulo se describe el primer artículo de investigación original, el cual fue enviado al Food Biophysics (factor de impacto de 1.7). El trabajo lleva por título *"Interrelation of Collagen Chemical Structure and Nanostructure with Firmness of Three Body Regions of Giant Squid (Dosidicus gigas)".*

En este trabajo se comparó la estructura, la estabilidad térmica y la textura del manto, aleta y tentáculos del calamar gigante. Para ello, se llevó acabo la purificación de la fracción soluble de colágeno, la cual fue caracterizada mediante análisis químicos, incluyendo el perfil de aminoácidos y contenido de piridonlina, análisis estructurales a través de técnicas espectrofotométicas de FTIR y RAMAN, perfiles electroforéticos y nanoestructurales a través de microscopia de absorción atómica. Los resultados obtenidos por dichos análisis fueron relacionados con la firmeza del músculo y el comportamiento térmico del colágeno. Con esta información se cumplió con parte de los objetivos planteados en la investigación.

El artículo mencionado al comparar la resistencia al corte del tejido de las tres regiones anatómicas se reportó el siguiente orden: tentáculo>aletas>manto. Este comportamiento se atribuyó al mayor contenido de colágeno, de iminoácidos, el mayor grado de hidroxilación de la prolina y lisina y la mayor concentración de piridinolina en el colágeno purificado de los tentáculos. Además, al comparar los densitogramas obtenidos por SDS-PAGE de colágenos purificados de cada región anatómica, se observó la mayor intensidad de la banda asociada a componentes entrecruzantes β en el colágeno proveniente de los tentáculos. El orden de intensidad de mayor a menor fue tentáculo>aleta>manto. También, los análisis espectrofotométricos de Infrarrojo Transformada por Fourier (FTIR), Resonancia

Magnética Nuclear de Protón (RMN^1H) y RAMAN y las observaciones a nivel nanoestrucutural por Miscroscopía de Absorción de Fuerza Atómica (AFM) indicaron mayor orden molecular del colágeno presente en los tentáculos que en manto y aletas. Estableciéndose una correlación significativa entre la concentración de piridinolina y firmeza (R^2 = 0.96)

Descripción del Capítulo II

Este capítulo corresponde a la información del segundo artículo enviado a la revista *Food Science and Biotechnology* (factor de impacto de 0.69). El trabajo lleva por título *"Muscle lysyl oxidase activity and structural/thermal properties of highly cross-linked collagen in jumbo squid (Dosidicus gigas) mantle, fins and arms".*

En este manuscrito se compara la actividad de LOX entre las tres regiones anatómicas del calamar gigante, su relación con la estructura y la estabilidad térmica de la fracción considerada más entrecruzada, la insoluble. El colágeno fue caracterizado mediante análisis térmico, contenido de aminoácidos, perfil electroforético, químicos estructurales a través de espectrofotometría de RAMAN y RMN de protón, concentración de LOX. En este estudio se correlacionó la concentración de piridinolina, la actividad de LOX y la estabilidad térmica del colágeno. Un punto relevante de este trabajo fue la detección a través de RAMAN además de la piridinolina de otra molécula entrecruzante la hidroximerodesmosina. Con este capítulo se cubren completamente los objetivos planteados en el presente trabajo.

En el artículo se detectó que el músculo de la aleta presentó la mayor actividad de LOX y que el colágeno purificado de esta región tenía las temperaturas de transición (T_{max}) y entalpías (ΔH), medido por Calorimetría de Barrido Diferencial, más bajas, que el manto y tentáculos. Además, se reportó que había una correlación significativa entre la actividad de la lisil oxidasa (LOX) con T_{max} (R^2 =-0.85) y el grado de hidroxilación de la prolina (R^2 =-0.97) y lisina (R^2 =-0.99). Si bien no se detectaron correlaciones altas entre Pyr con la T_{max}, la detección de un pico asociado a los protones de la hidroximerodesmosina por RMN^1H, sugiere que además de la Pyr existen otras moléculas que afectan la estabilidad térmica del colágeno.

Descripción del Capítulo III

El capítulo III describe un artículo original el cual lleva por título "*Spectroscopic Imaging: Nuclear Magnetic Resonance and Raman for the detection of collagen cross-linking from giant squid mantle, fins and tentacles tissues*", el cual fue sometido en la revista Instrumentation Science & Technology.

Debido a que mediante el uso de análisis flurométricos y de HPLC para la detección de la piridinolina se requiere el uso de reactivos y ante la nueva tendencia del uso de técnicas que no alteren la estructura molecular del componente químico a analizar, en este trabajo se comparó el uso de cuatro diferentes métodos a fin de detectar la presencia de la molécula en las fracciones solubles en pepsina e insolubles del colágeno extraído del manto, aletas y tentáculos del calamar gigante. Las técnicas que se compararon fueron fluorescencia, HPLC, RMN ^{1}H y RAMAN. Este manuscrito contribuyó a confirmar que, a mayor grado de entrecruzamiento del colágeno presente en los músculos de las regiones anatómicas, manto, aleta y tentáculo, evaluado a través de la molécula de piridinolina, mayor será la estabilidad térmica y firmeza del músculo que lo contiene.

En este manuscrito se estableció que por el método de HPCL solo se pudo detectar la presencia de piridinolina en la fracción insoluble del colágeno purificado de los tentáculos, mientras que por la técnica flurométrica la molécula se logró cuantificar únicamente en las fracciones solubles del colágeno obtenido de las tres regiones anatómicas estudiadas. Sin embargo, al realizar el análisis mediante las técnicas espectrofotométricas de RMN ^{1}H y RAMAN se detectaron señales asociadas a la piridinolina en las dos fracciones, soluble en pepsina e insoluble, obtenidas del músculo de las tres regiones anatómicas. Por ambos métodos se observó que la mayor intensidad de los picos asociados a dicha molécula se presentó en la fracción insoluble del colágeno extraído de los tentáculos, cuyos músculos mostraron a su vez una mayor estabilidad térmica y un menor decremento en su firmeza después de 30 min de cocción.

CAPÍTULO I

Interrelation of Collagen Chemical Structure and Nanostructure with Firmness of Three Body Regions of Giant Squid (*Dosidicus gigas*)

Artículo aceptado en *Food Biophysics*, DOI: 10.1007/s11483-017-9505-4
(ISSN 1557-1858),
indexada en JCR
Año 2017

Carta de recepción de las pruebas de galeras corregidas

De: CorrAdmin2@spi-global.com
Asunto: Confirmation mail for Article 10.1007/s11483-017-9505-4
Fecha: 10 de noviembre de 2017, 11:24
Para: josafat.ezquerra@unison.mx

Journal: Food Biophysics
DOI: 10.1007/s11483-017-9505-4
Title: Interrelation of Collagen Chemical Structure and Nanostructure with Firmness of three Body Regions of Jumbo Squid (Dosidicus gigas)

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Interrelation of Collagen Chemical Structure and Nanostructure with Firmness of three Body Regions of Jumbo Squid (*Dosidicus gigas*)

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Received: 31 July 2017 / Accepted: 7 November 2017

Abstract

The chemical structure, thermal denaturation and nanostructure of collagen, obtained from a cation-exchange separation of the mantle, fins and tentacles of jumbo squid (*Dosidicus gigas*), were comparatively studied. The main idea of this work, was to provide an in-depth understanding of the interdependence between pyridinoline (Pyr) content, helix chemical structure and nanostructure of squid collagen with squid tissue firmness. The tentacles required more shear force and its collagen presented the higher temperature and enthalpy of transition, than the mantle and fins. The tentacle firmness may be explained by the relatively higher imino amino acid content, proline and lysine hydroxylation degrees and Pyr content of its collagen. Moreover, among the regions studied, the collagen from the tentacles had a more intense β band chain. Also, the Fourier transform infrared analysis and Raman spectra, implied that the collagen in the tentacles, was more intermolecularly ordered than the mantle and fins. Consistent with these results, a comparative evaluation of the surface morphology of the three regions, with atomic force microscopy, suggested a more ordered collagen structure in the tentacles (lower roughness values). Based on the above, collagen from tentacles has a higher degree of molecular order that sustains a higher muscle firmness compared to that of other anatomical regions.

Keywords

Chemical structure
Firmness
Nanostructure

Squid collagen
Thermal denaturation

Introduction

Squid is considered as an important fishery resource in many countries' fishing industries, due to its high popularity among consumers [1]. The edible body-regions of this organism that are marketed are the mantle, fins, and tentacles. However, as with any seafood products, one of the challenges is to inhibit the changes in toughness and texture that happen during squid processing. Regarding the association between texture and proteins, numerous studies have shown that properties of jumbo squid connective tissue proteins can be used as benchmarks of squid muscle quality during freezing or cooking processes [2, 3, 4, 5, 6, 7, 8, 9].

Squid muscle connective tissue is stronger than that of fish [10] though, the major component of the connective tissues of both organisms is collagen [11]. Collagen is a complex protein that helps to maintain the union between cells [12]. Several types of collagen have been identified and the primary characteristic of all types are amino acid arrangements that are rich in proline and glycine. These arrays, form three chains that intertwine to create a triple helix that varies with composition and size [13]. Few studies about seafood muscle texture, have reported the interdependence of fillet firmness with the major cross-linking molecule, pyridinoline (Pyr), present in collagen structure. Li et al. [14] detected a significant correlation between Atlantic salmon fillet texture and Pyr concentration. Furthermore, Ramirez-Guerra et al. [9] proved that the textural behavior of squid mantle during ice storage, is related to Pyr concentration.

In cephalopods, the chemical composition of the muscle tissue depends, among other factors, on the anatomical region [15] due to the distinctive function muscles have in the live organism [16]. Accordingly, differences in collagen properties have been detected in the edible and non-edible anatomical regions of squid species [16, 17, 18, 19]. Moreover, in the Japanese pufferfish (*Fugu rubripes*), the Pyr concentration has been shown to differ between the muscular and skin collagen [20].

The main focus of this study was to compare the thermal denaturation, Pyr concentration, chemical structure and nanostructure of the collagen extracted from various anatomical regions of jumbo squid (*Dosidicus gigas*). The possible correlation between the chemical structure and nanostructure of collagen, with tissue firmness, was discussed. This work provides an in-depth understanding of the interdependence between squid firmness, and collagen chemical structure and nanostructure, and may be useful for future technological developments, such as squid-based convenience products. To our knowledge, a comparative study of the thermal denaturation of collagen, Pyr content, collagen chemical structure, collagen nanostructure, and muscle firmness properties, among the edible anatomical regions of squid species, has not been carried out before.

Materials and Methods

Samples and Sample Preparation

Ten jumbo squid specimens were hand-captured, by jigging, and were purchased from local fisherman (Bahia Kino in the Gulf of California, Mexico; 28.75° N, 112.25° W; water temperature 15–18 °C), during fall 2015. The squid were transported on ice to the laboratory, within 8 h of capture. The organisms were gutted, and the mantles were separated from the fins and tentacles. Each anatomical region was skinned, and the firmness was immediately measured. Approximately 250-g portions of each anatomical region, were packed in polyethylene bags and stored frozen (−25 °C), for no more than 7 days, until collagen extraction.

All reagents used in this work were reagent grade (Sigma Chemical Co., St. Louis, MO, USA). The solvents used in the amino acid analysis, were liquid chromatographic grade.

Muscle Firmness Analysis

Muscle portions (1 cm × 1 cm × 1 cm) from each fresh anatomical region (mantle, fins and tentacles), obtained from 10 jumbo squid specimens, were subjected to firmness analysis. The shear force required to cut the muscle sample was measured

using a TA-XT2 Plus Texturometer (Food Technology Corp., Sterling, VA, USA), equipped with a Warner-Bratzler shearing device, attached to a load cell (100 N), at a crosshead speed of 200 mm/min [9].

Extraction of Collagen

The connective tissue was extracted at 4 °C, by removing the myofibrillar and proteoglycan proteins from fresh muscle, using a 6 M urea solution containing 0.5 M sodium acetate (pH 6.8). Samples were stirred for 60 min and centrifuged at 39200×*g* for 40 min. The pellets were sequentially treated with 1 M Tris (pH 7.2, containing 0.05 M NaCl), 0.5 M acetic acid (1:5 *w/v*) and pepsin (10 mg/g tissue in 0.5 M acetic acid; 1:5 *w/v*). At each step, after stirring for 24 h, the samples were centrifuged at 39200×*g* for 40 min. Then, the collagen solutions were precipitated with 2 M NaCl and collected by centrifugation (39,200×*g* for 40 min). The precipitates were dissolved in 0.05 M acetic acid and dialyzed at 4 °C against water, using a cellulose membrane with a 10 kDa molecular weight cut-off. The collected samples were frozen at −40 °C and lyophilized [19].

The collagen was then purified by cation exchange column chromatography, using HiTrap CM Sepharose FF (GE Healthcare, Uppsala, Sweden) assisted by ÄKTApurifier chromatographic equipment (GE Healthcare, Uppsala, Sweden). Collagen samples (50 mg) were dissolved in 15 mL of 50 mM sodium acetate, pH 4.8, containing 6 M urea and, then, applied to a HiTrap CM FF column (5 × 1 mL), equilibrated with the same buffer. Fractions were eluted with a linear gradient of 0–0.5 M NaCl, in a total volume of 20 mL, at a flow rate of 1 mL/min. The effluent was monitored at 280 nm. The appropriate fractions were pooled and dialyzed against 0.05 M acetic acid, to prevent precipitation [21]. Finally, the sample was lyophilized and stored at −40 °C, for further analysis.

Amino Acid Profile

The amino acid composition of collagen was determined, using reverse-phase high-performance liquid chromatography (HPLC). The samples were hydrolyzed under reduced pressure in 6 M HCl, containing sodium thioglycolate (1:1 *v*/v), at 150 °C for 60 min. The hydrolyzed samples were derivatized at 60 °C for 5 min in

the presence of 0.5 M citrate, containing 2 mg/mL 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) in methanol and applied to an HPLC system (Agilent Technologies Inc., Palo Alto, CA) [22]. Analyses were performed in triplicate and the results were expressed as the number of residues per 1000 residues.

Determination of Purified Collagen Content

The hydroxyproline (Hyp) content, calculated by HPLC [22], was used as an index of collagen. The mass of Hyp was converted to collagen using the eq. 1 [23].

$$\text{Collagen} = \left[\frac{\text{Hyp} \times 8}{\text{crude protien}}\right] \times 100 \qquad 1$$

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to establish the purity of the obtained collagen, proteins were analyzed by SDS-PAGE [24] using a 5.5% acrylamide separating gel. Samples for electrophoresis were prepared homogenizing 10 mg of purified collagen in one mL of cold 0.05 M Tris, pH 7.4, containing 1% SDS. The homogenate was diluted 1:1 (*w/v*) in the sample buffer (0.5 M Tris-HCl pH 6.8, glycerol, 10% SDS, β-mercaptoethanol, 0.05% bromophenol blue), and a 20 µL aliquot of 12-µg protein was loaded onto a vertical electrophoresis gel, which was developed using a Mini Protean® III, Bio-Rad electrophoretic equipment (Hercules, CA). The electrophoretic separation was carried out according to manufacturer's procedure (120 V for 90 min). After electrophoresis, the gels were stained with Coomassie Blue R-250. Five electrophoresis gel images, for each extracted collagen were analyzed, using a densitometer (Bio-Rad Model GS-700, Hercules, CA), to obtain representative and statistically valid results.

Determination of Pyridinoline (Pyr)

Fluorescence spectrophotometry (Cary Eclipse G9800A Agilent Technologies, USA) was performed to determine the Pyr content in the collagen samples. Collagen was dissolved (0.5 absorbance units at 280 nm) with 50 mM sodium

acetate, containing 6 M urea, pH 4.8. The excitation wavelength was 295 nm and the emission signal was measured from 300–450 nm. The Pyr content was established from a standard Pyr (5–40 pmol) curve [25]. The Pyr content was estimated as moles per mole of collagen. The assays were performed in triplicate.

Differential Scanning Calorimetry (DSC)

The thermal profile of the collagen was analyzed using DSC, from 25–180 °C [19]. Thermal denaturation of collagen, was characterized by the enthalpy change (ΔH), estimated from the denaturation endotherm of collagen and maximum temperature of denaturation (T_{max}), which were measured, using a 1020 Series DSC thermal analysis system (Perkin Elmer, Norwalk, CN). Collagen samples (4–5 mg) were placed in DSC hermetic cells, and an empty capsule was used as a reference. Data acquisition was performed on a PE Nelson model 1022 (Perkin Elmer). Determinations were performed in triplicate.

Fourier Transform Infrared (FTIR) Analysis

FTIR spectra of extracted collagen, were obtained from pellets, prepared with 0.2 mg lyophilized sample and 10 mg potassium bromide [26]. The spectra were recorded using an infrared spectrophotometer (Perkin Elmer FT-IR Spectrum GX), with an average of 16 scans, over a spectral range of 4000 to 400 cm^{-1}, at a resolution of 4 cm^{-1}. Four FTIR spectra for each extracted collagen were analyzed, to acquire reliable, characteristic and statistically valid results.

Raman Spectroscopy

The purified collagen was analyzed by Raman spectrometry, using a Perkin Elmer GX instrument [27]. The excitation was performed at 1064 nm, using a Nd:YAG laser. The backscattered light was collected at 180 °C. The equipment was adjusted to 100 mW and 128 scans at 4 cm^{-1}, to avoid potential damage to the samples, due to the laser power. The bands of the Raman spectra were analyzed using the program equipment's analysis. Three Raman spectra for each extracted collagen were analyzed, to acquire reliable, characteristic and statistically valid results.

Atomic Force Microscopy (AFM)

The morphological characteristics of each collagen obtained from the mantle, fins and tentacles, were visualized by AFM. Each fraction was solubilized in 50 mM sodium acetate, containing 6 M urea, pH 4.8, at 5 mg/mL, then, placed on a glass slide and dried at room temperature (20–25 °C) for 30 min. Subsequently, the samples were washed with about 10–20 mL of water and allowed to dry again. The AFM images were obtained, with an alpha300 RA (WITec), in non-contact mode and under atmospheric conditions. The field scans were 5 × 5 μm for each sample, with 65,536 points per image. The images were processed with the program ProjectFour v4.1. At least 10 samples of each extracted collagen were examined, to acquire trusty, characteristic and statistically valid results.

Statistical Analyses

Data were analyzed by one-way analysis of variance (ANOVA), to explore differences among the samples. Differences between least-squares means were examined by Tukey-Kramer's honestly significant difference (HSD) test ($p < 0.05$, in all cases). Correlation values among firmness, proline and lysine hydroxylation degrees' data, β-component (SDS-PAGE), Pyr content, maximum temperature, ratio of bands 1240/ 1451 (FTIR), ratio of bands 1270/1450 (RAMAN), and surface roughness (AFM) were also calculated. All analyses were carried out using PC-SAS version 6.08 (SAS Institute Inc., 1994). The results were presented as mean ± standard deviation.

Results and Discussion

Firmness

Muscle firmness was measured by recording the force required to penetrate the muscles of the mantle, fins and tentacles, respectively. The highest firmness ($p < 0.05$), was detected in the tentacles (86.5 N), followed by the fins (33.6 N) and mantles (29.6 N). The higher firmness detected in tentacles could be attributed to their specific functions during swimming mechanisms [16]. The firmness detected in mantle muscle concurred with that previously reported for fresh mantle of the

same squid species [9].

Concentration of Collagen

The extracted collagen (expressed as grams of dry connective tissue per 100 g of each anatomical region), varied significantly ($p < 0.05$) among the evaluated anatomical regions. The greatest yield was obtained from the tentacles (20.3%), followed by the fins (12.7%) and mantle (7.7%). These results concurred with those previously obtained from the same squid species [19] and *Todaropsis eblanae* [16] but were higher than those found in *Loligo patagonica*, *Illex argentinus* and *Illex coindetii* [16, 28]. Several exogenous factors including the time of year, water temperature, pressure and diet, may influence the connective tissue extracted [16]. Concerning the percentage of collagen, the tentacles had a significantly higher concentration ($p < 0.05$) (91.3%) than the fins (85.5%) and mantle (83.1%). This trend agrees with previous works, which found that the muscle collagen content varies, depending on the anatomical region [11, 16, 19, 29, 30].

Amino Acid Analysis

The amino acid compositions of the purified collagen from the mantle, fins and tentacles (Table 1), revealed a high prevalence of glycine (Gly), representing around 28% of the total collagen residues in all fractions. These Gly contents are typical for collagen. In addition, low proportions of leucine (Leu), Lysine (Lys) and serine (Ser) and high proportions of alanine (Ala) and hydroxyproline (Hyp) were detected. Morales Morales and others et al. [16] observed the same amino acid distribution patterns, in the collagen isolated from the mantle and arms of some cephalopods.

Table 1

Amino acid composition (number of residues/1000 amino acids) of collagen purified from the mantle, fins and tentacles of jumbo squid*

Amino acid	Mantle	Fins	Tentacles
Hyp	92 ± 1.1	89 ± 1.3	114 ± 1.4

Asp	40 ± 1.1	50 ± 1.3	50 ± 1.2
Thr	29 ± 0.4	29 ± 0.1	30 ± 0.4
Ser	38 ± 0.3	39 ± 0.4	20 ± 0.4
Glu	70 ± 1.1	90 ± 1.3	70 ± 0.3
Pro	140 ± 1.2	100 ± 1.2	130 ± 1.3
Gly	280 ± 1.2	290 ± 1.3	290 ± 1.2
Ala	130 ± 1.3	140 ± 2.4	130 ± 1.3
Val	30 ± 0.4	26 ± 0.2	24 ± 0.2
Met	10 ± 0.2	10 ± 0.1	10 ± 0.4
Ile	10 ± 0.2	15 ± 0.3	10 ± 0.4
Leu	10 ± 0.1	10 ± 0.1	10 ± 0.2
Tyr	3 ± 0.2	3 ± 0.4	2 ± 0.3
Phe	20 ± 0.4	20 ± 0.2	20 ± 0.2
His	10 ± 0.1	9 ± 0.1	11 ± 0.2
Hyl	15 ± 0.5	16 ± 0.6	18 ± 0.8
Lys	16 ± 0.8	20 ± 0.3	15 ± 0.6
Arg	59 ± 0.7	58 ± 0.5	50 ± 0.9
Imino acid (Pro + Hyp)	230 ± 1.1^{b}	180 ± 1.7^{c}	240 ± 1.2^{a}
Pro hydroxylation (%)	39 ± 0.6^{c}	44 ± 0.3^{b}	46 ± 0.3^{a}
Lys hydroxylation (%)	48 ± 0.2^{b}	46 ± 0.3^{c}	55 ± 0.3^{a}
Collagen content ** (%)	83 ± 0.6c	85 ± 1.3b	91 ± 2.4a

*Values are the mean of triplicates ± standard deviation ($n = 3$).** Collagen content = [hydroxyproline × 8/crude protein] × 100 (AOAC, Method 990.26, 2000). Different letters in imino acid (Pro + Hyp), Pro hydroxylation, Lys hydroxylation and true collagen denote significant difference ($p < 0.05$)

Comparing the total imino amino acid [Proline (Pro) + Hyp] content among the

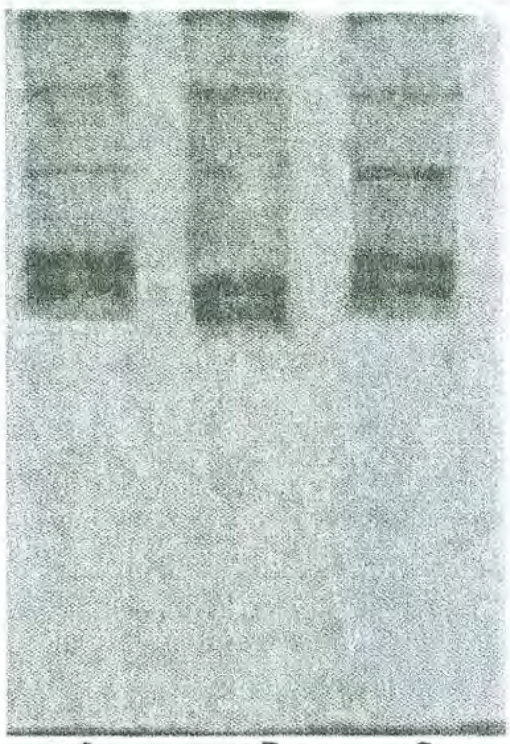
anatomical regions, the collagen from the tentacles showed the highest value. This sample also showed the highest degree of Pro and Lys hydroxylation. Therefore, under the conditions of this study, this result demonstrated that the tissue collagen derived from the tentacles possessed a higher degree of crosslinkage than that of mantle and fins.

SDS-PAGE Electrophoresis

According to the electrophoretic patterns of the purified collagen samples obtained from each anatomical region (Fig. 1), the collagen comprised α-chains and inter- and intramolecular crosslinked components: β (dimer) and γ (trimer), indicating that lysine-derived crosslinks were present [31]. Comparable electrophoretic patterns of the mantle, fins and tentacles, have been reported for collagen type I of *Illex coindetii*, *Todaropsis eblanae* and *Todarodes pacificus* [16, 32]. The similarity in the electrophoretic migration and amino acid composition, suggested the presence of type I collagen in the analyzed tissues. The band optical intensity (OD) of the crosslinked chain (β dimer) was significantly higher in collagen ($p < 0.05$) from the tentacles (2128 OD) than fins (1851 OD) and mantle (1701 OD).

Fig. 1

Gel electrophoretic profile of collagens, purified from jumbo squid (*Dosidicus gigas*). Lane **a** mantle; **b** fins; **c** tentacles



Pyr Content

Another considerable difference between the mantle, fins and tentacles, was detected in the Pyr content ($p < 0.05$). Consistent with the above-mentioned results, the collagen of the tentacles had a significantly higher Pyr content ($p < 0.05$) (2.09 mmol/mol collagen) compared to the fins (1.22 mmol/ mol collagen) and mantle (0.91 mmol/mol collagen). These differences indicated that the collagen from each anatomical region, differs in crosslinking degree, which is controlled by the number of bonds prone to form hydroxylamine (and likewise their solubility in salt solutions and buffers) [29]. The tentacles perform a grasping function in the live organism [16]. Therefore, its muscular collagen might require more Pyr than the mantle and fins.

As documented previously in fish and squid muscles [9, 14] the Pyr content is closely associated with squid muscle firmness. Pyr is formed through the oxidation of hydroxylysine (Hyl) by lysyl oxidase (LOX), and the activity of this enzyme has been shown to affect the physical strength of tissue fibers [3]. Our results showed the collagen derived from the tentacles had a higher Hyl content than that from the

mantle and fins (Table 1). However, it is also possible that the LOX activity may vary in each body anatomical region studied, affecting its firmness and thermal stability. Therefore, further research on LOX, in squid tissue is needed.

DSC

DSC analysis of the collagen, produced one endothermic transition peak, with a denaturation temperature above 100 °C, in all instances, in concurrence with other reports [7, 19, 33]. The peak temperature (T_{max}) is indicative of the stability of collagen during heating [34], whereas the ΔH is due to endothermic processes, such as the breaking of bonds [35]. The collagen with the highest T_{max} and ΔH was derived from the tentacles (Table 2). Similarly, Torres-Arreola et al. [19] also reported that collagen from the tentacles showed both the highest T_{max} and ΔH. These results imply that collagen from the tentacles has a more stable helix structure than the fin and mantle collagens [3, 26]. Unlike Pro, the Hyl content is closely correlated with the thermal stability of collagen [36]. However, the differences in hydroxylation (%) of Lys (Table 1), might suggest that Pyr itself contributed partially to the collagen thermal stability. Several factors that dictate the collagen thermal stability, such as LOX activity [3], are intertwined in a complex manner [37], requiring more studies to decipher the exact intercorrelations.

Table 2

Thermal profile of collagen from the mantle, fins and tentacles of jumbo squid

Anatomical region	T_o (°C)	T_{max} (°C)	ΔH (J/g)
Mantle	133 ± 1.4^a	150.4 ± 1.4^b	21.8 ± 1.5^b
Fins	129 ± 1.7^a	142.8 ± 1.7^c	16.5 ± 1.4^c
Tentacles	131 ± 1.6^a	159.9 ± 1.7^a	25.8 ± 1.7^a

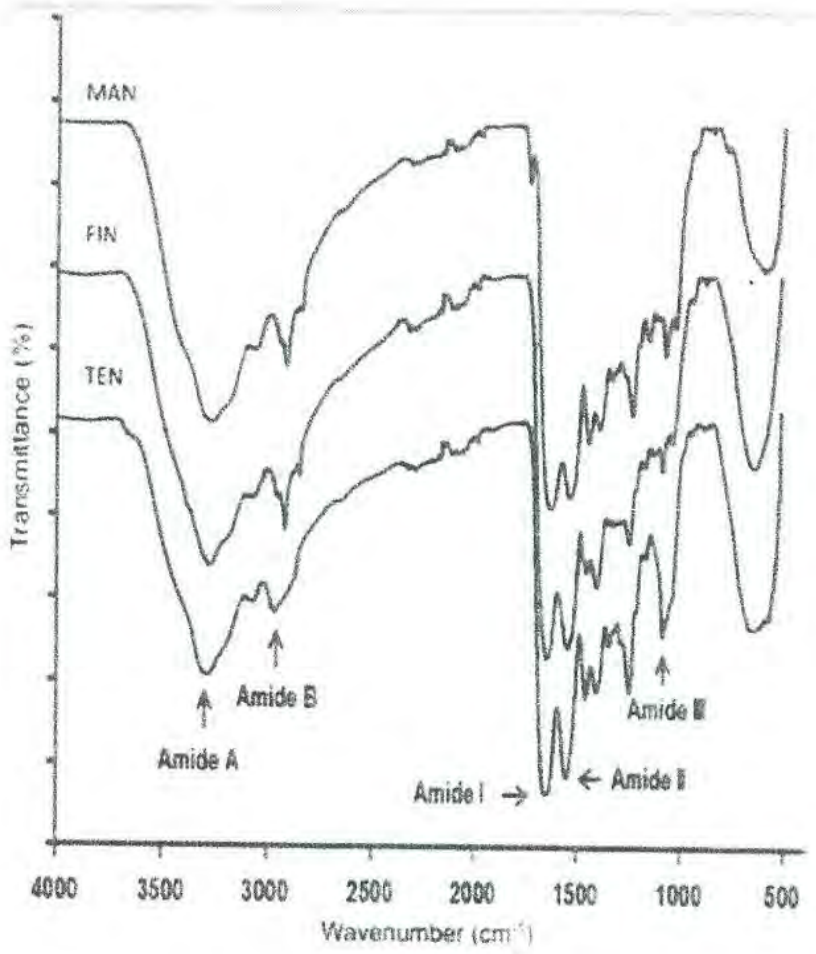
T_o = Onset temperature; T_{max} = Temperature at maximum heat flow; ΔH = Total enthalpy change in melting/gelation of collagen. Values are mean of triplicates ± standard deviation (n = 3). Different letter for each column are statistically different ($p < 0.05$)

FTIR Analysis of Collagen

The FT-IR (3500–400 cm^{-1}) spectra of the three collagen specimens (Fig. 2), represented those reported for other marine collagens [26, 38, 39]. The spectra of the collagens derived from jumbo squid mantle, fins, and tentacles, differed in the amide I and amide II peaks, which in the mantle, appeared at a lower frequency. A shift of these peaks to lower frequencies, implies a lower degree of molecular order [40]. Moreover, the ratio between amide III and the region near 1440–1460 cm^{-1}, is associated with a helical structure (index ratio (IR) = % transmittance at 1240 cm^{-1}/% transmittance at 1451 cm^{-1}) [41, 42]. The IR values detected in the collagen of each anatomical region were 1.35 (mantle), 1.28 (fins) and 1.05 (tentacles). The IR values close to 1.0, detected in the collagen of the tentacles, confirmed that a considerable extent of intermolecular structure exists in this region [41]. This behavior coincides with the Pyr content, which was lower in the collagen samples derived from the mantle than the fins and tentacles. Therefore, it appears that there are fewer intermolecular cross-links in mantle collagen compared to that from tentacles and fins. In the present study, the IR values positively correlated with the squid muscle firmness. Thus, the ratio between amide III and 1440–1460 cm^{-1}, should be considered as one of the explanatory variables, contributing to squid firmness in addition to the Pyr content.

Fig. 2

Fourier transform infrared spectra of collagen purified from the mantle (MAN), fins (FIN) and tentacles (TEN) of jumbo squid



Raman Spectroscopy Analysis of Collagen

Raman analysis was also applied to analyze the structure of the purified collagens. In general, relatively neutral bonds (CC, CH, C = C) are strong Raman scatterers. The differences in the shape and intensity of the bands, observed between the 1700–800 cm^{-1} regions of the Raman spectra of the obtained collagens (Fig. 3), were associated with the differences in the imino acid (Hyp and Pro) concentrations detected.

Fig. 3

Raman spectra of collagen from the mantle (MAN), fins (FIN) and tentacles (TEN) of jumbo squid, in 1700–600 cm^{-1} region

possible to suggest that due to the chemical structure characteristics of collagen from squid fins, its flesh in this region has a lower heat resistance than the mantle and tentacles, which is among the consumers and seafood producers' concerns.

Table 3

Correlation analysis between collagen chemical structure and thermal properties and na and muscle firmness of jumbo squid

	SF	Hyp	Hyl	Pro + Hyp	SDS	Pyr	DSC	IR
Hyp	0.76*							
Hyl	0.92**	0.56						
Pro + Hyp	0.58	*ND*	*ND*					
SDS	0.91**	0.84*	0.71*	0.32				
Pyr	0.96**	0.76*	0.79*	0.41	0.99**			
Tmax	0.75*	0.33	0.94**	0.91**	0.94**	0.75*		
FTIR	−0.97**	0.73*	0.82*	−0.44	0.98**	0.99**	−0.77*	
RAMAN	0.82*	0.43	0.97**	0.86*	0.75*	0.81*	0.99**	−0.83*
AFM	−0.59	0.95**	0.36	0.31	0.80*	0.74*	0.12	0.72*

*Significant correlation values ($p < 0.05$)

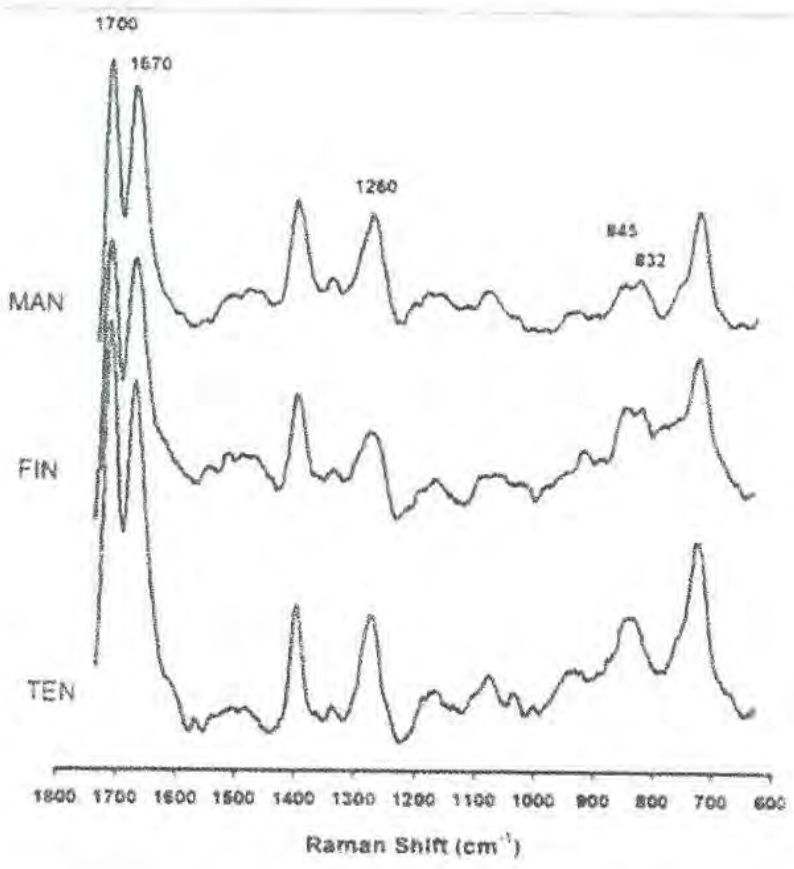
**Significant correlation values ($p < 0.01$)

Observations 9

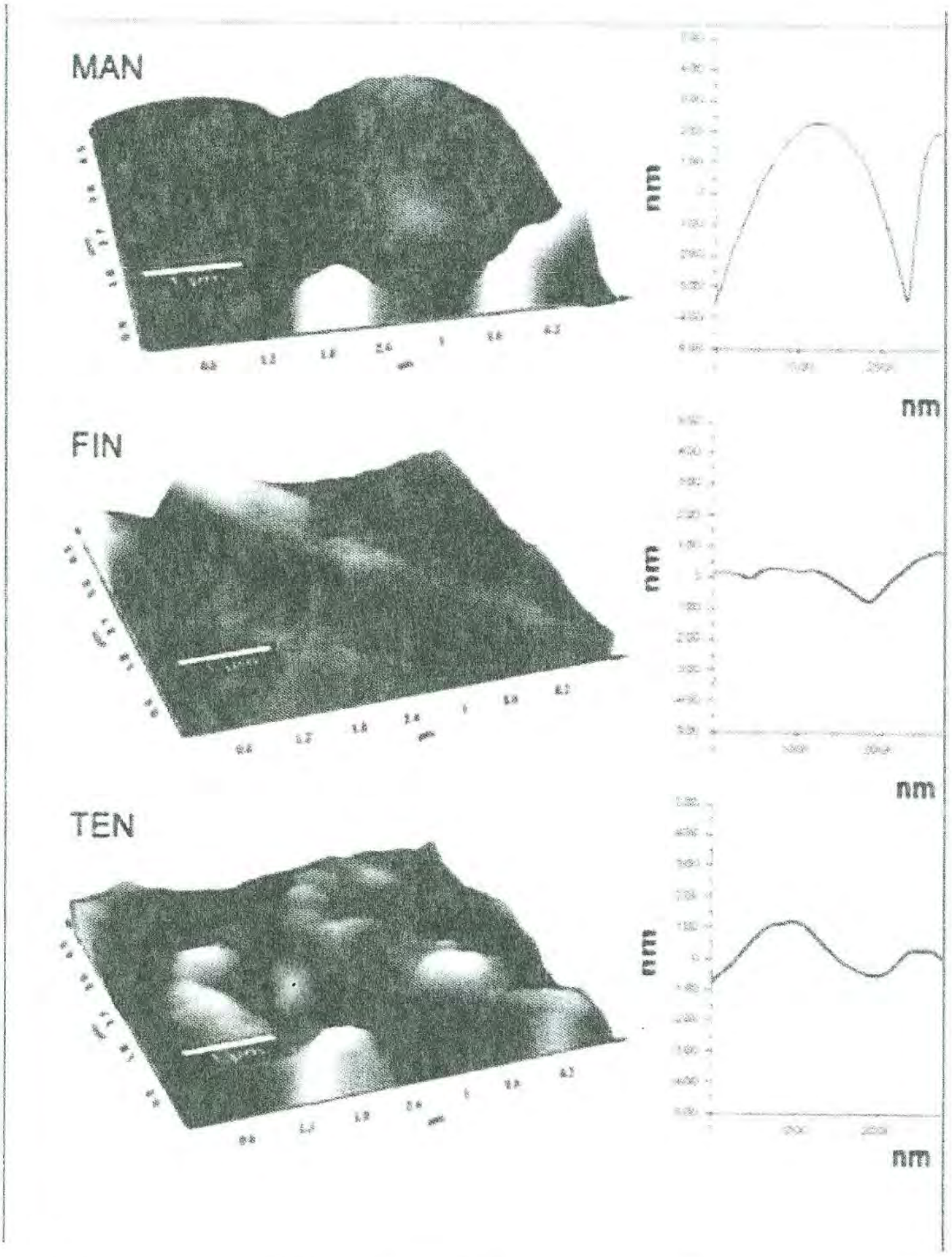
SF, firmness; Hyp, % proline hydroxylation; Hyl, % lysine hydroxylation; SDS, β-com intensity band; Pyr, pyridnoline content; DSC, maximum temperature; IR, ratio of banc 1240/1451; RAM, ratio of bands 1270/1450; AFM, surface roughness

ND Not determined

AFM of Collagen



The Raman spectra indicated structural differences in the collagen from jumbo squid mantle, fins and tentacles. In all purified collagens a very intense band at 1670 cm^{-1} was present and attributed to amide I. The peak detected at 1270 cm^{-1} is assigned to amide III [27, 43]. Fewer intense bands were observed in the fin collagen than the mantle and tentacles. The absorption between the amide III and 1452 cm^{-1} bands demonstrates the existence of the helical structure [44]. As in FTIR, the ratio between the 1270/1450 cm^{-1} bands intensity, which is also associated with the helical structure [44] was 0.96, 0.64 and 0.46, for the tentacles, mantle and fin collagen, respectively. Lower values imply a less stable molecule [44]. Other considerable differences were in the Pro residue activity (830–910 cm^{-1}) and the Hyp (845 cm^{-1}). Among the three collagen specimens, these bands were more prominent in the collagen from the tentacles, in accordance with the amino acid profile. The thermal stability of collagen has been directly correlated with the content of imino amino acids (Pro + Hyp) [45]. In the present study, the Pro + Hyp content and the ratio of 1270/1450 cm^{-1} bands intensity ratio were strongly associated with the collagen thermal stability (Table 3). Thus, it is


MAN
nm
FIN
nm
TEN
nm
nm

This work attempted to compare the nanostructure of collagen with flesh firmness. Important micro-properties of the cell fibers, like contour length, height and radius of the collagen monomer, were omitted in the analysis. Thus, such parameters must be included in future studies, to provide a more comprehensive analysis of squid collagen structural factors that determine the firmness of squid muscle. Presumably, an important part of data variability, would explain the thermal properties, chemical structure and nanostructure differences, among the main jumbo squid edible anatomical regions. Nevertheless, this experiment showed that the significant differences evident in the nanostructure of collagen fibers, must be considered in future works of the firmness properties of squid tissue, during cold management and thermal processing, including sensory analysis. The research has shown an association between the collagen chemical structure, nanostructure and firmness (Table 3) of squid tissue but a more detailed study on the properties of processing squid muscle, is still needed.

Conclusion

Under the conditions of this work, it was proven that each anatomic region from jumbo squid has different firmness. The T_{max} and ΔH, indicated that the fibers of tentacle collagen, are stronger than that of the mantle and fins. The differences detected, are due to the different crosslinked helix structure among the collagens derived from the mantle, fins and tentacles. The surface morphology, examined with AFM, indicated a higher degree of molecular order in the tentacle collagen molecules, compared to the other anatomical regions, which was consistent with the results of muscle firmness and collagen thermodynamic values. These results may serve as a fundamental basis for the proper management and processing aspects, of jumbo squid tissues.

Acknowledgments

This research was supported by CONACYT, under grant 180214. Author Sarabia-Sainz acknowledges the doctoral fellowship received from CONACYT. The authors are also grateful to Ivan Tolano, for his valuable help with the DSC measurements.

Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflicts of interest.

References

1. Food and Agriculture Organization of the United Nations, Globefish-analysis and information on world fish trade. (Market reports 2017). Available from: http://www.fao.org/in-action/globefish/market-reports/cephalopods/en/ . Accessed 17 Mar 2017

2. Y.E. Ueng, C.J. Chow, Textural and Histological Changes of Different Squid Mantle Muscle during Frozen Storage. J Agric Food Chem **46**(11), 4728–4733 (1998). https://doi.org/10.1021/jf9803278

3. M. Ando, M. Ando, M. Makino, Y. Tsukamasa, Y. Makinodan, M. Miyoshi, Interdependence Between Heat Solubility and Pyridinoline Contents of Squid Mantle Collagen. J Food Sci **66**(2), 265–269 (2001). https://doi.org/10.1111/j.1365-2621.2001.tb11329.x

4. C. Ruiz-Capillas, A. Moral, J. Morales, P. Montero, The effect of frozen storage on the functional properties of the muscle of volador (Illex coindetii). Food Chem **78**(2), 149–156 (2002). https://doi.org/10.1016/S0308-8146(01)00392-2

5. R.R. Olivas, O.R. Sández, N.F. Haard, R.P. Aguilar, J.M.E. Brauer, Eur Food Res Technol **219**, 312 (2004)

6. M.E. Paredi, H.A. Roldan, M. Crupkin, Changes in myofibrillar proteins and lipids of squiD (ILLEX ARGENTINUS) during frozen storage. J Food Biochem **30**(5), 604–621 (2006). https://doi.org/10.1111/j.1745-4514.2006.00088.x

7. A.Z. Valencia-Pérez, M.H. García-Morales, J.L. Cárdenas-López, J.R.

Herrera-Urbina, O. Rouzaud-Sández, J.M. Ezquerra-Brauer, Food Chem **107**, 137 (2008)

8. M. Raman, S. Mathew, JIARM **2**, 452 (2014)

9. H.E. Ramirez-Guerra, M.J. Fimbres-Romero, A.E. Tapia-Vazquez, J.M. Ezquerra-Brauer, E. Márquez-Ríos, G.M. Suarez-Jimenez, W. Torres-Arreola, Relationship between lysyl oxidase activity, pyridinoline content and muscle texture during ice storage of jumbo squid (Dosidicus gigas). Int J Food Sci Technol **50**(12), 2700–2706 (2015). https://doi.org/10.1111/ijfs.12951

10. I. Kołodziejska, Z.E. Sikorski, C. Niecikowska, Food Chem **66**, 15 (1999)

11. Z.E. Sikorski, I. Kołodziejska, The composition and properties of squid meat. Food Chem **20**(3), 213–224 (1986). https://doi.org/10.1016/0308-8146(86)90174-3

12. K. Gelse, E. Pöschl, T. Aigner, Collagens—structure, function, and biosynthesis. Adv Drug Deliv Rev **55**(12), 1531–1546 (2003). https://doi.org/10.1016/j.addr.2003.08.002

13. J.H. Muyonga, C.G.B. Cole, K.G. Duodu, Characterisation of acid soluble collagen from skins of young and adult Nile perch (Lates niloticus). Food Chem **85**(1), 81–89 (2004). https://doi.org/10.1016/j.foodchem.2003.06.006

14. X. Li, R. Bickerdike, E. Lindsay, P. Campbell, D. Nickell, A. Dingwall, I.A. Johnston, Hydroxylysyl Pyridinoline Cross-Link Concentration Affects the Textural Properties of Fresh and Smoked Atlantic Salmon (Salmo salarL.) Flesh. J Agric Food Chem **53**(17), 6844–6850 (2005). https://doi.org/10.1021/jf050743+

15. R. Kreuzer, in Cephalopods: handling, processing and products, (FAO, 1984), p. 108

16. J. Morales, P. Montero, A. Moral, Isolation and partial characterization of two types of muscle collagen in some cephalopods. J Agric Food Chem **48**(6), 2142–2148 (2000). https://doi.org/10.1021/jf990711k

17. S. Mizuta, R. Yoshinaka, M. Sato, M. Sakaguchi, Isolation and partial characterization of two distinct types of collagen in the squid Todarodes pacificus. Fish Sci **60**(4), 467–471 (1994). https://doi.org/10.2331/fishsci.60.467

18. A. Thanonkaew, S. Benjakul, W. Visessanguan, Chemical composition and thermal property of cuttlefish (Sepia pharaonis) muscle. J Food Compos Anal **19**(2-3), 127–133 (2006). https://doi.org/10.1016/j.jfca.2005.04.008

19. W. Torres-Arreola, R. Pacheco-Aguilar, R.R. Sotelo-Mundo, O. Rouzaud-Sández, J.M. Ezquerra-Brauer, CyTA-J Food **6**, 10 (2008)

20. M. Ando, Y. Nakagishi, K. Yoshida, M. Nakao, Y. Nakagawa, Y. Makinodan, Y. Tsukamasa, K. Kawasaki, Pyridinoline concentrations in muscular and skin collagen of fish and relationship between collagen solubility and pyridinoline concentration in fish muscular collagen. Fish Sci **72**(5), 1104–1108 (2006). https://doi.org/10.1111/j.1444-2906.2006.01262.x

21. M. Nishimoto, S. Mizuta, R. Yoshinaka, Characterization of molecular species of collagen in muscles of Japanese amberjack, Seriola quinqueradiata. Food Chem **84**(1), 127–132 (2004). https://doi.org/10.1016/S0308-8146(03)00183-3

22. F.A. Vázquez-Ortiz, G. Caire, I. Higuera-Ciapara, G. Hernández, High performance liquid chromatographic determination of free amino acids in shrimp. J Liq Chromatogr Relat Technol **18**(10), 2059–2068 (1995). https://doi.org/10.1080/10826079508013960

23. AOAC, Official Methods of Analysis, Gaithersberg, MD: Association of Official Analytical Chemist, 17th ed. (2000)

24. U.K. Laemmli, Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature **227**(5259), 680–685 (1970). https://doi.org/10.1038/227680a0

25. N. Arakawa, M. Kim, M. Otsuka, An Improved High-Performance Liquid Chromatographic Assay for the Determination of Pyridinoline in Connective Tissues. J Nutr Sci Vitaminol **38**(4), 375–380 (1992). https://doi.org/10.3177/jnsv.38.375

26. P. Singh, S. Benjakul, S. Maqsood, H. Kishimura, Isolation and characterisation of collagen extracted from the skin of striped catfish (Pangasianodon hypophthalmus). Food Chem **124**(1), 97–105 (2011). https://doi.org/10.1016/j.foodchem.2010.05.111

27. T. Ikoma, H. Kobayashi, J. Tanaka, D. Walsh, S. Mann, Physical properties of type I collagen extracted from fish scales of Pagrus major and Oreochromis niloticas. Int J Biol Macromol **32**(3-5), 199–204 (2003). https://doi.org/10.1016/S0141-8130(03)00054-0

28. Z.E. Sikorski, J.A. Borderias, 1994. In Seafood Proteins, ed. By Z.E. Sikorski, B.S. Pan, F. Shahidi (Chapman & Hall, New York, 1994), p. 58–70, DOI: https://doi.org/10.1007/978-1-4615-7828-4_5

29. M. Sadowska, Z.E. Sikorski, Collagen in the tissues of squid illex argentinus and loligo patagonica - contents and solubility. J Food Biochem **11**(2), 109–120 (1987). https://doi.org/10.1111/j.1745-4514.1987.tb00116.x

30. J.E. Chan-Higuera, R.M. Robles-Sánchez, A. Burgos-Hernández, E. Márquez-Ríos, C.A. Velázquez-Contreras, J.M. Ezquerra-Brauer, Squid by-product gelatines: Effect on oxidative stress biomarkers in healthy rats. Czech J Food Sci **34**(2), 105–110 (2016). 10.17221/320/2015-CJFS

31. S. Kimura, Y. Miyauchi, N. Uchida, Comp. Biochem. Physiol. Part B,

Biochem. Mol Biol **99**, 473 (1991)

32. P. Kittiphattanabawon, S. Nalinanon, S. Benjakul, H. Kishimura, J. Chem. (2015)

33. W.S. Otwell, D.D. Hamann, Textural characterization of squid (loligo pealei lesuer): scanning electron microscopy of cooked mantle. J Food Sci **44**(6), 1629–1635 (1979). https://doi.org/10.1111/j.1365-2621.1979.tb09104.x

34. D.A. Ledward, R. Chizzolini, R.A. Lawrie, Int J Food Sci Technol **10**, 349 (1975)

35. G. Bruylants, J. Wouters, C. Michaux, Curr Med Chem **12** (2011, 2005)

36. A. Miki, S. Inaba, T. Baba, K. Kihira, H. Fukada, M. Oda, Structural and physical properties of collagen extracted from moon jellyfish under neutral pH conditions. Biosci Biotechnol Biochem **79**(10), 1603–1607 (2015). https://doi.org/10.1080/09168451.2015.1046367

37. C.A. Miles, N.C. Avery, V.V. Rodin, A.J. Bailey, The increase in denaturation temperature following cross-linking of collagen is caused by dehydration of the fibres. J Mol Biol **346**(2), 551–556 (2005). https://doi.org/10.1016/j.jmb.2004.12.001

38. R. Duan, J. Zhang, X. Du, X. Yao, K. Konno, Properties of collagen from skin, scale and bone of carp (Cyprinus carpio). Food Chem **112**(3), 702–706 (2009). https://doi.org/10.1016/j.foodchem.2008.06.020

39. M.H. Uriarte-Montoya, H. Santacruz-Ortega, F.J. Cinco-Moroyoqui, O. Rouzaud-Sández, M. Plascencia-Jatomea, J.M. Ezquerra-Brauer, Giant squid skin gelatin: Chemical composition and biophysical characterization. Food Res Int **44**(10), 3243–3249 (2011). https://doi.org/10.1016/j.foodres.2011.08.018

40. K.J. Payne, A. Veis, Fourier transform ir spectroscopy of collagen and

gelatin solutions: Deconvolution of the amide I band for conformational studies. Biopolymers **27**(11), 1749–1760 (1988). https://doi.org/10.1002/bip.360271105

41. A.M.D. Guzzi Plepis, G. Goissis, D.K. Das-Gupta, Dielectric and pyroelectric characterization of anionic and native collagen. Polym Eng Sci **36**(24), 2932–2938 (1996). https://doi.org/10.1002/pen.10694

42. F. Pati, B. Adhikari, S. Dhara, Isolation and characterization of fish scale collagen of higher thermal stability. Bioresour Technol **101**(10), 3737–3742 (2010). https://doi.org/10.1016/j.biortech.2009.12.133

43. B.G. Frushour, J.L. Koenig, Raman scattering of collagen, gelatin, and elastin. Biopolymers **14**(2), 379–391 (1975). https://doi.org/10.1002/bip.1975.360140211

44. M. Unal, H. Jung, O. Akkus, Novel raman spectroscopic biomarkers indicate that postyield damage denatures bone's collagen. J Bone Miner Res **31**(5), 1015–1025 (2016). https://doi.org/10.1002/jbmr.2768

45. P.L. Privalov, Stability of proteins. Adv Protein Chem Struct Biol **35**, 1 (1982). https://doi.org/10.1016/S0065-3233(08)60468-4

46. H. Yang, Y. Wang, S. Lai, H. An, Y. Li, F. Chen, Application of atomic force microscopy as a nanotechnology tool in food science. J Food Sci **72**(4), R65–R75 (2007). https://doi.org/10.1111/j.1750-3841.2007.00346.x

47. A. Sionkowska, J. Skopinska-Wisniewska, M. Gawron, J. Kozlowska, A. Planecka, Chemical and thermal cross-linking of collagen and elastin hydrolysates. Int J Biol Macromol **47**(4), 570–577 (2010). https://doi.org/10.1016/j.ijbiomac.2010.08.004

48. T.R. Thomas, *Rough Surfaces, Ed. By T.R. Thomas* (World Scientific Publishing Co. Pte. Ltd, London, 1999)

49. Z.E. Sikorski, D.N. Scott, D.H. Buisson, R.M. Love, The role of collagen in the quality and processing of fish. Crit Rev Food Sci Nutr **20**(4), 301–343 (1984). https://doi.org/10.1080/10408398409527393

CAPÍTULO II

Muscle lysyl oxidase activity and structural/thermal properties of highly cross-linked collagen in jumbo squid (*Dosidicus gigas*) mantle, fins and arms

Artículo aceptado en *Food Science and Biotechnology* (ISSN 1226-7708),
indexada en JCR
Año 2017

Carta de aceptación

De: "FOSB Editorial Office" <em@editorialmanager.com>

Fecha: 27 de septiembre de 2017, 00:14:46 GMT-7

Para: "Wilfrido Torres-Arreola" <wilfrido.torres@guayacan.uson.mx>

Asunto: Your Submission FOSB-D-17-00374R1

Responder a: FOSB Editorial Office <rica.hierco@springer.com>

Dear Dr. Torres-Arreola,

We are pleased to inform you that your manuscript, "Muscle lysyl oxidase activity and structural/thermal properties of highly cross-linked collagen in jumbo squid (*Dosidicus gigas)* mantle, fins and arms", has been accepted for publication in

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You will receive an e-mail in due course regarding the production process.

Please remember to quote the manuscript number, FOSB-D-17-00374R1, whenever inquiring about your manuscript.

With kind regards,

Eunok Choe

Editor in Chief of Food Science and Biotechnology

Muscle lysyl oxidase activity and structural/thermal properties of highly cross-linked collagen in jumbo squid (*Dosidicus gigas*) mantle, fins and arms

Abstract

Muscle from mantle, fins and arms of squid (*Dosidicus gigas*) were compared based on lysyl oxidase activity (LOX), chemical/structural and thermodynamic properties of highly cross-linked collagen. The arms collagen presented the highest temperature (Tp) and enthalpy of transition. The arms collagen thermic properties may be explained by the higher imino amino acid content, proline and lysine hydroxylation degrees. Moreover, among the regions, the collagen from the arms had a more intense ⚮ band chain, hydroxymerodesmosine peak in the resonance magnetic nuclear spectra and pyridinoline peak in the Raman spectra. Fins showed the highest LOX activity. The LOX activity was associated with the Tp, proline and lysine hydroxylation degrees. These results implied that the collagen in the arms was more intermolecularly ordered than the mantle and fins, and may provide a theoretical basis for a better understanding of the thermal behaviour of squid tissues during management and processing.

Keywords: Chemical structure; hydroxymerodesmosine; lysyl oxidase; squid collagen; thermal denaturation.

Introduction

According to the Food and Agricultural Organisation (FAO), jumbo squid (*Dosidicus gigas*) is one of the most important cephalopods captured in many countries' fishing industries (1). The main globally traded goods are the mantle, fins and arms. Fresh squid muscle has a viscous thickness and is hard to chew. Moreover, the textural behaviour of squid during ice storage and cooking differs from other seafood products (2, 3). These properties are attributed to the unique thermodynamic characteristics of its connective tissue (2), proteinaceously rich in collagen (4).

Moreover, due to the distinct function of the muscles in the live organism (5), the physicochemical properties of extracted squid connective tissue proteins depend on their anatomical origin (3, 5, 6, 7). Such properties can be attributed to the highly variable crosslinking degree that depends, among other factors, on the tissue evaluated (8).

The principal collagen fibre crosslinking mechanism reportedly occurs through the oxidation of hydroxylysine (Hyl) by lysyl oxidase (LOX), which is formed from pyridinoline (Pyr) (9). Pyr is an aromatic molecule that can be covalently linked to up to three collagen chains (10) and its concentration is closely associated with squid muscle thermal stability (11). However, several factors that dictate the collagen thermal stability, such as LOX activity (12), are intertwined in a complex manner (13). Thus, it is possible that Pyr is not the only molecule that affects the collagen thermal stability, yet there is scarce information available in the literature, to draw a definite

conclusion. Another molecule that could affect the collagen thermal stability is hydroxymerodesmosine (HMD). HMD is also formed through LOX activity (14, 15) and is linked to the interaction between α-chains, which form dimers or trimers.

Different fractions of collagen obtained from jumbo squid muscle, have been partially characterized, measuring Pyr concentration and LOX activity (7, 16, 17); however, these studies have primarily focused on soluble collagen fractions, which is not enough to elucidate the different cross-linking mechanisms in collagen fibers. A comparative research study of the collagen thermal denaturation, Pyr presence, and LOX muscle activity, among the highly cross-linked collagen from the edible anatomical regions of squid species, has not yet been performed. Therefore, the main focus of this study was to compare the thermal denaturation and chemical structure of highly cross-linked collagen, extracted from various anatomical regions of the jumbo squid (*D. gigas*). The possible correlation between the thermal properties of collagen, with tissue LOX activity, was discussed. This work provides an in-depth understanding of the interdependence between squid LOX activity, and collagen structure and thermal stability that can be used to direct future research on squid muscle management and processing.

Material and methods

Samples and sample preparation

Ten jumbo squid (*D. gigas*) (weight 5–6 kg, length 45–50 cm), captured during the fall of 2016 in the Gulf of California (28.75° N, 112.25° W), were purchased from local fisherman. The gutted organisms were transported on ice to the laboratory, within 8 h of capture. The organisms were skinned. Then, the mantles were separated from the fins and arms, and all samples immediately stored frozen (-25 °C), for no more than 7 days, until collagen and LOX extraction.

All reagents used in this work, were reagent grade (Sigma Chemical Co., St. Louis, MO, USA). Liquid chromatographic grade solvents were used in the amino acid analysis.

Collagen extraction

The connective tissue from the main anatomical regions of jumbo squid muscle (mantle, fins and arms) was extracted with 0.1 M NaOH (1:3 w/v, 4 °C, 24 h), followed by washing with Milli-Q water (to remove excess NaOH). Once centrifuged (32,200 *g*), the connective tissue was frozen at -40 °C, lyophilised and, then, fractionated with pepsin solution (10 mg g^{-1} tissue in 0.5 M acetic acid, 1:5 w/v, 4 °C, 24 h). After centrifugation (32,200 *g*, 40 min), the precipitate (50 mg) was homogenised (4 °C, 24 h) in 15 mL of 50 mM sodium acetate, pH 4.8, containing 6 M urea. The sample was passed through a cation exchange (18) HiTrap CM Sepharose FF column (GE Healthcare, Uppsala, Sweden), coupled to a ÄKTA purifier system (GE Healthcare). Highly cross-linked collagen was eluted with a linear gradient from 0–0.5 M NaCl, in a

total volume of 20 mL (flow rate 1.0 mL/min). The effluent was monitored at 280 nm. The fractions were pooled, dialysed against 0.05 M acetic acid, lyophilised and stored at -40 °C, until further analysis.

Gel electrophoresis

The purity of collagen was analysed by sodium dodecyl sulphate 7.5% polyacrylamide gel electrophoresis (SDS-PAGE) (19). The Highly cross-linked collagen (12 µg) was dissolved in 0.05 M Tris, pH 7.4, containing 1% SDS. The separation gel used, contained 5.5% acrylamide. The electrophoresed gels were stained with Coomassie Blue R-250 and imaged, using a densitometer (Bio-Rad GS-700, Hercules, CA).

Amino acid analysis

The amino acid composition of collagen, was determined by reverse-phase high-performance liquid chromatography (Agilent Technologies Inc., Palo Alto, CA) (20). The samples were hydrolysed in 6 M HCl containing sodium thioglycolate (1:1, v/v), at 150 °C for 60 min. After hydrolysis, the samples were derivatised at 60 °C for 5 min, in the presence of 0.5 M citrate containing 2 mg mL^{-1} 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole in methanol. The analysis was performed in triplicate and the results were expressed in % (w/w).

Raman spectroscopy

A Raman spectrometer (Perkin-Elmer GX), equipped with a Nd:YAG laser (excitation wavelength 1064 nm), was used to analyse the collagen (21) and to detect the presence of Pyr in the samples (22). The backscattered light was collected at 180 °C. The equipment was adjusted to 100 mW and 128 scans were collected at 4 cm^{-1}. The spectra were analysed using the program equipment's analysis.

^{1}H nuclear magnetic resonance (^{1}H NMR)

^{1}H NMR analyses of the collagen were measured at 25 °C on a Bruker Avance 400 spectrometer (Billerica, MA, USA), operating at 400 MHz (23). Lyophilised samples (1 mg) were dissolved in 0.5 mL of deuterated water (D_2O at 99.6% atom 2H, Sigma-Aldrich, St Louis, MO,USA) and 0.5 mL of deuterated potassium hydroxide solution (KOD 40% in D_2O). Dimethylsilapentane-5-sulphonic acid (DSS) was used as the reference, using a 20-ppm spectral window, 90° pulse and an acquisition time of 3.98 s for 128 scans.

Differential scanning calorimetry (DSC)

The thermal profile of the collagen was analysed, using DSC, from 25–180 °C (7). The transition temperature, onset temperature (To) and peak temperature (Tp), and the transition enthalpy (ΔH) of the collagen fractions, were measured using a 1020 Series DSC thermal analysis system (PerkinElmer, Norwalk, CN). Data acquisition and evaluation were done with a computer, using a Perkin Elmer PE Nelson 1022 integrator.

LOX extraction

LOX was sequentially extracted from the fresh muscle of the mantle, fins and arms, with phosphate buffer (pH 7.8) containing 16 mM KH_2PO_4, 16 mM K_2HPO_4, 0.4 M NaCl, and phosphate-buffered saline (1:2 w/v, 4 °C), followed by 4 M urea (4 °C, 3 h) (24). The homogenate was centrifuged (39,000 *g*, 40 min) and the supernatant was precipitated with 4 M urea. The urea fraction was resuspended in phosphate-buffered saline and frozen at -80 °C.

LOX activity

A Cary Eclipse G9800A fluorescence spectrophotometer (Agilent Technologies, USA) was used, to determine the fluorescent Amplex red (*N*-acetyl-3,7 dihydroxyphenoxazine) compound, generated from the LOX activity in the samples (16). The reaction mixture (1000 µL) consisted of 10 mM 1,5-diaminopentane (synthetic substrate), 50 mM sodium borate (pH 8.2), 1.2 M urea, Amplex red and 1 U mL^{-1} horseradish peroxidase. For the reaction, 50 µL of the enzymatic extract was added to the mixture, followed by incubation at 37 °C for 30 min. The reaction was stopped by immersion in an ice bath for 5 min. The excitation wavelength was 560 nm and the emission wavelength was 590 nm. The amount of hydrogen peroxide (H_2O_2) produced by the action of LOX, was determined by comparing the fluorescence intensity with an H_2O_2 standard curve. The specific activity was expressed as enzyme units per mg of protein (U mg^{-1}). Each trial was performed in triplicate.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA), to establish differences among the samples of the mantle, fins and arms. The normality of the

data was assessed by the Kolmogorov–Smirnov test. Differences between least-squares means were examined by Tukey–Kramer's honestly significant difference (HSD) test ($p < 0.05$, in all cases). JMP version 4.04 (Statsoft, Tulsa, OK) was used for all statistics. Correlation values for LOX activity with Tp, and proline (Pro) and lysine (Lys) hydroxylation degrees, were calculated. All assays were performed in triplicate and the results were presented as mean ± standard deviation.

Results and discussion

Highly cross-linked collagen concentration

The yields of connective tissue in mantle, fins and arms were 0.8, 1.3 and 2.0 g/100 g muscle, respectively. In jumbo squid, connective tissue concentrations usually vary between 1 and 18 g depending on the anatomical region and the age of the organism (4). In these extracts, the highly cross-linked collagen concentration varied significantly ($p < 0.05$). The greatest yield was obtained from the arms (51%), followed by the mantle (28.7%) and fins (22.3%). This behaviour is similar to that observed in previous works, which found that, depending on the anatomical region, there is variability in muscle collagen content (4, 5, 7).

SDS-PAGE electrophoresis

The electrophoretic patterns of the purified collagen samples obtained from each anatomical region (Figure 1), demonstrated that the collagen comprised α- and β- chains as the main constituents, with a low content of γ-chains, indicating that Lys-derived crosslinks were present (26). The similarity in the electrophoretic migration and amino acid composition (Table 1) for collagen type I of *D. gigas* with *Illex coindetii*, *Todaropsis eblanae* and *Todarodes pacificus* (5, 27), confirmed the presence of type I collagen in the analysed tissues. The band intensity (optical density) of the crosslinked chain (β dimer) was higher ($p < 0.05$) in collagen from the arms (3,040 ± 211) than fins (2,280 ± 210) and mantle (1,520 ± 214). This trend was confirmed by the Pyr Raman analysis.

Amino acid analysis

The amino acid compositions of purified collage from the mantle, fins and arms, are shown in Table 1. The glycine (Gly) content represented 29–30% of the total amino acids residues in all the collagen samples. These Gly contents are typical for collagen (25). In addition, low proportions of leucine (Leu), Lys and serine (Ser) and high proportions of alanine (Ala) and hydroxyproline (Hyp) were detected. Based on the study by Morales *et al.* (5), these results implied that the three anatomical regions contain type I collagen.

The analysis of the total imino acid (Pro + Hyp) content revealed that, collagen from the arms had a higher ($p < 0.05$) Pro + Hyp content than that obtained either from mantle or fins. Furthermore, the Pro and Lys hydroxylation extents (%), tended

to be higher ($p < 0.05$) in the arms collagen. The differences in the percentage of hydroxylation of these amino acids, suggest that each anatomical region possesses different covalent crosslinks. Therefore, under the conditions of this study, this result demonstrated that the tissue collagen derived from the arms, possessed a higher degree of crosslinks than the mantle and fins, as was confirmed by SDS-PAGE and the intensity of the Pyr band, by Raman spectroscopy.

Raman spectroscopy analysis

Raman spectroscopy was applied to analyse the structure of the purified collagens (Figure 2). In general, relatively neutral bonds (CC, CH, C=C) are strong Raman scatterers. The bands observed at about 1663 and 830 cm^{-1}, are associated with Pyr. The differences in the shape and intensity of these bands, suggest structural differences among the samples (22). The higher intensity of these bands in collagen from the arms compared to mantle and fins implies a higher proportion of highly cross-linked amino acids in this collagen.

Bands observed in the high-frequency region (~1246 cm^{-1}) are assigned to amide III. Fewer intense bands in this region were observed in the collagen from the mantle and fins than from the arms, suggesting fewer chain interactions through the hydrogen bonds of the collagen triple helix (21, 28), as well as a lower thermal stability.

The peak at 1326 cm^{-1} (22) is assigned to Pro and 845 cm^{-1} to Hyp (29). These bands were more prominent in the collagen from the arms than mantle and fins, in accordance with the amino acid profiles. The highest Hyp activity, which was

associated with the arms collagen, confirmed that the collagen in this anatomical region possesses a more stable triple helix, and therefore, would be expected to display the highest thermal stability, among the samples.

The 815–935 cm^{-1} region is associated with vibrations of C-C and C-O-C bonds present in covalent crosslinks of collagen molecules including, besides Pyr, HMD, 5-hydroxylysinonorleucine, and glucosyl-galactosylhydroxylysinonorleucine (14, 30, 31). These types of bonds derive from the condensation between Lys and histidine (His) residues of the protein side chains. The oxidation of the adjacent Lys residues by LOX activity, results in the condensation of both residues that subsequently react with His residues, to form histidino-dihydrohydroxymerodesmosine (15).

^{1}H NMR

The chemical structure of collagen from the squid mantle, fins and arms, were also analysed by ^{1}H NMR (Figure 3), allowing the assignment of amino acid side chain protons. All extracts were prepared under identical conditions to avoid variability in the NMR spectra. Consequently, any differences in spectra can be attributed to the anatomical region evaluated.

The ^{1}H NMR analysis of all samples studied, confirmed the presence of water molecules (4.8 at 4.6 ppm) (32). Proton alpha-Pro (4.5 ppm) chemical displacements were similar in all samples analysed. However, the intensity of this peak varied. Although the method cannot be used for amino acid quantification, a relative estimation can be made. In general, a smaller Pro peak was observed in collagen

from the fins than mantle and arms, which is consistent with the above-discussed amino acid compositions. Moreover, the peak at 3.8 ppm, attributed to protons present in hydroxylated carbon and allyl methylene adjacent to the imino group of HMD (14), was more intense in the arms than the other anatomical regions evaluated, suggesting that relatively more Lys residues had been oxidised by LOX, in the collagen from the arms, to form this molecule.

Thermal analysis

The thermal stability of collagen, purified from the mantle, fins and arms, was determined using DSC, by measuring the beginning (To) and the peak temperatures (Tp) during the protein transition (Table 2). The denaturation temperature was above 100 °C in all cases, in agreement with previous reports (2, 33); To and Tp of collagen extracted from arms were higher ($p < 0.05$) than the transition temperatures of the mantle and fin collagens, indicating that the collagen extracted from the arms was more thermally stable than the other collagens. However, the necessary energy (ΔH) to disrupt the structure of the collagen extracted from the mantle was greater ($p < 0.05$) than in the collagen of fin and arms. In DSC, a single peak may consist of several endothermic and exothermic interactions. Thermally induced protein denaturation results from a mixture of reactions such as the endothermic disruption of hydrogen bonds, the exothermic breaking of hydrophobic interactions, and others events occurring during heating (34). These results showed that the helices of the mantle collagen had a greater number of conformational changes than those of the other collagens.

Among the samples, the lowest Tp and ΔH values were observed in the collagen from fins, consistent with the lower Pro and degree of hydroxylation of Pro and Leu, detected in this anatomical region. Likewise, Torres-Arreola *et al.* (7) reported that among the anatomical regions, collagen from the fins showed both the lowest Tp and ΔH and can be attributed to a less stable triple helix structure (12, 35).

LOX activity

The specific LOX activity determined in each anatomical region was significantly different ($p < 0.05$). The highest LOX activity was measured in the fins (6.8×10^{-3} U mg^{-1}), followed by mantle (3.5×10^{-3} U mg^{-1}) and arms (2.3×10^{-3} U mg^{-1}). Among the anatomical regions, the highest LOX activity detected in fins muscle, might suggest that less Lys residues have been oxidised by this enzyme, as confirmed by the relatively lower Hyl content and the lower band intensity of Pry and HMD, by Raman and ^{1}H NMR, respectively, in this sample.

Finally, significant correlations between Hyl content (R2= -0.99), Hyp content (R2= -0.97) and Tp (R2= -0.85) of collagen, with LOX activity, were observed. Thus, the differences in hydroxylation (%) of Lys and Pro (Table 1), and HMD band intensity (^{1}H NMR) (Figure 3), might suggest that Pyr itself, partially contributed to the collagen thermal stability. Several factors that dictate the collagen thermal stability, such as LOX activity (12), are interconnected in a complex manner (13), requiring more studies to decipher the exact intercorrelations.

Under the conditions of this work, it was proven that the collagen from each jumbo squid anatomical region has a different chemical structure. The Tp and ΔH

indicated that the fibres of arms collagen are stronger than that of the mantle and fins. The differences detected are due to variations in the crosslinked helix structure, among the collagens derived from the mantle, fins and arms. The detection of the HMD band by Raman indicated that the Pyr is not the only molecule involved in the crosslinking of collagen, which was consistent with the results of the collagen thermodynamic values. Despite the lower LOX activity detected in arms muscle, its collagen seems to have a higher degree of crosslinking than the mantle and fins. However, analysis using additional techniques is important to corroborate this finding. These results may serve as a fundamental basis, for further investigation into the management and processing of jumbo squid tissues.

Acknowledgments

This research was supported by CONACYT under grant 180214. Author Sarabia-Sainz acknowledges doctoral fellowship from CONACYT. The authors are also grateful to Javier Valdez for his valuable help during the study.

References

1. FAO. Global production statistics–fisheries and aquaculture. Available from: http://www.fao.org/fishery/statistics/global-aquaculture-production/en. Accessed May. 27, 2017.

2. Valencia-Pérez AZ, García-Morales MH, Cárdenas-López JL, Herrera-Urbina JR, Rouzaud-Sández O, Ezquerra-Brauer, JM. Effect of thermal process on connective tissue from jumbo squid (*Dosidicus gigas*) mantle. Food Chem. **107**: 1371-1378 (2008).

3. Osuna-Amarillas PS, Márquez-Ríos E, Rouzaud-Sandez O, Suarez-Jiménez GM, Cota-Arriola O, Ocaño-Higuera VM, Arvizu-Flores AA, Torres-Arreola W. Physicochemical changes of connective tissue proteins in jumbo squid (*Dosidicus gigas*) muscle during ice storage. J. Food Process. Preserv. **41**(1) (2017).

4. Sikorski ZE, Kołodziejska I. The composition and properties of squid meat. Food Chem. 20(3): 213-224 (1986).

5. Morales J, Montero P, Moral A. Isolation and partial characterization of two types of muscle collagen in some cephalopods. J. Agric. Food Chem. 48(6): 2142-2148 (2000).

6. Thanonkaew A, Benjakul S, Visessanguan W. Chemical composition and thermal property of cuttlefish (*Sepia pharaonis*) muscle. J. Food Compost. Anal. 19(2): 127-133 (2006).

7. Torres-Arreola W, Pacheco-Aguilar R, Sotelo-Mundo RR, Rouzaud-Sández O, Ezquerra-Brauer JM. Partial characterization of collagen from mantle, fin, and arms of jumbo squid (*Dosidicus gigas*). CyTA-J Food 6(2):101-108 (2008).

8. Gómez-Guillén MC, Sarabia AI, Solas MT, Montero P. Effect of microbial transglutaminase on the functional properties of megrim (*Lepidorhombus boscii*) skin gelatin. J. Sci. Food Agr. 81(7): 665-673 (2001).

9. Butler E, Hardin J, Benson S. The role of lysyl oxidase and collagen crosslinking during sea urchin development. Exp. Cell. Res. 173(1): 174-182 (1987).

10. Eyre D. Collagen crosslinking amino acids. Methods Enzymol. 144: 115–139 (1987).

11. Young OA, Braggins TJ, Barker GJ. Pyridinoline in ovine intramuscular collagen. Meat Sci. 37(2): 297-303 (1994).

12. Ando M, Makino M, Tsukamasa Y, Makinodan Y, Miyoshi M. Interdependence between heat stability and pyridinoline contents of squid mantle collagen. J. Food Sci. 66: 265–269 (2001).

13. Miles CA, Avery NC, Rodin VV, Bailey AJ. The increase in denaturation temperature following cross-linking of collagen is caused by dehydration of the fibres. J. Mol. Biol. 346(2): 551-556 (2005).

14. Masuda M, Karube S, Hayashi Y, Shindo H, Igarashi M. Direct measurement of collagen crosslinks with automatic amino acid analyzer—identification of peaks due to crosslinks. FEBS Lett. 63(2): 245-249 (1976).

15. Voet D, Voet JG, Pratt CW. Fundamentals of Biochemistry: Life at the Molecular Level 2nd ed. Hoboken pp. 142 (2006).

16. Torres-Arreola W, Ezquerra-Brauer JM, Figueroa-Soto CG, Valenzuela-Soto EM, Garcia-Sanchez G, Marquez-Rios E, Pacheco-Aguilar R. Lysyl oxidase from

jumbo squid (*Dosidicus gigas*) muscle: detection and partial purification. Int. J. Food Sci. Technol. 46(8): 1711-1715 (2011).

17. Ramirez-Guerra HE, Fimbres-Romero MJ, Tapia-Vazquez AE, Ezquerra-Brauer JM, Márquez-Ríos E, Suarez-Jimenez GM, Torres-Arreola W. Relationship between lysyl oxidase activity, pyridinoline content and muscle texture during ice storage of jumbo squid (*Dosidicus gigas*). Int. J. Food Sci. Technol. 50(12): 2700-2706 (2015).

18. Nishimoto M, Mizuta S, Yoshinaka R. Characterization of molecular species of collagen in muscles of Japanese amberjack, *Seriola quinqueradiata*. Food Chem. 84(1): 127-132 (2004).

19. Laemmli U. Cleavage of structural proteins during assembly of the head bacteriophague T4. Nature 227: 680–685 (1970).

20. Vázquez-Ortiz FA, Caire G, Higuera-Ciapara I, Hernández G. High performance liquid chromatographic determination of free amino acids in shrimp. J. Liq. Chrom. Relat. Tech. 18(10): 2059-2068 (1995).

21. Ikoma T, Kobayashi H, Tanaka J, Walsh D, Mann S. Physical properties of type I collagen extracted from fish scales of *Pagrus major* and *Oreochromis niloticas*. Int. J. Biol. Macromolec. 32(3): 199-204 (2003).

22. Gamsjaeger S, Robins SP, Tatakis DN, Klaushofer K, Paschalis EP. Identification of pyridinoline trivalent collagen cross-links by Raman microspectroscopy. Calcif. Tissue Int. 100(6): 565-574 (2017).

23. Uriarte-Montoya MH, Santacruz-Ortega H, Cinco-Moroyoqui FJ, Rouzaud-Sández O, Plascencia-Jatomea M, Ezquerra-Brauer JM. Giant squid skin gelatin: chemical composition and biophysical characterization. Food Res. Int. 44(10): 3243–3249 (2011).

24. Kagan H, Cai P. Isolation of active site peptides of lysyl oxidase. Methods Enzymol. 258: 122–132 (1995).

25. Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. Collagen: the fibrous proteins of the matrix. Molecular Cell Biology, 4, Section 22.3, New York, USA: W. H. Freeman (2000).

26. Kimura S, Miyauchi Y, Uchida N. Scale and bone type I collagens of carp (*Cyprinus carpio*). Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 99(2): 473-476 (1991).

27. Kittiphattanabawon P, Nalinanon S, Benjakul S, Kishimura H, Characteristics of pepsin-solubilised collagen from the skin of splendid squid (*Loligo formosana*). J. Chem. (2015).

28. Frushour BG, Koenig JL. Raman scattering of collagen, gelatin, and elastin. Biopolymers 14(2): 379-391(1975).

29. Zhang Q, Andrew Chan KL, Zhang G, Gillece T, Senak L, Moore DJ, Mendelsohn R, Flach CR. Raman microspectroscopic and dynamic vapor sorption characterization of hydration in collagen and dermal tissue. Biopolymers 95(9): 607-615 (2011).

30. Borel JP. Les collagènes. l'Eurobiologiste 25(195): 247-271 (1991).

31. Leroy M, Labbé, JF, Ouellet M, Jean J, Lefèvre T, Laroche G, Auger M, Pouliot R. A comparative study between human skin substitutes and normal human skin using Raman microspectroscopy. Acta Mater. 10(6): 2703-2711 (2014).

32. Fullerton, GD, Nes E, Amurao M, Rahal A, Krasnosselskaia L, Cameron I. An NMR method to characterize multiple water compartments on mammalian collagen. Cell Biol. Int. 30(1): 66-73 (2006).

33. Mizuta S, Tanaka T, Yoshinaka R. Comparison of collagen types of arm and mantle muscles of the common octopus (*Octopus vulgaris*). Food chem. 81(4): 527-532 (2003).

34. Stanley DW, Yada RY. 1992. Physical consequences of thermal reactions in food protein systems. Chap 16 in: Physycal Chemistry of Foods. Editors H.G. Schwartzberg & R.W. Hartel. Marcel Dekker Inc. EUA. Pg 693.

35. Singh P, Benjakul S, Maqsood S, Kishimura H. Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*). Food Chem. 124(1): 97-105 (2011).

Figure captions

Figure 1 SDS–PAGE patterns of collagen obtained from the mantle (A), fins (B) and arms (C) of giant squid.

Figure 2 Raman spectra of collagen obtained from the mantle (A), fins (b) and arms (C) of giant squid, showing the high-frequency fingerprint regions (500 and 3000 cm^{-1}).

Figure 3 ^{1}H NMR spectra of collagen obtained from the mantle (A), fins (b) and arms (C) of giant squid. Amino acids indicated by their corresponding peaks; Lysine, (Lys), threonine (Thr), isoleucine (Ile), glutamate (Glu), proline (Pro), histidine (His) and hydroxymerodesmosine (HMD).

Table 1. Amino acid composition (%) of highly cross-linked collagen purified from the mantle, fins and arms of jumbo squid*.

Amino acid	Mantle	Fins	Arms
Hyp	9.1±0.1	7.9±0.3	11.2±0.4
Asp	5.7±0.1	5.3±0.3	5.4±0.2
Thr	2.9±0.4	2.5±0.1	2.0±0.4
Ser	2.9±0.3	3.9±0.4	2.0±0.4
Glu	8.0±0.5	9.2±0.7	6.8±0.3
Pro	11.0±0.6	10.6±0.6	13.1±0.7
Gly	29.5±0.6	28.9±0.7	30.0±0.6
Ala	12.2±0.7	10.4±1.2	12.4±0.7
Val	2.9±0.4	2.9±0.2	3.0±0.2
Met	1.0±0.2	1.0±0.1	1.0±0.4
Ile	1.1±0.2	1.2±0.3	1.0±0.4
Leu	1.2±0.1	1.9±0.1	1.0±0.2
Tyr	0.4±0.02	0.4±0.04	0.2±0.03
Phe	1.2±0.4	1.2±0.2	1.0±0.2
His	1.0±0.1	1.9±0.1	1.0±0.2
Hyl	1.8±0.5	1.7±0.6	2.0±0.8
Lys	1.9±0.8	2.3±0.3	1.9±0.6
Arg	6.2±0.7	6.1±0.5	5.0±0.9
Imino acid (Pro + Hyp)	20.1 ± 0.4^{b}	18.5 ± 0.3^{c}	24.3 ± 0.6^{a}
Pro hydroxylation (%)	45.3 ± 0.6^{c}	42.7 ± 0.3^{c}	46.1 ± 0.3^{a}
Lys hydroxylation (%)	49.3 ± 0.2^{b}	43 ± 0.3^{c}	51 ± 0.3^{a}
True collagen content/crude protein** (%)	84 ± 0.6^{c}	75 ± 1.3^{b}	93 ± 2.4^{a}

*Values are the mean of triplicates±standard deviation (n=3). **True collagen content/crude protein = [hydroxyproline x 8/crude protein] × 100 (AOAC, Method 990.26, 2000). Different letters in imino acid (Pro+Hyp), Pro hydroxylation, Lys hydroxylation and true collagen denote significant difference ($p < 0.05$).

Table 2. Transition temperatures and enthalpies of collagen obtained from mantle, fins and arms of giant squid

Anatomical region	To (°C)	Tp (°C)	ΔH (J/g)
Mantle	137±0.6[b]	153.2±0.6[b]	33.8±1.4[a]
Fins	135±0.4[c]	145.9±0.6[c]	23.3±1.5[b]
Arms	138±0.7[a]	176.1±0.4[a]	25.8±1.5[b]

Values are mean of three replicates. Different letter for each column are statistically different ($p < 0.05$).

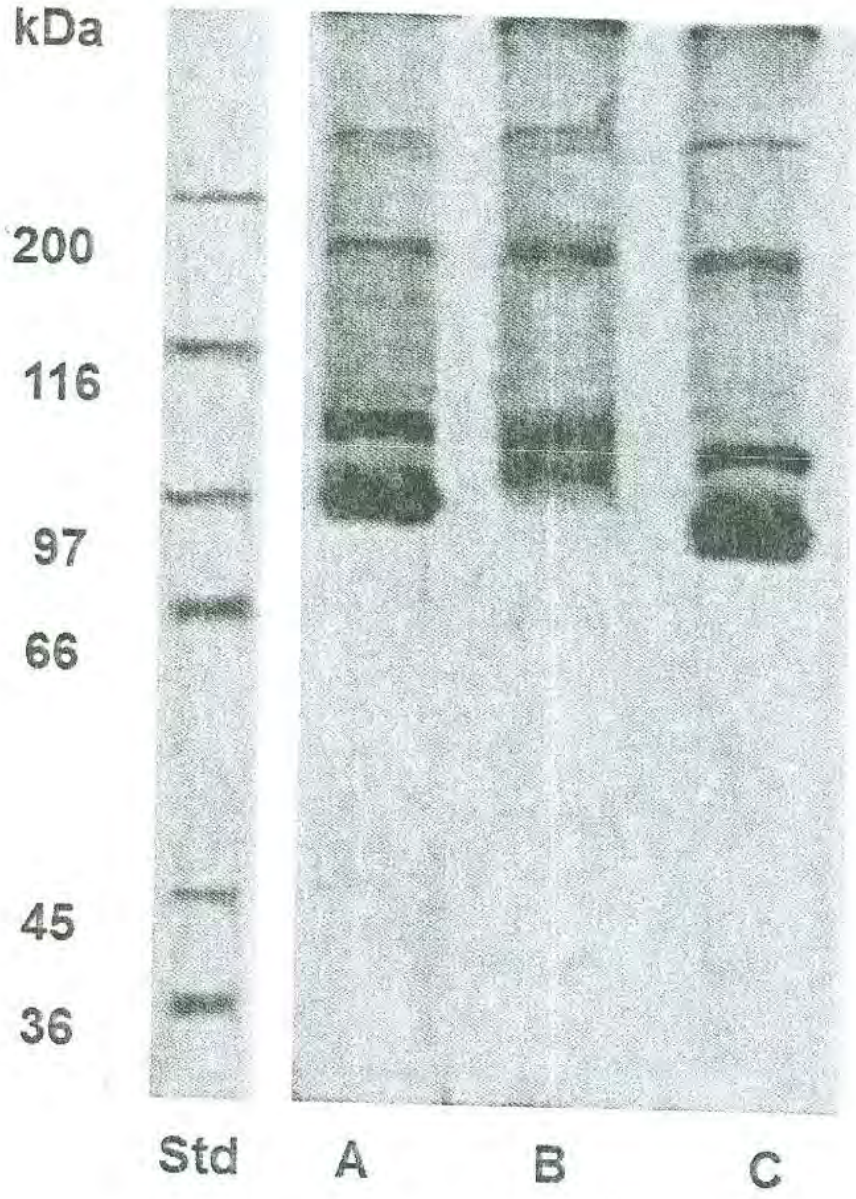

Figure 1.

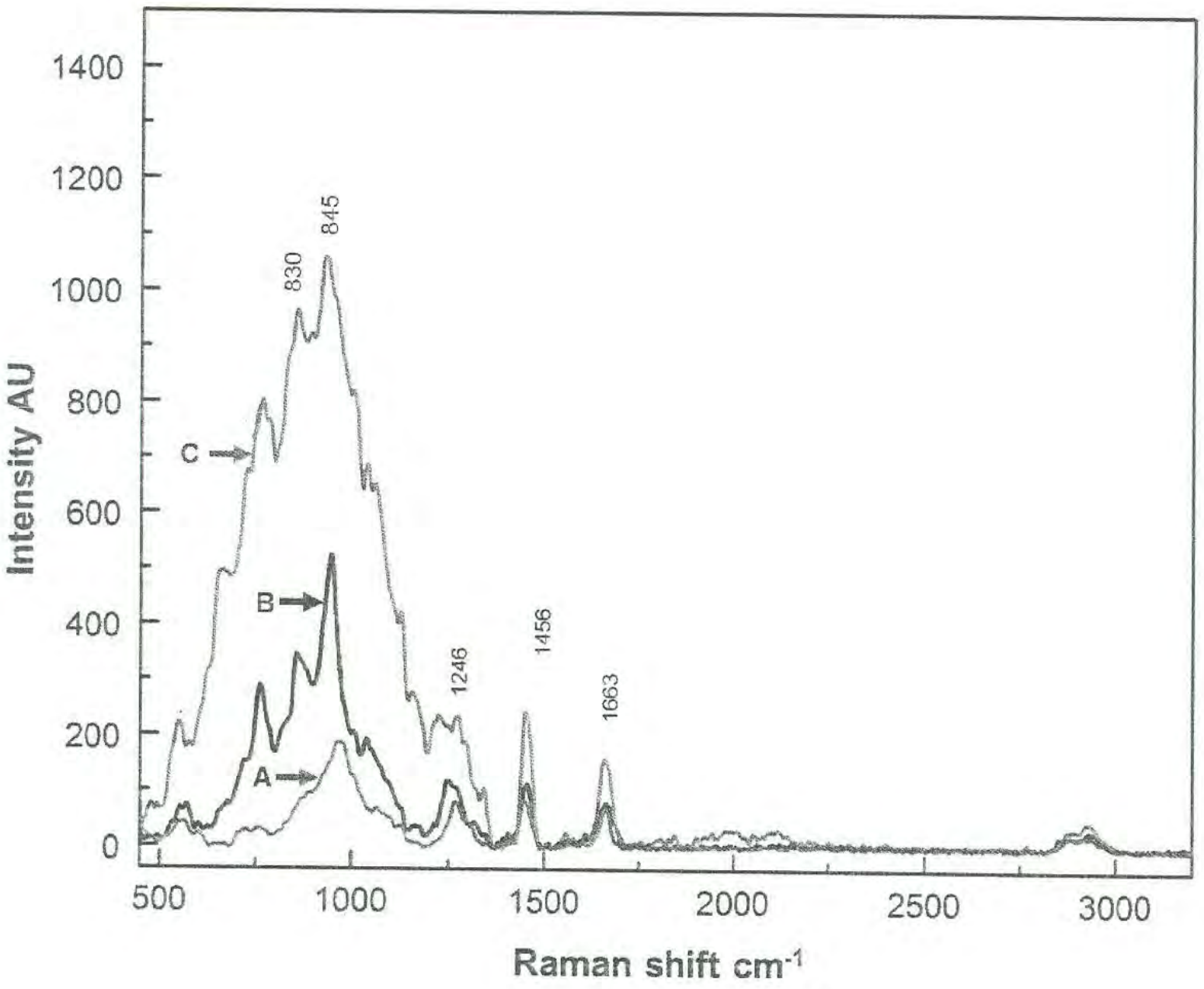
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Figure 2.

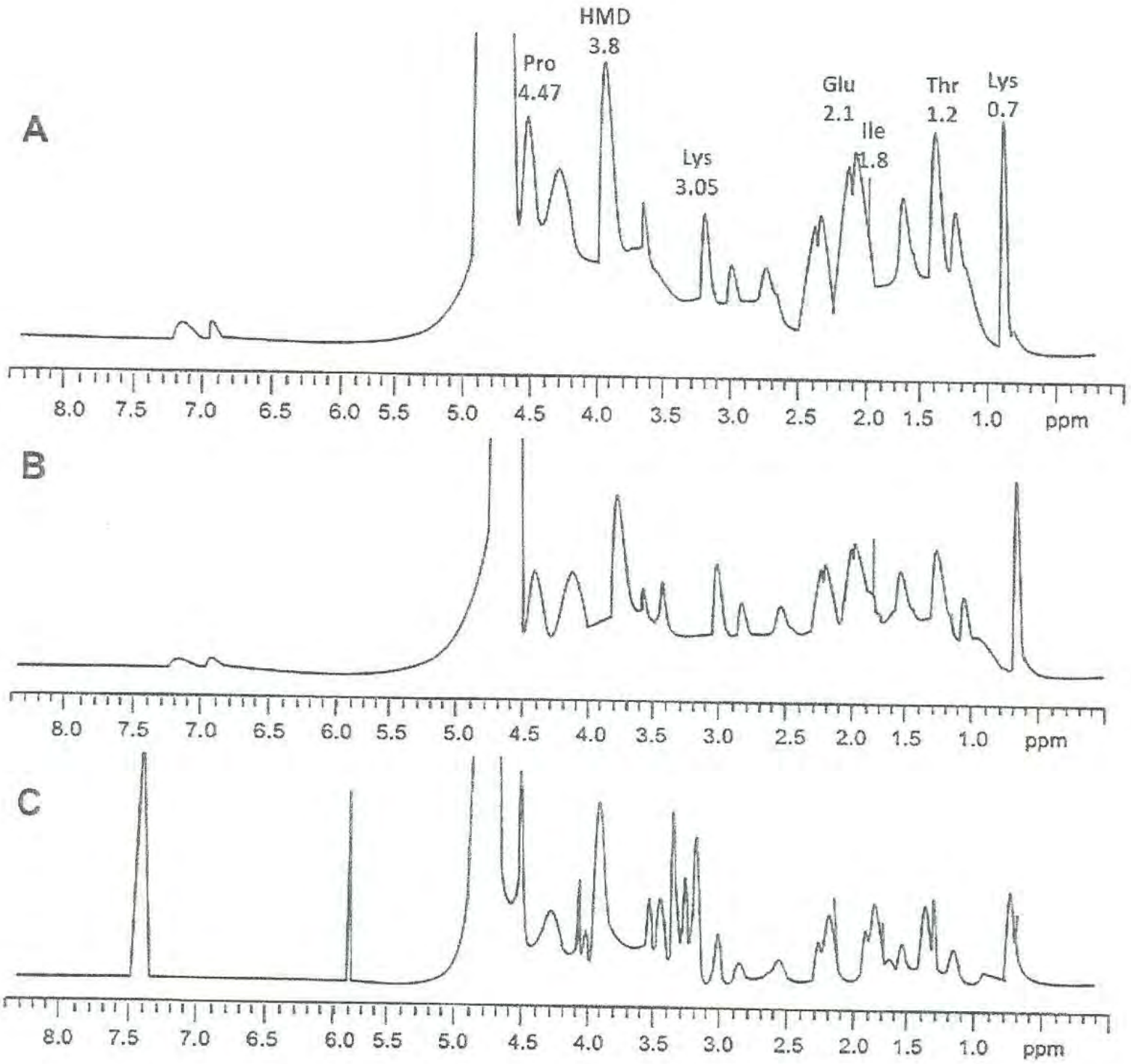
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Figure 3.

CAPÍTULO III

Spectroscopic Imaging: Nuclear Magnetic Resonance and Raman for the detection of collagen cross-linking from giant squid mantle, fins and tentacles tissues

Manuscrito sometido a *Instrumentation Science & Technology* (ISSN 1073-9149), *indexada en JCR*

Año 2017

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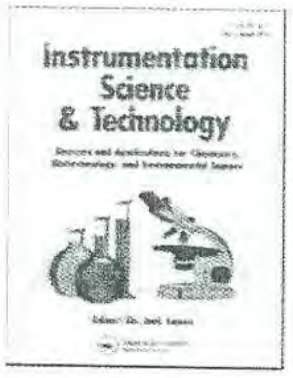

Spectroscopic imaging: nuclear magnetic resonance and Raman for the detection of collagen cross-linking from giant squid mantle, fin, and tentacle tissues

Journal:	*Instrumentation Science & Technology*
Manuscript ID	Draft
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Sarabia-Sainz, Héctor ; Universidad de Sonora Division de Ciencias Biologicas y de la Salud, Departamento de Investigación y Posgrado en Alimentos Torres-Arreola, Wilfrido; Universidad de Sonora Division de Ciencias Biologicas y de la Salud, Departamento de Investigación y Posgrado en Alimentos Ezquerra-Brauer, Josafat; Universidad de Sonora Division de Ciencias Biologicas y de la Salud, Departamento de Investigación y Posgrado en Alimentos
Keywords:	Dosidicus gigas, HPLC, 1H-NMR, Pyridinoline, Raman

Spectroscopic imaging: nuclear magnetic resonance and Raman for the detection of collagen cross-linking from giant squid mantle, fin, and tentacle tissues

Runing Head: Spectroscopic analysis of pyridinoline from squid collagen

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Acknowledgements

This research was supported by CONACYT under grant 180214. Author Sarabia-Sainz acknowledges a doctoral fellowship from CONACYT. The authors are also grateful to Monica Acosta Elias for her valuable help with Raman measurements.

Spectroscopic imaging: nuclear magnetic resonance and Raman techniques for the detection of collagen cross-linking from squid (*Dosidicus gigas*) mantle, fin, and tentacle tissues

Abstract

The application of proton nuclear magnetic resonance (^{1}H-NMR) imaging and Raman spectrometry is described in this work, for the detection and structural analysis of collagen cross-linking (pyridinoline, or Pyd) in soluble (SCF) and insoluble collagen fractions (ICF) obtained by cation-exchange separation of the mantle, fins, and tentacles of squid (*Dosidicus gigas*). The Pyd was detected using fluorometric analysis only in SCF fractions. However, the Pyd was detected when using high performance liquid chromatography (HPLC) only in ICF from tentacles. Meanwhile, in all samples, Pyd was detected by ^{1}H-NMR and Raman spectrometry. The higher peak intensities in both methods were observed in ICF from tentacles, the muscles of which showed a lower decrease in shear force after 30 min of cooking treatment. ^{1}H-NMR and Raman gives an assay which is more sensitive than fluorescence and HPLC for Pyd detection in squid tissues, suggesting much greater potential as analysis techniques for different types of tissue and collagen fractions.

Keywords: *Dosidicus gigas*; HPLC; ^{1}H-NMR; Pyridinoline; Raman

Introduction

Recent years have seen increased interest in identifying and characterising bioactive proteins derived from marine sources. Among them, squid by-products are valuable underutilised sources of protein, where the predominant protein is collagen. Collagen is a fibrous insoluble protein mostly found in skin, cartilage, and connective tissues, whose primary function is to provide support; this protein maintains the union between cells[(1)]. Several types of collagen have been identified; the primary

characteristic of all types are amino acid arrangements that are rich in proline and glycine. These arrays form three chains that intertwine to create a triple helix, which varies in composition and size[2]. The principal collagen fibre cross-linking mechanism reported is the oxidation of hydroxylysine by lysyl oxidase, which results in pyridinoline (Pyd)[3]. Pyd is an aromatic molecule that can be covalently linked to up to three collagen chains[4].

In squid, collagen is a very important component since it significantly contributes to its viscous and hard muscle. It is also responsible for unusual textural change behaviour during ice storage or the cooking process, which is different to the textures of other seafood products that have been attributed to Pyd[5–6]. Likewise, squid gelatine, a product derived from collagen that it is a common ingredient in various industries including food, pharmaceuticals, and cosmetics among others, has been extracted and characterised[7–9]. Squid gelatine physicochemical properties are generally related to Pyd[9]. Consequently, more emphasis has been put on detection studies of squid by-products such as Pyd to explain the physicochemical characteristics of the raw material and its collagen functional properties[5, 6, 10].

The fluorescence properties of Pyd, caused by the hydroxylated pyridinium ring, have been applied to detect Pyd in some fish and squid species collagen sources, mainly muscle and skin[5, 10, 11]. Although Pyd was detected in the edible regions of *Dosidicus gigas*, *Pagrus major*, and *Seriola quinqueradiata*, this molecule could not be found in their skins, which are rich in connective tissue[5, 10–11].

The Pyd content varies broadly with the type of tissue and its sparse occurrence makes detection very difficult[12]. In fish muscle, collagen exists in endomysium, perimysium, and myocommata. Myocommata is required for much physical strength because it must connect myomeres continuously. However, endomysium might be not required for such physical strength[10]. Therefore, because the required strength is different depending on site location, collagens with several concentrations of Pyd might exist. Moreover, Pyd crosslinks would have a close relationship to collagen solubility[5, 10]. Consistent with this, a considerable amount of Pyd predominates in insoluble collagens, rather than soluble ones, as detected in several squid species[5].

For the Pyd analysis, several techniques can be applied, although high performance liquid chromatography (HPLC) is the method of reference[13]. However, it can be time consuming[14] and, depending on the initial steps of Pyd isolation, the characteristic fluorescence might not be detected[15]. Moreover, although the spectroscopic methods are not as selective as HPLC, they are feasible for analysis of Pyd[16, 17]. Proton nuclear magnetic resonance (^{1}H-NMR) imaging and Raman spectroscopy are non-destructive and highly versatile analytical techniques. They display considerable potential for *in vivo* and *in vitro* analysis of biological tissues and they do not require previous extraction[18] or preparation.

To the best of our knowledge, ^{1}H-NMR imaging with Raman spectroscopy has not been applied to biochemical characterisation of collagen cross-linked peptides of squid tissues from three different anatomical regions, either from collagen soluble or

insoluble fractions. The purpose of the present study is to probe if it is feasible to compare the Pyd content in the soluble and insoluble collagen fractions extracted from the mantle, fins, and tentacles of squid (*Dosidicus gigas*) by both ^{1}H-NMR and Raman spectroscopic methods. Also, the shear force required to shear the squid muscle and its collagen thermal properties, after a 30 min cooking treatment, was determined. The present study contributes information regarding some aspects of Pyd analytical methods for squid collagen tissues, which might lead to a more suitable and reliable method for Pyd analysis.

Materials and methods

Reagents

All reagents used were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Samples and sample preparation

Ten squid (*Dosidicus gigas*) specimens, hand-captured by jigging, were purchased from local fisherman (Bahia Kino in the Gulf of California, Mexico, 28.75° N, 112.25° W, water temperature 15–18°C). The squid were transported in ice to the laboratory within 8 hours of capture. The organisms were gutted, and the mantles were separated from the fins and tentacles. Each anatomical region was skinned and chopped. Approximately 250 g portions of each anatomical region were packed in polyethylene bags and stored at freezing temperature (-25°C) for no more than seven days until collagen extraction.

Extraction and fractionation of collagen

Collagen was extracted at 4°C. Non-collagenous material was removed by extraction with a 6 M urea solution containing 0.5 M sodium acetate (pH 6.8), stirred for 60 min, and centrifuged at 39,200 x g for 40 min. The precipitates were stirred in 1 M Tris (pH 7.2), containing 0.05 M NaCl, for 24 h. After stirring, the mixtures were centrifuged at 39,200 x g for 40 min. The residues were homogenised with 0.5 M acetic acid (ratio 1:5 w/v), incubated with agitation for 24 h, and centrifuged at 39,200 x g for 40 min. The precipitate was mixed with pepsin solution (10 mg/g tissue in 0.5 M acetic acid; 1:5 w/v) and incubated for 24 h. The homogenate was centrifuged at 39,200 x g for 40 min to produce a supernatant, designated the pepsin-solubilised collagen (PSC)[19].

The soluble and insoluble fractions from the PSC were prepared by adding NaCl to a final concentration of 2 M, and the precipitate was collected by centrifugation at 39,200 x g for 40 min. The resulting supernatant was designated the soluble collagen fraction (SCF), and the precipitate was designated the insoluble collagen fraction (ICF). Both fractions were dissolved in 0.05 M acetic acid and dialysed at 4 °C with water in a cellulose membrane with a 10 kDa cutoff[20]. The collected samples were frozen at -40 °C and lyophilised.

Purification of collagen fractions

Purification of collagen from the PSC and ICF were further purified by cation exchange column chromatography[21] using a HiTrap CM Sepharose FF (GE Healthcare, Uppsala, Sweden) assisted by ÄKTA Purifier chromatographic equipment (GE Healthcare, Uppsala, Sweden). Each fraction (50 mg) was dissolved in 15 mL of

50 mM sodium acetate (pH 4.8) containing 6 M urea and applied to a HiTrap CM FF column (5 x 1 mL) equilibrated with the same buffer. Fractions were eluted with a linear gradient from 0 to 0.5 M NaCl over a total volume of 20 mL at a flow rate of 1.0 mL/min. The effluent was monitored at 280 nm. The appropriate fractions were pooled and dialysed in 0.05 M acetic acid to prevent precipitation. Finally, the sample was lyophilised and stored at -40°C for further analysis. The results were expressed as the percentage of collagen, calculated by determining the amount of hydroxyproline in the SCF and ICF[22].

Determination of Pyridinoline (Pyd)

Fluorescence spectrophotometry (Cary Eclipse G9800A Agilent Technologies, U.S.A.) was performed to determine the Pyd content in the collagen samples. Collagen was dissolved (0.5 absorbance units at 280 nm) with 50 mM sodium acetate, containing 6 M urea and with pH 4.8. The excitation wavelength was 295 nm and the emission signal was measured from 300–450 nm. The Pyd content was established from a standard Pyd (5–40 pmol) curve[13]. The assays were performed in triplicate.

Determination of Pyd by HPLC

The Pyd was quantified on each lyophilised collagen[11]. The collagen fractions were hydrolysed with 6 M HCl at 150 ° C for 2 h in sealed tubes. The hydrolysate was dissolved in 3 ml of 90% acetic acid. 2.5 mL of acetonitrile was added and the mixture was filtered through a cellulose packed bed (10 mm x 80 mm) column (CF-11; Whatman, Maidstone, UK) to remove contaminants. The column was washed with five volumes of the mobile phase (n-butanol: acetic acid: distilled water, 8:1:1 (v/v/v),

and then 400 µL of milli-Q water. Finally, the Pyd was eluted with 200 µL N-heptafluorobutyric acid (HFBA) and collected in a single fraction.

From the collected fractions, the quantification of Pyd was carried out by HPLC[5]. For this, a gradient system made by Agilente Technologies (U.S.A.) was used in a reverse phase column (Microsorb MV, 5C18, 4.6 mm in diameter x 250 mm; Rainin Company, Woburn, MA, U.S.A.). Detection of Pyd was performed using an F-1000 spectrofluorometer (excitation at 295 nm, emission at 395 nm). 50 µl (from the Pyd extract in 200 µL in HFBA) was taken and injected into the chromatograph at a flow rate of 1 mL min^{-1}. The mobile phase consisted of two solutions: 0.12% HBFA in distilled water (solution A) and 50% acetonitrile in distilled water (solution B). The column was pre-equilibrated with 20% of solution B and 80% of solution A. Elution was carried out by a linear gradient of 20–30% of solution B. The Pyd was quantified according to the retention time and compared to an external standard purchased from Merck (Darmstadt, Germany). Analysis of each sample was done in triplicate and the content was expressed as mmol Pyd mol^{-1} collagen.

^{1}H nuclear magnetic resonance imaging (^{1}H-NMR)

^{1}H-NMR of the SCF and ICF was performed at 25°C on a Bruker model Avance 400 (Billerica, MA, U.S.A.) operating at 400 MHz[9]. Lyophilised samples (1 mg) were dissolved in 0.5 mL of deuterated water and 0.5 ml of deuterated potassium hydroxide. Dimethylsilapentane sulfonic acid was used as the reference using a 20-ppm spectral window.

1 ***Raman spectroscopy***

2 The purified collagen samples were analysed by Raman spectrometry using a
3 Perkin-Elmer GX instrument[22,23]. The excitation was performed at 1064 nm using a
4 Nd:YAG laser, and backscattered light was collected at 180° angle of reflection. The
5 incident power was 100 mW, and 128 scans at 4 cm^{-1} were taken to avoid damage to
6 the samples due to laser power. The bands of the Raman spectra were analysed
7 using the program team's analysis.

8 ***Texture and collagen thermic properties after the cooking process***

9 Ten skinned mantle, fin, and tentacle portions from fresh (0 min) and cooked
10 (for 30 min) samples were analysed for texture and collagen thermal properties. The
11 texture was measured by recording the force required to penetrate the material using
12 a Chatillon 2-3b texturometer (Empire Scale Co., Santa Fe Springs, CA, U.S.A.) with
13 a cylindrical plunger 0.6 cm diameter. The shearing direction was set perpendicular to
14 the orientation of the muscle cells [24]. Collagen was extracted with 6 M urea in a
15 sodium acetate buffer (0.5 M, pH 6.8) as previously described [19]. The thermal
16 behaviour of 4–5 mg of lyophilised collagen, placed in hermetic pans, was studied
17 with differential scanning calorimetry from 20 to 150°C (1020 Series DSC7 Thermal
18 Analysis System, Perkin-Elmer, Norwalk, CT, U.S.A.). The transition temperatures
19 and enthalpies were established with a computer (PE Nelson Model 1022, Perkin-
20 Elmer). The instrument was calibrated for temperature baseline using indium as a
21 standard, and the test was run at a heating rate of 10°C min^{-1}.

Statistical analysis

Data obtained for the Pyd concentration obtained by fluorescence, texture, and calorimetric analysis were subjected to one-way ANOVA to explore differences among the samples; the comparison of means was performed using the least-squares Tukey-Kramer test. Differences among samples were considered significant for a confidence interval at the 95% level ($p<0.05$) in all cases. The analyses were performed using the SAS program 6.08 for PCS (SAS Institute Inc., 1994). Descriptive statistics was used to analyse the ^{1}H-NMR and Raman spectra.

Results and discussion

Extraction and purification of collagen fractions

The percentage of collagen detected in both SCF and ICF varied significantly ($p<0.05$) between mantle, fins, and tentacle samples. In SCF fractions, the order of obtained values was tentacles (2.37±0.04 mg) > mantle (1.94±0.06 mg)> fins (1.72±0.05 mg), whereas in ICF it was tentacles (2.45±0.02 mg) > mantle (1.94±0.07 mg) = fins (1.94±0.03 mg). These differences suggest that collagen from each anatomical region differs in cross-linking degree, which is controlled by the number of bonds prone to form hydroxylamine (and likewise their solubility in salt solutions and buffers)[25]. This behaviour is similar to that observed in previous studies, which found that, depending on the anatomical region, there is variability in muscle collagen content[19, 20, 25].

Fluorescence

The typical fluorometric peak emission of Pyd at 358 nm[26] was observed only in ICF from tentacles and in SCF from the three anatomical regions (Table 1). Among

these samples, the greatest intensity was observed in the ICF. Comparing the SCF fractions, the intensity from high to low was tentacles> fins> mantle. A standard concentration curve of 15 to 250 pMol μL^{-1} was constructed to determine the Pyd concentration. The regression analyses for the standard curve (y = 0.1904x - 33.334) gave a coefficient of determination (R^2) of 0.99 and a coefficient of variation (CV) <1% for all measurements. Thus, the accuracy of the set points and the linearity of the calibration curve reliably described the analytical quantification of Pyd.

Since the fractionation procedure did not involve drastic treatments expected to cause the hydrolysis of peptides bonds, our results possibly suggest that a significant proportion of Pyd is in free form in tentacles and in the ICF.

HPLC analysis

To establish the exact proportion of Pyd in each analysed sample, HPLC was performed after acid hydrolysis. However, the fluorescence spectra of Pyd obtained by HPLC were not in agreement with the fluorescence analysis. A Pyd peak was only detected in the IFC from tentacles (Figure 1). Using this method, the quantitative analysis of the IFC from tentacles gave a value of about 3.8 mmol mol^{-1} collagen.

The Pyd fluorescence is affected by the concentration of Pyd in the solution[15]. At lower or higher Pyd concentrations the normal fluorescence is fully quenched and the obtained spectra is different[15]. Therefore, it is possible that in samples where the characteristic Pyd peak was not detected, the reason may be partly due to lower Pyd concentrations.

^{1}H-NMR analysis

NMR analysis probes the chemical environment of the hydrogen protons to determine their position and the environment that surrounds them[27]. The ^{1}H-NMR spectra from the all lyophilised collagens are shown in Figure 2. All collagen fractions were analysed under the same conditions to avoid variability in the results. The signals associated with the aromatic protons of Pyd (7.94–7.78 ppm)[28] were observed in all analysed samples. Moreover, protons of water (4.6–4.8 ppm) were detected in all samples, and a different behaviour was observed between the spectra obtained from fraction of ICF with respect to other analysed collagens. The most marked difference is in the region of 3.19 ppm to 3.22 ppm, which corresponds to chemical shifts in aromatic rings[26].

Although, the exact concentration of Pyd in the samples was not established, the intensity of the peak associated with Pyd suggests that the ICF and collagen from tentacles has the highest Pyd content. This trend was confirmed by the Pyr Raman analysis.

Raman analysis

An analysis of the Raman measurements was performed analyse the structure of purified collagens. The bands associated with Pyd, at about 1663 and 830 cm^{-1}[29], were detected in both fractions (SCF and ICF) from the three regions analysed (mantle, fins, and tentacles) (see Figure 3). The differences in band shapes and the intensities observed in these regions of the spectra are associated with structural differences. The higher intensity of these bands in collagen from ICF and tentacles imply higher proportions of highly cross-linked in these collagen samples.

In all purified collagens, a very intense band at 1670 cm^{-1} was present and attributed to amide I. The peak detected at 1270 cm^{-1} is assigned to amide III. Fewer intense bands were observed in the PSC fractions than in the ICFs. The lower activity detected in amide III suggests fewer chain interactions through the hydrogen bonds of the collagen triple helix[23, 30].

Proline residue activity was observed at 1326 cm^{-1}[17] while hydroxyproline was observed at 845 cm^{-1}[31]. These bands were more prominent in the ICFs, in accordance with the Pyd peak. The highest hydroxyproline activity confirmed that ICF possesses a more stable triple helix.

Firmness analysis after cooking treatment

Textural changes were observed after 30 min of cooking (Table 1). Significant differences among muscles from different anatomical regions were detected in the uncooked samples, but after 30 min of cooking, muscle from mantles showed a greater decrease in shear force than that from tentacles and fins. A lower Pyd was also detected in the SCF from this anatomical region, and a lower peak intensity was observed in Raman analysis of both of its collagen fractions. In agreement with these results, the highest decrease in both transition temperature and enthalpy after cooking, was detected in collagen from mantle.

Finally, considering that the insoluble fraction from collagen is highly cross-linked, the correlation analysis was applied to the Raman Pyd peak of this fraction, with firmness and transition temperature (Tmax) decreasing after cooking. Significant correlation values between decreasing firmness (R^2 = -0.89 to -0.96), decreasing enthalpy (R^2 = -0.71 to -0.79), and decreasing Tmax (R^2 = -0.86 to -0.93) and the

Raman Pyd peak (Table 3). Although the intensity of a spectrum could be directly proportional to the concentration of any molecule, a calibration procedure will be necessary to determine the relationship between peak intensity and Pyd concentration. Then, a routine analysis of Pyd measurements could be performed on seafood products.

Conclusion

The fluorometric and HPLC methods allowed us to establish the concentration of Pyd in the analysed samples. However, this quantification could not be performed in all cases due to the absence of signals associated with this molecule. From ^{1}H-NMR and Raman spectra, it was possible to identify bands associated with the Pyd molecule in all samples and to at least qualitatively compare the presence of this molecule between the fractions analysed and the regions studied. Moreover, both NMR and Raman studies were very useful to detect differences in the collagen chemical structure of the obtained collagen fractions and the regions studied. The decrease in shear force after cooking treatment indicated that the fibres of tentacle collagen are stronger than those of the mantle and fins. The differences detected are due to the different crosslinked helix structure of the collagens derived from mantle, fins, and tentacles. These results will serve as a useful tools in studies concerning the collagen molecule and, specifically, to the detection of Pyd.

References

[1] Gelse, K.; Pöschl, E.; Aigner, T. Collagens-structure, function, and biosynthesis. Adv. Drug Deliv. Rev. 2003, 55 (12), 1531–1546.

[2] Muyonga, J.H.; Cole, C.G.B.; Duodu, K.G. Characterisation of acid soluble

collagen from skins of young and adult Nile perch (Latesniloticus). Food Chem. 2004, 85 (1), 81–89.

[3] Butler, E; Hardin, J; Benson, S. The role of lysyl oxidase and collagen crosslinking during sea urchin development. Exp. Cell. Res. 1987, 173 (1), 174–182.

[4] Eyre, D. Collagen crosslinking amino acids. Methods Enzymol. 1987, 144, 115–139.

[5] Ando, M.; Makino, M.; Tsukamasa, Y.; Makinodan, Y.; Miyosh, M. Interdependence between heat solubility and pyridinoline contents of squid mantle collagen. J. Food Sci. 2001, 66 (2), 265–269.

[6] Ramirez-Guerra, H.E.; Fimbres-Romero, M.D.J.; Tapia-Vazquez, A.E.; Ezquerra-Brauer, J.M.; Márquez-Ríos, E.; Suarez-Jimenez, G.M.; Torres-Arreola, W. Relationship between lysyl oxidase activity, pyridinoline content and muscle texture during ice storage of jumbo squid (*Dosidicus gigas*). Int. J. Food Sci. Tech. 2015, 50 (12), 2700–2706.

[7] Gómez-Guillén, C.; Turnay, J.; Fernández-Díaz, M.; Ulmo, N.; Lizarbe, M.; Montero-García, P. Structural and physical properties of gelatin extracted from different marine species: A comparative study. Food Hydrocoll. 2002, 16 (1), 25–34.

[8] Giménez, B.; Gómez-Estaca, A.; Alemán, M.C.; Gómez-Guillen, M.C.; Montero, M.P. Physico-chemical and film forming properties of giant squid (Dosidicus gigas) gelatin. Food Hydrocoll. Year, 23 (3), 585–592.

[9] Uriarte-Montoya, M.H.; Santacruz-Ortega, H.; Cinco-Moroyoqui, F.J.; Rouzaud-Sández, O.; Plascencia-Jatomea, M.; Ezquerra-Brauer, J.M. Giant squid skin gelatin:

chemical composition and biophysical characterization Food Res. Int. 2011, 44 (10), 3243–3249.

[10] Ando, M.; Nakagishi, Y.; Yoshida, K.; Nakao, M.; Nakagawa, T.; Makinodan; Tsukamasa, Y.; Kawasaki, K.I. Pyridinoline concentrations in muscular and skin collagen of fish and relationship between collagen solubility and pyridinoline concentration in fish muscular collagen. Fish. Sci. 2006, 72 (5), 1104–1108.

[11] Ramírez-Guerra, H.E., Mazorra-Manzano, M.A., Ezquerra-Brauer, J.M., Carvajal-Millan, E., Pacheco-Aguilar, R., Lugo-Sánchez, M.E., Ramírez-Suárez, J.C. Hydroxylysyl-pyridinoline occurrence and chemical characteristics of collagen present in jumbo squid (Dosidicus gigas) tissues. J. Food Compos. Anal. 2015, 44, 10–17.

[12] Ogawa, T.; Kawanishi, Y. Simple and direct determination of pyridinoline and 2′-deoxypyridinoline by automatic analysis. J. Chromatogr. A, 1986, 354, 495–497.

[13] Meddah, B.; Kamel, S.; Giroud, C.; Brazier, M. Method for the isolation and purification of pyridinoline and deoxypyridinoline rosslinks from bone by liquid chromatographic techniques. Prep. Biochem. Biotechnol. 1999, 29 (1), 63–75.

[14] Arakawa, N.; Kim, M.; Otsuka, M. An improved high-performance liquid chromatographic assay for the determination of pyridinoline in connective tissues. J. Nutr. Sci. Vitaminol. 1992, 38 (4), 375–380.

[15] Robins, S.P. Cross-linking of collagen. Isolation, structural characterization and glucosylation of pyridinoline. Biochem. J. 1983, 215 (1), 167–173.

[16] Paschalis, E.P.; Gamsjaeger, S.; Tatakis, D.N.; Hassler, N.; Robins, S.P.; Klaushofer, K. Fourier transform infrared spectroscopic characterization of

mineralizing type I collagen enzymatic trivalent cross-links. Calcif. Tissue Int. 2015, 96 (1), 18–29.

[17] Gamsjaeger, S.; Robins, S.P.; Tatakis, D.N.; Klaushofer, K.;Paschalis, E.P. Identification of pyridinoline trivalent collagen cross-links by Raman microspectroscopy. Calcif. Tissue Int. 2017, 100 (6), 565–574.

[18] Matthäus, C.; Chernenko, T.; Newmark, J.A.; Warner, C.M.; Diem, M. Label-free detection of mitochondrial distribution in cells by nonresonant Raman microspectroscopy. Biophys J. 2007, 93 (2), 668–673.

[19] Torres-Arreola, W.; Pacheco-Aguilar, R.; Sotelo-Mundo, R.R.; Rouzaud-Sández, O.; Ezquerra-Brauer, J.M. Partial characterization of collagen from mantle, fin, and arms of jumbo squid (Dosidicus gigas). CYTA-J Food, 2008, 6 (2), 101–108.

[20] Morales, J.; Montero, P.; Moral, A. Isolation and partial characterization of two types of muscle collagen in some cephalopods. J. Agric. Food Chem. 2000, 48 (6), 2142–2148.

[21] Nishimoto M.; Mizuta S.; Yoshinaka, R. Characterization of molecular species of collagen in muscles of Japanese amberjack, Seriola quinqueradiata. Food Chem. 2004, 84 (1), 127–132.

[22] Leach, A.A. Notes on a modification of the Neuman & Logan method for the determination of the hydroxyproline. Biochem. J. 1960, 74 (1), 70–end.

[23] Ikoma, T.; Kobayashi, H.; Tanaka, J.; Walsh, D.; Mann, S. Physical properties of type I collagen extracted from fish scales of *Pagrus major* and *Oreochromis niloticas*. Int. J. Biol. Macromolec. 2003, 32 (3), 199–204.

R . 190014

[24] Dunajski, R. Texture of fish muscle. J. Texture Stud. 1980, 10 (4), 301–318.

[25] Sadowska, M.; Sikorski, Z.E. Collagen in the tissues of squid *Illex argentinus* and *Loligo patagonica*-Content and solubility. J. Food Biochem. 1987, 11, 109–120.

[26] Robins, S.P.; Duncan, A.; Wilson, N.; Evans, B.J. Standardization of pyridinium crosslinks, pyridinoline and deoxypyridinoline, for use as biochemical markers of collagen degradation. Clinical Chem. 1996, 42 (10), 1621–1626.

[27] Fullerton, G.D.; Nes, E.; Amurao, M.; Rahal, A.; Krasnosselskaia, L.; Cameron, I. An NMR method to characterize multiple water compartments on mammalian collagen. Cell Biol. Int. 2006, 30 (1), 66–73.

[28] Sell, D.R.; Monnier, V.M. Structure elucidation of a senescence cross-link from human extracellular matrix: Implication of pentoses in the aging process. J. Biol. Chem. 1989, 3264 (36), 21597–21602.

[29] Bundi, A.; Wuthrich, K. 1H NMR parameters of the common amino-acid residues measured in aqueous solutions of the linear tetrapeptides H-Gly-Gly-X-Ala-OH. Biopolymers 1979, 18, 285–297.

[30] Frushour, B.G.; Koenig, J.L. Raman scattering of collagen, gelatin, and elastin. Biopolymers 1975, 14 (2), 379–391.

[31] Zhang, Y.; Liu, W.; Li, G.; Shi, B.; Miao, Y.; Wu, X. Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (Ctenopharyngodon idella). Food Chem. 2007, 103 (3), 906–912.

Figure Caption

Figure 1. Determination of pyridinoline by HPLC. Insoluble collagen hydrolysate (ICF) from squid (*Dosidicus gigas*) tentacle.

Figure 2. ^{1}H-NMR spectra of pepsin soluble collagen (A) and insoluble collagen (B) fractions from squid (*Dosidicus gigas*) mantle (MAN), fins (FIN), and tentacles (TEN). Activity of the proline proton (Pro) and the α proton of the C-4 of pyridinoline ring (Pyd).

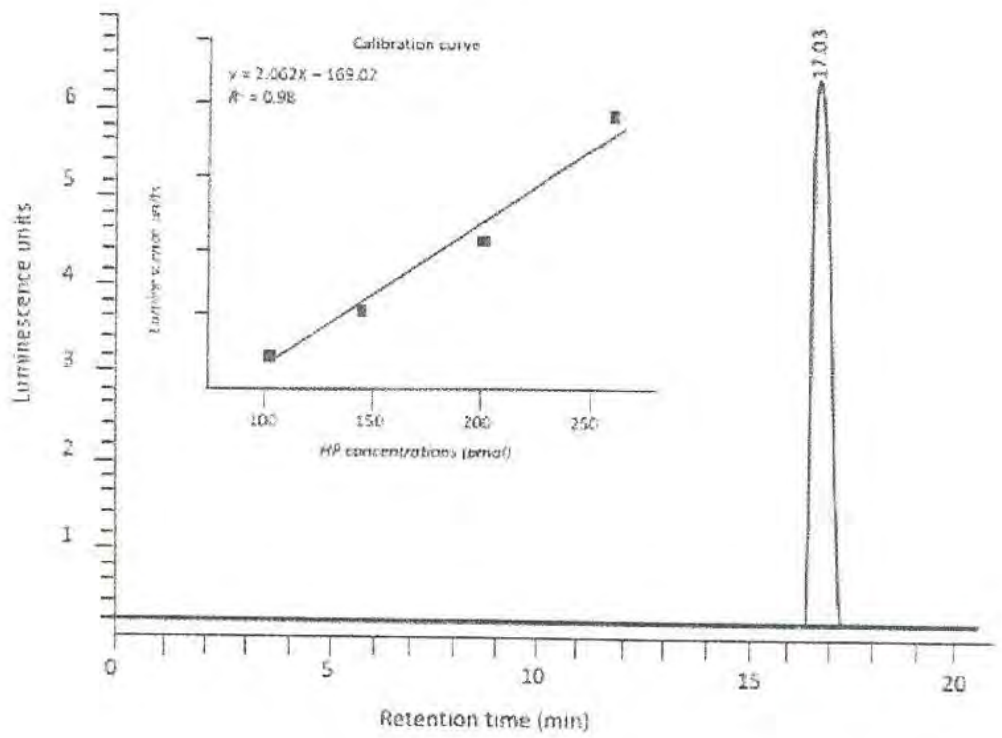
Figure 3. Raman spectra of insoluble collagen (A) and pepsin soluble collagen (B) fractions from squid (*Dosidicus gigas*) mantle (MAN), fins (FIN), and tentacles (TEN). The 1200–1800 cm^{-1} region is shown.

Table Caption

Table 1. Pyridinoline (Pyd) content of the purified soluble collagen (SCF) and insoluble collagen (ICF) fractions from squid (*Dosidicus gigas*) mantle, fins, and tentacles by fluorometric analysis and muscle texture change after 30 min of cooking treatment.

Table 2. Change in denaturation enthalpy (ΔH) and endothermic transition temperature (Tmax) after 30 min of cooking treatment of collagen extracted from squid (*Dosidicus gigas*) mantle, fins, and tentacles[1].

Table 3. Correlation analysis between Raman pyridinoline peak intensity (Pyd) and shear force, and thermal properties, of squid muscle after 30 min of co



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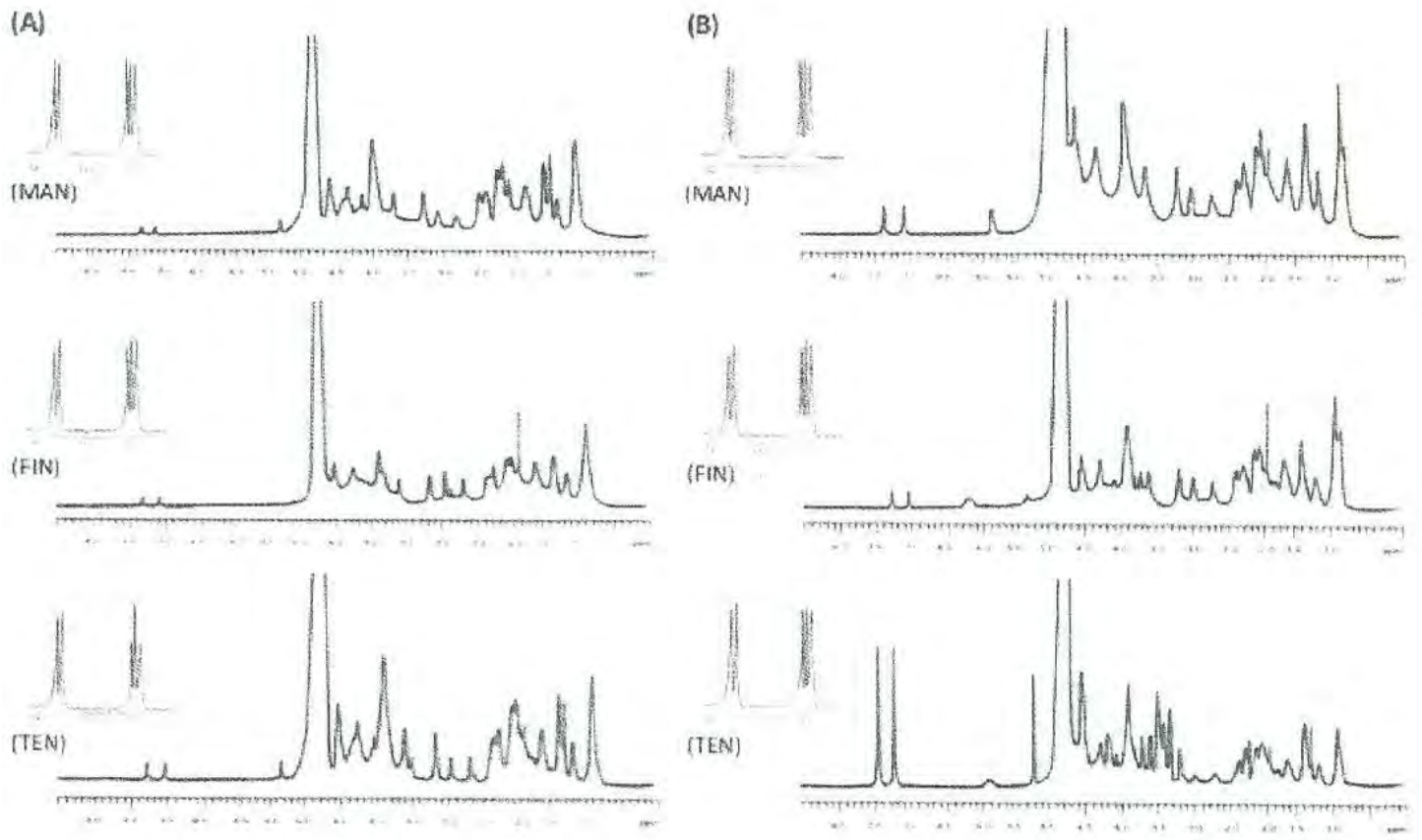

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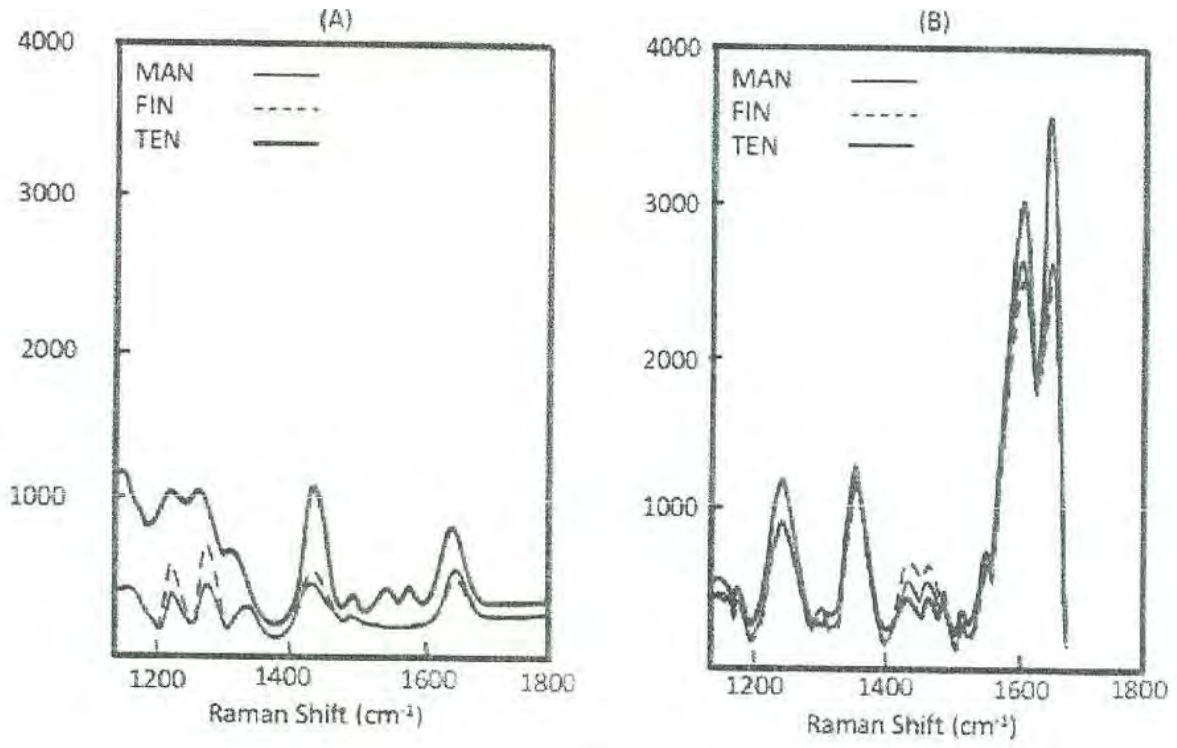

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Anatomical region	Collagen fraction	Pyd (Pmol μL^{1})[1]	Time 0 min Shear force (Lbf)[2]	Time 30 min Shear force (Lbf)[2]	ΔSF (%)[3]
Mantle			6.7±0.5[c]	3.8±0.1[c]	44[a]
	SCF	90.5±1.5[d]			
	ICF	ND			
Fins			7.6±0.4[b]	4.8±0.4[b]	37[b]
	SCF	122.0±1.4[c]			
	ICF	ND			
Tentacles			29.6±0.5[a]	7.9±0.5[a]	27[c]
	SCF	208.9±2.5[b]			
	ICF	226.1±2.8[a]			

[1]Each fraction was adjusted to a concentration of 0.5 absorbance at 280 nm. The Pyd content was determined from the intensity (UA) and compared with a standard Pyd concentration curve (R^2 = 0.99). ND: signal not detected.
[2]N= 10 determinations per sample. Different letters (a to c) in the same column for Pyd and shear force indicate significant differences ($p< 0.05$).
[3]ΔSF = Decreasing shear force.

Table 2. Change in denaturation enthalpy (ΔH) and endothermic transition temperature (Tmax) after 30 min of cooking treatment of collagen extracted from squid (*Dosidicus gigas*) mantle, fins, and tentacles.[1]

Anatomical region	ΔH (Jg^{-1})			Tmax (°C)		
	Cooking time (min)		ΔSH (%)[2]	Cooking time (min)		ΔST (%)[3]
	0	30		0	30	
Mantle	21.8±1.4[b]	7.1±1.4[c]	67.5[a]	148.3±0.6[b]	103.2±0.1[c]	30[a]
Fins	16.3±1.5[c]	12.1±1.5[b]	21[b]	143.4±0.6[c]	107.6±0.4[b]	25[b]
Tentacles	26.2±1.5[a]	23.6±1.5[a]	9.8[c]	161.9±0.1[a]	131.1±0.5[a]	19[c]

[1]Values are the mean of triplicates±standard deviation (n=3). Different letters (a to c) in the same column for ΔH or Tmax (°C) indicate significant differences ($p< 0.05$).

[2]ΔSH = Decreasing ΔH.

[3]ΔST = Decreasing Tmax.

Table 3. Correlation analysis between Raman pyridinoline peak intensity (Pyd) and shear force, and thermal properties, of squid muscle after 30 min of cooking time

	SCF[1]	ICF[2]
	Pyd	Pyd
ΔSF[3]	-0.89*	-0.96**
ΔSH[4]	-0.71*	-0.79*
ΔST[5]	-0.86*	-0.93**

*Significant correlation values ($p<0.05$).

**Significant correlation values ($p<0.01$).

Nine observations.

[1]SCF = Soluble collagen fraction.

[2]ICF = Insoluble collagen fraction.

[3]ΔSF = Decreasing shear force.

[4]ΔSH = Decreasing ΔH.

[5]ΔST = Decreasing Tmax.

CONCLUSIONES

Bajo las condiciones de este trabajo, se demostró que la actividad de la lisil oxidasa no influyó directamente en la concentración de piridinolina, pero se estableció que a mayor orden molecular, mayor estabilidad térmica y firmeza del músculo del calamar gigante (*Dosidicus gigas*).

La temperatura máxima de transición, así como los valores de entalpía, indicaron que las fibras de colágeno de los tentáculos poseen mayor resistencia térmica que el proveniente del manto y las aletas. Las diferencias detectadas se deben a la diferencia estructural y al grado de entrecruzamiento de la hélice entre los colágenos provenientes del manto, las aletas y los tentáculos.

La detección de la banda de hidroximerodesmosina indicó que la piridinolina no es la única molécula implicada en el entrecruzamiento del colágeno.

Estos resultados pueden servir como base teórica para una mejor comprensión del comportamiento que presente el músculo de las principales regiones que se comercializan del calamar gigante y para que en un futuro se lleven estudios enfocados al desarrollo de nuevos productos a base de calamar.

RECOMENDACIONES

El colágeno purificado de cada una de las tres regiones anatómicas, presenta características que lo pueden asociar a un colágeno tipo I y V, para corroborar exactamente el tipo es necesario llevar a cabo estudios proteómicos y genómicos.

Se recomienda la secuenciación de aminoácidos mediante espectrometría de masas para establecer las diferencias de composición de cada fracción de colágeno.

La medición del tamaño de las fibras de colágeno y el orden en el cual se reparten en el músculo intacto podrían estar asociadas con la textura. Un análisis mediante microscopia de barrido podría proporcionar esta información adicional.

La piridinolina no es la única molécula involucrada en el entrecruzamiento del colágeno, por lo que se recomienda estudiar la incidencia de los distintos tipos de entrecruzamiento y su relación con la actividad de la lisil oxidasa.

Se recomienda una investigación para el uso potencial del colágeno del calamar en el cual se aproveche sus características fisicoquímicas y su alto rendimiento de obtención, para optimizar los productos a base calamar gigante.