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Programa de Posgrado en Ciencias y Tecnología de Alimentos

"El saber de mis hijos hará mi grandeza"

Efecto de la adición del suero de leche sobre las propiedades fisicoquímicas de una película a base de proteína miofibrilar de manto de calamar gigante (*Dosidicus gigas*)

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APROBACIÓN

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"To grow a memory. To grow a good memory. A happy memory.
It doesn't matter what you plant.
But you must plant it with love"

-Gregory Maguire (Out of Oz)-

"El amor y el aprendizaje son similares,
nunca se desperdician"

-Hope Jahren (La memoria secreta de las hojas)-

CONTENIDO

RESUMEN

El presente trabajo de investigación tuvo como objetivo la elaboración de biopelículas empleando proteína miofibrilar obtenida del manto de calamar gigante (*Dosidicus gigas*), adicionadas con proteínas el suero de leche (concentrado proteico). El experimental fue dividido en tres etapas para su desarrollo.

Etapa 1: Consistió en establecer las condiciones para la elaboración de las películas, así como la elección del polialcohol más adecuado para fungir como agente plastificante, teniendo como opciones glicerol, sorbitol, manitol, maltitol y xilitol. Se empleó manto de calamar gigante como materia prima para la obtención de proteína miofibrilar, ésta se utilizó para la obtención de las soluciones formadoras de película (15 % de concentrado proteico w/w), preparándose varias soluciones con distintos plastificantes: glicerol (40%), sorbitol (30%), manitol, maltitol y xilitol (20%). Los resultados mostraron variación en cuanto a compatibilidad con la proteína miofibrilar, no obstante, ninguna película mostró transmisión a la luz en la región ultravioleta del espectro. Finalmente, solo glicerol y sorbitol permitieron obtener películas flexibles y transparentes, con un comportamiento más elástico en el caso de glicerol, elongación de 920% y esfuerzo tensil (TS) de 0.94 MPa, y más plástico para sorbitol, con una elongación de 511% y TS de 4.41 MPa.

Etapa 2: Se incorporó la proteína de suero de leche a la formulación del material, manejándose las películas con mejor desempeño mecánico (valores más altos en elongación y esfuerzo tensil) de la etapa anterior. Al adicionar el concentrado proteico de suero de leche se obtuvieron 9 películas diferentes, tres concentraciones de suero (5, 10 y 15%) y tres plastificantes: glicerol, sorbitol y glicerol-sorbitol (1:1). Algo destacable fue la desaparición del "blooming" presente en las películas con sorbitol, además, la adición de suero disminuyó los valores de permeabilidad al vapor de agua (algo que se pretendía), obteniendo el valor más bajo al adicionar un 15 % de suero en las películas de sorbitol, obteniendo un valor de 18.9 $g/m^2\ d^{-1}$. En cuanto a la estructura, los espectros de infrarrojo por transformada de Fourier (FT-IR) sugieren un cambio de α-hélice a hoja β, de acuerdo con el desplazamiento de la banda correspondiente a la Amida I, posiblemente debido a la ruptura de puentes de hidrógeno por acción de la proteína del suero, en efecto conjunto al plastificante.

Etapa 3: Consistió en evaluar la estabilidad de las películas generadas en la segunda etapa. Las 9 películas obtenidas durante la etapa 2 fueron almacenadas en condiciones de refrigeración (4-7 °C) y evaluadas cada mes para determinar la magnitud de los cambios en sus propiedades funcionales. Los resultados sugieren cambios en la matriz proteica debido a un incremento en las interacciones proteína-proteína, lo cual puede observarse en el espectro FT-IR en las bandas Amida A y Amida III. Dichos cambios tuvieron como efecto un aumento en la solubilidad en agua de las películas durante su almacenamiento, así como un aumento de su transparencia.

Los resultados obtenidos permiten concluir que el empleo de proteína miofibrilar de calamar gigante adicionada con proteína de suero de leche permite la obtención de materiales con propiedades funcionales y estabilidad suficientes para su posible empleo como envase de alimentos, específicamente la elaboración de biopelículas.

ABSTRACT

The aim of this work was the elaboration of biofilms made of myofibrillar proteins from giant squid (*Dosidicus gigas*) mantle, added with whey protein concentrate. The experiment was divided in three stages.

Stage 1: Establishment of conditions for film elaboration, as well as selection of the most adequate poly alcohol to serve as plasticizer, for which glycerol, sorbitol, mannitol, maltitol and xylitol were used. Myofibrillar protein fraction was used for the film forming solutions (15 % protein concentrate w/w), and different poly alcohols were tested as plasticizer agents: glycerol (40%), sorbitol (30%), mannitol, maltitol and xylitol (20%). Results shown variation of compatibility with the myofibrillar protein, however, any film had light transmission on the UV region of the spectra. Finally, only glycerol and sorbitol served to obtain flexible see-through films, with a more elastic behavior for glycerol, elongation of 920% and tensile strength (TS) of 0.94 MPa, and more plastic for sorbitol, elongation of 511% and TS of 4.41 MPa.

Stage 2: Whey protein was added to the material formulation, choosing from the previous stage the films with better performance. When whey protein concentrate was added, 9 films were obtained, three whey concentrations (5, 10 and 15%) and three plasticizers: glycerol, sorbitol and glycerol-sorbitol (1:1). Something remarkable was the absence of blooming on sorbitol films, moreover, whey addition (15%) caused a decrease in water permeability values of sorbitol films down to 18.9 $g/m^2\ d^{-1}$. Regarding structure, infrared spectra (FT-IR) suggest a change in amide I from α helix to β sheet, maybe due to hydrogen bond rupture by whey proteins action alongside the plasticizer agent.

Stage 3: The films were stored and refrigerated (4-7 °C) for 3 months to evaluate their stability. The 9 films obtained on stage 2 were stored in refrigeration (4-7 °C) and evaluated monthly to define how much their properties changed over time. The results suggest a change in the protein matrix due to an increment in protein-protein interaction, observed in the FT-IR spectra Amide A and Amide III bands. These changes had an impact in film solubility, with higher values after storage, an equal behavior could be seen on transparency.

INTRODUCCIÓN

En últimos años, los deshechos generados debido a los empaques alimenticios se han convertido gradualmente en un problema grave, de ahí que la búsqueda de alternativas y posibles soluciones haya ganado auge. Dentro de las opciones que cuentan con mayor popularidad destaca el empleo de materiales biodegradables, los cuales, a diferencia de los polímeros de uso habitual son más amigables con el medio ambiente (Wihodo y Moraru, 2013).

Los materiales de empaque cumplen con múltiples funciones, siendo la principal proteger al alimento de su entorno, debido a la pérdida o ganancia de humedad, oxigeno (rancidez oxidativa), o incluso contaminación por microorganismos, asegurando así su calidad y una mayor vida de anaquel (Ansorena et al., 2016). En el caso particular de las películas biodegradables, las cuales entraran en contacto directo con el alimento, es además necesario cumplan con propiedades sensoriales aceptables, estabilidad bioquímica y microbiológica (Debeaufort et al., 1998). Por lo que resulta ser una buena opción el empleo de polímeros de origen natural, como las proteínas.

Una propuesta podrían ser las proteínas de origen marino, debido a la creciente necesidad de la industria pesquera de disminuir los residuos que se generan (FAO, 2018). Es aquí donde entra en juego el calamar gigante, pues si bien la producción es elevada, no se aprovecha el organismo completo, de ahí que las proteínas extraídas de su músculo puedan ser empleadas para la obtención de biopelículas, ya sea del tejido conectivo o miofibrilar. No obstante, si bien las películas obtenidas de proteínas miofibrilares presentan buenas propiedades mecánicas, en general son una barrera pobre al vapor de agua debido a la naturaleza hidrofílica de los aminoácidos así como de los plastificantes, que deben emplearse para disminuir la rigidez inherente del material (Prodpran et al., 2007). Por lo anterior, se pueden emplear mezclas de proteínas en una misma formulación, o incluso probar con distintos plastificantes, de modo que se obtengan las propiedades más adecuadas para la futura aplicación del material generado. Con base en esto, el presente trabajo pretende elaborar una película de proteína miofibrilar de manto de calamar gigante, con el fin de dar un uso a los subproductos generados durante su procesamiento, no obstante se espera además disminuir la permeabilidad al vapor de agua del material obtenido, por lo que se propuso la adición de

proteínas del suero de leche, las cuales al poseer un peso molecular menor (seroalbúmina es la mayor con 66kDa) podrán complementar la red proteica formada por las proteínas miofibrilares de mayor tamaño (200 kDa para miosina). Además, al poseer un punto isoeléctrico similar (ambas alrededor de 5) no habrá problemas de incompatibilidad al elegir algún pH en particular durante la elaboración de la solución formadora de película. Finalmente, se espera contribuir con a la futura disminución de empaques convencionales, al presentar una alternativa más a las ya existentes.

ANTECEDENTES

La investigación en biomateriales, contrario a lo que se pueda pensar, data de antes de la segunda guerra mundial, en que la demanda de recursos actuó como catalizador para el desarrollo científico y tecnológico de polímeros a base de proteína. Entre los productos disponibles comercialmente se encontraban películas, recubrimientos e incluso textiles (ej. La zeína de maíz mezclada con algodón se empleó para producir la fibra textil conocida como Vicara) (Kiplinger 2003). Sin embargo, el uso y producción de estos materiales declinó después de la guerra debido al desarrollo de productos a base de petróleo, que eran más baratos, fáciles de producir y en general con un desempeño superior.

En la actualidad, el interés en los biomateriales ha retomado auge gracias a la demanda de los consumidores por productos amigables al medio ambiente que disminuyan la dependencia de fuentes no renovables (Comstock et al., 2004).

Biopelículas

Generalmente, se define como biopelícula a una capa delgada de material (puede o no ser comestible) de origen natural formado sobre el alimento (recubrimiento) o pre-formado (película). El propósito de estos materiales es inhibir la migración de humedad, oxígeno, dióxido de carbono, aromas, lípidos, etc.; como acarreadores de ingredientes (antioxidantes, antimicrobianos, sabores) y/o mejorar la integridad del alimento durante su manejo (Krochta y De Mulder-Jhonston,1997).

Las biopelículas ofrecen una alternativa a los métodos de envase convencional, si bien, no tienen la finalidad de reemplazarlos totalmente, tienen, el potencial para reducir la cantidad de empaque empleada en los alimentos, así como limitar la migración de humedad, aromas, y lípidos entre el alimento y el ambiente o incluso entre componentes de este (algo que los envases tradicionales no pueden lograr).

Fuentes de Obtención

En últimos años se han propuesto materias primas de fuentes agrícolas y renovables para la producción de plásticos que puedan reemplazar en un futuro a los envases de un solo uso, especialmente los empleados en la industria alimentaria (Flieger et al., 2003; Chalermthai et

al., 2019). Sin embargo, debido a que una gran cantidad de dichos materiales son derivados de plantas, es decir, materia prima de primera generación, esto puede presentar un riesgo a futuro para la seguridad alimentaria, por lo que el empleo de materias primas de segunda generación resulta más adecuado, como es el caso de sub-productos, residuos o incluso deshechos derivados de algún proceso (Chalermthai et. al., 2019).

Los materiales empleados para la producción de biopelículas se dividen en tres categorías principales según su origen y producción: categoría 1) polímeros extraídos/removidos directamente de biomasa (polisacáridos y proteínas); categoría 2) polímeros producidos por síntesis química clásica utilizando monómeros de base biológica (ácido poliláctico), y categoría 3) polímeros producidos por microorganismos o bacterias genéticamente modificadas. Ahora bien, de manera general la fabricación de las biopelículas se realiza con tres componentes principales: el biopolímero, plastificante y solvente, los cuales influirán en las propiedades del material final (junto al método de elaboración). No obstante, de los biopolímeros disponibles, solo las proteínas liberan nitrógeno durante su degradación, pudiendo actuar así como fertilizantes, beneficio que no poseen el resto de las fuentes. (Murrieta-Martínez et al., 2017).

Películas a Base de Proteína

De los polímeros disponibles, las proteínas son las más empleadas, debido a su abundancia, habilidad para formar películas, así como su valor nutricional. Además, sus propiedades inherentes las hacen una excelente materia prima, pues gracias a su estructura (basada en 20 monómeros diferentes) es posible obtener distinto tipos de uniones en diversas posiciones, lo que da lugar a un amplio rango de propiedades funcionales, formando también una matriz cohesiva gracias a la distribución de cargas (Kaewprachu et al., 2016). Una característica destacable es la posibilidad de diseñar la película, los grupos funcionales presentes pueden ser alterados por métodos físicos, químicos o enzimáticos, con la consecuente variación y ajuste de propiedades funcionales según se requiera (Gómez-Estaca et al., 2016).

En general la formación de una película a base de proteína se basa en tres pasos principales: formación de uniones intermoleculares de baja energía, las cuales estabilizan al polímero en estado nativo; segundo, las cadenas de polímero se reacomodan y orientan, y tercero, la formación de la red tridimensional (Hammann y Schmid, 2014). Esto se puede llevar a cabo

por dos procesos distintos: 1) húmedo o "casting", en el que la proteína es disuelta en un solvente adecuado para obtener la solución formadora de película, a la cual se adiciona el plastificante o incluso aditivos como antimicrobianos o nanopartículas, la solución es entonces vertida en una superficie plana y el solvente evaporado. 2) seco, en presencia de agentes plastificantes, bajos niveles de humedad y altas temperaturas, se somete a presión y fuerzas de cizalla, mediante las que se obtiene un carácter viscoelástico. Se puede emplear la extrusión o el termoformado (Gómez-Estaca et al., 2016).

Enfocándonos en el aprovechamiento de subproductos o deshechos generados en la industria, se tratarán dos fuentes de proteína. La primera es el calamar gigante, una pesquería de importancia a nivel nacional, pero de poco aprovechamiento y bajo valor. La segunda son las proteínas del suero de leche, producto generado en grandes cantidades y no aprovechado del todo.

Producción y aprovechamiento del calamar gigante

El calamar gigante es una importante pesquería en México, siendo Sonora su principal productor y, si bien ha disminuido a nivel local en años recientes, a nivel mundial va en aumento, siendo Perú el país con mayor captura, con un promedio de 500 a 600 mil toneladas anualmente (Mereguetti, 2017). En el caso de la producción nacional, la mayoría está destinada a la exportación, principalmente en presentaciones de bajo valor comercial como son fresco-congelado, precocido-congelado, "bailarina" (cabeza con tentáculos) y aleta (Montaño-Méndez et al., 2015), que se convierten en materia prima para la elaboración de productos como aros, deditos, calamar en su tinta etc. Por lo que es necesario comenzar a procesar y dar valor agregado para mejorar su precio en el mercado; al hacer esto consecuentemente se generaran "deshechos" (como pudieran ser pequeños trozos de músculo) a partir de los cuales se podría extraer proteína miofibrilar para la elaboración de biopelículas, ya que las cantidades requeridas son bajas.

En el caso de las proteínas miofibrilares las películas obtenidas son usualmente muy rígidas, atribuido a la presencia de enlaces covalentes, especialmente puentes disulfuro, aunque esto se soluciona fácilmente empleando algún agente plastificante (Rocha et al., 2013), generando entonces una matriz más elástica con buenos valores de elongación y esfuerzo tensil. Otra

característica en este tipo de materiales es su desempeño bajo como barrera al vapor de agua, debido al carácter hidrofílico de los aminoácidos, sumados al plastificante (Prodpran et al., 2007).

Proteínas miofibrilares de calamar gigante en la elaboración de películas

Son varias las especies acuáticas que se han empleado para la obtención de películas, peces principalmente, pero en este caso es de interés particular las obtenidas a partir de calamar. Hay pocos estudios reportados con proteína de calamar, uno de ellos es el realizado por Leerahawong et al., (2011) con manto del calamar japonés común (*Todadores pacificus*). El estudio se centró en determinar la sal más adecuada para la solubilización de las proteínas previo a la elaboración de la película, teniendo como opciones: cloruro de sodio (NaCl), citrato trisódico dihidratado (Na- citrato), benzoato de sodio (Na-benzoato), acetato de sodio (Na-acetato) y tartrato de sodio (Na-tartrato). El trabajo reporta la obtención de películas transparentes y con excelente barrera a la luz UV. En lo referente al desempeño mecánico, los valores más elevados se obtuvieron al emplear Na-citrato, reportando un esfuerzo tensil (TS) de 6.68MPa y porcentaje de elongación de 135%. En cuanto a la permeabilidad al vapor de agua, los valores más bajos se obtuvieron con Na-tartrato ($0.54x10^{-10}$ g/m s Pa) y los más elevados, con NaCl ($1.26x10^{-10}$ g/m s Pa). Posteriormente, estos mismos autores (Leerahawong et al., 2012) continuaron con el estudio, pero esta vez evaluando la estabilidad de la película en almacenamiento, reportando un aumento progresivo del esfuerzo tensil, pero sin cambios significativos en el porcentaje de elongación. En tanto que la permeabilidad al vapor de agua disminuyó, relacionado seguramente con el mayor esfuerzo tensil causado por un aumento en las interacciones cadena-cadena en la película al irse perdiendo el plastificante. Por otra parte, el trabajo realizado por Blanco-Pascual et al. (2013) empleó manto de calamar gigante (*Dosidicus gigas)* con el objetivo de comparar distintas formas de extracción de proteína miofibrilar (lavados con agua, condiciones ácidas y alcalinas, y empleo de sales) para obtener una película con propiedades funcionales adecuadas. Los mejores resultados se obtuvieron al usar condiciones ácidas y básicas, asumiendo que la desnaturalización parcial favoreció la formación de uniones más fuertes en la red proteica, generando así películas con buenas propiedades mecánicas, alcalinas (TS= 3.10Mpa) y ácidas (TS= 0.85MPa). Los valores de permeabilidad al vapor fueron de 1.55 y $1.71x10^{-7}$ g/m s Pa para películas alcalinas

y ácidas, respectivamente. En general, reportan la obtención de películas transparentes, flexibles y con buena estabilidad microbiológica. Al igual que en el trabajo con calamar japones, Blanco-Pascual et al. (2014) evaluaron la estabilidad en almacenamiento de los materiales, observando un incremento en la solubilidad en las películas alcalinas, mientras que en las ácidas sucedió lo contrario, aumentando además su TS. Con base en lo reportado por estos autores, se puede considerar a las proteínas miofibrilares del calamar como adecuadas para la elaboración de películas. No obstante, es necesario realizar más investigación al respecto con la finalidad de establecer los componentes y condiciones más favorables para la obtención de este tipo de materiales.

Producción y aprovechamiento del suero de leche

La producción mundial de suero en 2008 fue de 160 millones de toneladas, incrementando a 200 en 2011, y mostrando un incremento anual constante del 2% (Smithers, 2008, Illianes, 2011). Tomando en cuenta que, en general, para la obtención de 1 kg de queso se generan 9 L de suero (0.55% de proteína: ~50%, beta-lactoglobulina y ~20%, alfa-lactoalbumina), no hay que dejar de lado que solo alrededor del 60% del suero es utilizado, descartando el resto usualmente al drenaje, lo que puede llegar a causar problemas ambientales (Guimarães et al., 2010). Vale la pena mencionar que se ha sugerido a la leche y productos lácteos expirados, de los cuales grandes cantidades son desechadas diariamente por los supermercados, pudiendo llegar hasta las 7 toneladas (Saad, 2017) como otra fuente para la obtención de proteínas del suero, teniendo así un alto potencial para la producción de bioplásticos (Chalermthai et al., 2019).

Proteínas del suero de leche en la elaboración de películas

Ya se han producido con éxito películas y recubrimientos comestibles a partir de las proteínas del suero, los cuales han demostrado propiedades mecánicas y de barrera superiores a las de otras fuentes proteicas (cereales y polisacáridos), además de generar materiales transparentes, flexibles y carentes de color y olor (Ramos et al., 2013). Los trabajos reportados se dividen en aquellos que emplean aislado proteico del suero (WPI) y los que utilizan concentrado proteico del suero (WPC), los cuales se diferencian por la cantidad de proteína presente, siendo mayor en el aislado. Ramos et al. (2013) llevaron a cabo una comparación entre ambas

clases de película. Entre sus resultados reportan un ligero color amarillento al usar WPC, en cuanto a propiedades como el contenido de humedad, densidad y actividad de agua, al usar WPI se obtuvieron menores valores de humedad y actividad de agua, y mayores en densidad. De igual modo, menores valores de permeabilidad al vapor de agua fueron obtenidos para WPI en comparación con WPC, 1.55 y 7.54x10 g m^2 d, respectivamente. En lo referente a propiedades mecánicas, WPI obtuvo mayores valores de TS, aunque en elongación fueron muy semejantes. No obstante, debido a los métodos de obtención de WPI (más costosos), el empleo de WPC suele ser más común, pensando a largo plazo.

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HIPÓTESIS

La adición de proteína de suero de leche a una solución de proteínas miofibrilares de calamar gigante (*D. gigas*), permitirá obtener una película estructuralmente mejor organizada, en comparación con una elaborada únicamente de proteína miofibrilar, repercutiendo en sus propiedades fisicoquímicas y su estabilidad de manera positiva.

OBJETIVO GENERAL

Evaluar el efecto de la adición de un concentrado de proteína del suero de leche en la elaboración de una película de proteína miofibrilar de calamar gigante (*D. gigas*).

Objetivos Específicos

- Establecer las condiciones para la elaboración de una biopelícula a partir de proteína miofibrilar de calamar gigante (*D. gigas*) empleando distintos polialcoholes (Glicerol, Sorbitol, Maltitol, Manitol y Xilitol) como agentes plastificantes.
- Determinar el efecto de los distintos plastificantes en las propiedades funcionales (ópticas, mecánicas, solubilidad, permeabilidad, térmicas) y estructura (FT-IR) de las películas obtenidas.
- Incorporar proteína del suero de leche y evaluar su efecto en las propiedades y estructura de las películas.
- Evaluar la estabilidad de las películas por un periodo de 3 meses.

DESARROLLO DEL TRABAJO DE INVESTIGACIÓN

El trabajo experimental fue dividido en tres etapas.

Primera Etapa

Se partió del manto de calamar gigante (*Dosidicus gigas*), del cual se trabajó únicamente con la fracción de proteína miofibrilar. Se establecieron las condiciones para la obtención de las películas empleando distintos polialcoholes como agentes plastificantes (glicerol, sorbitol, manitol, maltitol y xilitol). Una vez elaboradas, se evaluaron sus propiedades funcionales (ópticas, mecánicas, solubilidad, permeabilidad y térmicas) y de estructura (FT-IR).

Segunda Etapa

Se trabajó con las películas elegidas por su mejor desempeño en la primera etapa, las elaboradas utilizando glicerol y sorbitol. A éstas se les adicionó el concentrado proteico del suero de leche. Se obtuvieron un total de 9 películas diferentes, manejando tres concentraciones de suero (5, 10 y 15%) y tres de plastificante (glicerol 40%, sorbitol 30% y gli/sor 50:50). Nuevamente se evaluaron sus propiedades funcionales y estructura.

Tercera Etapa

Finalmente, las 9 películas elaboradas en la segunda etapa fueron almacenadas en condiciones de refrigeración por un periodo de 3 meses. Sus propiedades funcionales y estructurales fueron monitoreadas de forma mensual para evaluar la estabilidad del material.

El trabajo realizado en la presente investigación quedó documentado en los capítulos presentados a continuación.

Descripción del Capítulo 1

Estado del arte del aprovechamiento industrial de las proteínas del calamar gigante. Así también, de proteínas de diversas fuentes y su potencial uso como biopelículas.

El primer capítulo contiene dos artículos de revisión. El primero titulado: Squid protein characteristics and their potential industrial applications, publicado en la revista *Interciencia* (ISSN 0378-1844). En este manuscrito se aborda de manera breve y concisa las diversas características que distinguen a las proteínas del calamar gigante, y como pueden ser aprovechadas por la industria en general. El segundo artículo se titula: Edible protein films: Sources and behavior, publicado en la revista *Packaging Technology and Science* (ISSN 1099-1522). En este documento se hace una revisión de las distintas clases de proteínas que pueden emplearse para la elaboración de biopelículas, ya sea que se extraigan de cereales, leche, y tejidos musculares o conectivos, y qué podemos esperar en cada caso.

Descripción del Capítulo 2

Obtención y caracterización de películas de proteína miofibrilar de calamar gigante empleando distintos polialcoholes como agente plastificante.

En el segundo capítulo se presenta un artículo de investigación titulado: Effect of different polyalcohols as plasticizers on the functional properties of squid protein film (*Dosidicus gigas*), publicado en la revista *Coatings* (ISSN 2079-6412). En este trabajo se describe cómo el uso de distintos polialcoholes: glicerol, sorbitol, manitol, maltitol y xilitol; influye en la formación y propiedades de las películas elaboradas con proteínas miofibrilares de calamar.

Descripción del Capítulo 3

Evaluación del efecto de la adición de concentrado proteico del suero de leche en las propiedades y estructura de las películas de proteína miofibrilar de calamar gigante y evaluación de la estabilidad de las películas obtenidas.

En el tercer capítulo se presenta el artículo de investigación titulado: Development, characterization and stability of blend films based on squid protein (*Dosidicus gigas*) and whey protein concentrate, el cual será enviado a la revista *Progress in Organic Coatings* (ISSN 0300-9440). En este manuscrito se presentan los resultados generados al mezclar proteína miofibrilar de calamar gigante y proteína del suero de leche para la elaboración de una biopelícula, así como la caracterización del material obtenido. Además, de su almacenamiento durante un periodo de 3 meses, con una caracterización mensual de sus propiedades para determinar su cambio con el paso del tiempo.

CAPITULO 1

Squid protein characteristics and their potential industrial applications

Claudia Lizeth Murrieta-Martínez, Víctor Manuel Ocaño Higuera, Guadalupe Miroslava Suárez Jiménez, Enrique Márquez Ríos (2016). *Interciencia.* ISSN: 0378-1844.

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SQUID PROTEIN CHARACTERISTICS AND THEIR POTENTIAL INDUSTRIAL APPLICATIONS

CLAUDIA LIZETH MURRIETA-MARTÍNEZ, VÍCTOR MANUEL OCAÑO-HIGUERA, GUADALUPE MIROSLAVA SUÁREZ-JIMÉNEZ AND ENRIQUE MÁRQUEZ RÍOS

SUMMARY

The global increase in population has caused a rise in the food demand and seafood is no exception. This has led to an overexploitation of commonly consumed species, and new species have been proposed to satisfy current demands. One such species is the giant squid. This species has a great potential due to its intrinsic characteristics. This fishing resource is one of the most important in Mexico, but only 10-20% of the catch is consumed domestically. The rest is exported to Asian countries, with minimal added value. In this sense, integral uses of this resource must be considered, as the mantle, fins, head and viscera can be used for different purposes. In this review, we summarize the major characteristics of squid proteins and their application as food ingredients, in aquaculture and in other practical applications, as well as what has been and is being done in the industry.

The giant squid (*Dosidicus gigas*) is an abundant resource found in the pelagic zone of the Eastern Pacific and is the largest and most abundant squid species from Chile to the Northwest coast of the United States (Nigmatullin *et al.*, 2001). In Mexico, it is abundant in the Gulf of California (Markaida, 2005).

This species has many outstanding characteristics that are important to consumers, including high yields after gutting because the viscera only account for 10%, easy evisceration, lean white flesh without bones or scales, and low cost (Campo-Deaño *et al.*, 2009). These features make the giant squid an attractive cephalopod species for product development (Encinas-Arzate *et al.*, 2014). However, the local population prefers other species such as shrimp, shark (*cazón*), ray, and other fish, and thus giant squid is highly underutilized, with only 10-20% of the catch being consumed in Mexico. The rest is exported fresh, fresh-frozen or cooked-frozen to other countries, mainly to Asian markets, with minimum processing and very low added value. It is therefore important to add value to this resource, so as to gain a better perspective of consumption and better prices. Recent efforts have developed value-added products in which jumbo squid could be used (Campo-Deaño *et al.*, 2009). The characteristics of the squid's mantle (white, lean, no bones or scales) make it a good alternative for protein concentrates, which could be used for manufacturing surimi. The mantle and by-products could also be used to obtain protein hydrolysates, collagen and pigments, while the viscera are rich in enzymes and lipids. Numerous studies have been made of these by-products to reach a more integrated approach to this resource, but more research is still necessary, especially in the development of value-added products. The aim of this review is to summarize some of the studies leading to solutions for a better use of squid proteins and by-products regarding the current state of the industry.

KEYWORDS: / *Dosidicus gigas* / Gelation / Giant Squid / Functionality / Proteins /

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Giant Squid Generalities

Squid muscle is a source of high-quality protein because it is readily digestible and possesses all of the essential amino acids. Appropriate processing of jumbo squid muscle can result in enriched products. However, attempts to process jumbo squid have failed because of scarce knowledge of its physiological and intrinsic characteristics (De La Fuente-Betancourt *et al*., 2009). The high proteolytic activity in the mantle (Konno *et al*., 2003) results in functional differences of protein concentrates made of squid mantle compared to other marine species. However, most studies reporting a rapid quality loss worked with squid that was frequently subjected to poor post-capture management; for example, specimens left in piles without evisceration or cooling treatment for several hours, sometimes reaching temperatures up to 35ºC (Márquez-Ríos *et al*., 2007).

Mantle composition

The squid mantle is composed of five different tissue layers (Martínez *et al*., 2000), where bands are divided into two types: circumferential, composed of fibers running around the entire circumference of the mantle cone, and radial, composed of fibers that connect two tunics of connective tissue. The exterior tunic is made of collagen fiber layers adjacent to an external layer of connective tissue fibers, which are located under the skin (Otwell and Giddings, 1980).

The general chemical compositions of the mantle, fins and tentacles are similar to those of non-fat fishes. However, squid muscle fibers and connective tissue are stronger than those of fish muscle and their arrangement is also very different (Stanley and Hultin, 1984; Sugiyama *et al*., 1989). It contains a different class of myofibrillar proteins that are more water soluble and have a different organization, being less susceptible to freezing and more prone to thermal denaturation (Kolodziejska *et al*., 1999).

Protein composition

Proteins from squid mantle muscle differ from those of marine vertebrates. The myofibrillar fraction constitutes ~75-85% of it, with myosin as its major component followed by actin and paramyosin, the latter being particularly common in marine invertebrates and can represent up to 25% of themyofibrillar proteins (Sikorski and Kolodziejska, 1986; Cortez-Ruiz *et al*., 2008). Sarcoplasmic proteins represent ~15% of the total, and has endogenous proteolytic activity that could be responsible for the high rate of autohydrolysis (Arias-Moscoso *et al*., 2014; Sánchez-Sánchez *et al*., 2014). An important difference in relation to other marine organisms is the higher stromal fraction, composed primarily of connective tissue and representing 11% of all proteins (in fish muscle it amounts to 2-3%). These differences are primarily related to the physiological needs of the jumbo squid, such as their high energy movements needed for locomotion (Macgillivray *et al*., 1999; Kier and Curtin, 2002; Kier and Thompson, 2003). To propel itself through the water using jet propulsion, an elastic but also strong mantle is necessary, which results in the fiber crosslinking and the high amounts of collagen found in the skin and mantle.

Protein Characteristics and Opportunities

High proteolytic endogenous activity

Cephalopods typically have a high level of proteolytic activity, higher than most fish species (Stanley and Hultin 1984; Kolodziejska *et al*., 1987; Hurtado *et al*., 1999). For this reason *D. gigas* muscle undergoes intense proteolysis immediately after capture (Nagashima *et al*., 1992; Gómez-Guillén *et al*., 1996). However, the high endogenous enzymatic activity reported in giant squid muscle (Ayensa *et al*., 2002; Ezquerra-Brauer *et al.,* 2002; Gómez-Guillén *et al.,* 2002; Ruiz-Capillas *et al.,* 2003) could be taken advantage of in order to recover soluble proteins from squid by-product by auto hydrolysis, which could be used for aquaculture feed.

Obtention of auto hydrolysates is advantageous since one of the most expensive ingredients in feed production is protein, and different protein sources are used to reduce costs. Protein hydrolysates possess healthy and nutraceutical properties and have been used as food supplements (Haard, 2001). This would be particularly valuable in a starter diet for fish larvae since they are unable to digest and assimilate nutrients efficiently (Lian *et al.,* 2005). Sánchez-Sánchez *et al.* (2014) compared the hydrolysates of head, tentacles and skin from jumbo squid by-products, obtained by auto-hydrolysis (55ºC, pH 5.0) and a chemical-enzymatic process (45ºC, pH 2.5 and pepsin). After 90min, 18-30kDa proteins were observed in both processes, indicating that endogenous enzymatic activity in giant squid is enough to obtain hydrolysates with good characteristics. This was confirmed by Arias-Moscoso *et al.* (2014), who obtained hydrolysates of skin, head and fins jumbo squid, at two different pH values (5.0 and 7.0) by means of its endogenous proteases. Both treatments exhibited similar degradation patterns, with <45kDa proteins observed after 120min of hydrolysis. Endogenous proteases in squid by-products can produce hydrolysates with useful properties, resulting in a higher yield at pH 7.0. It was hypothesized that this type of hydrolysate could be used in shrimp feed due to its characteristics (González-Félix *et al.,* 2014). The authors evaluated the use of squid hydrolysates obtained by acid-enzymatic hydrolysis and auto hydrolysis as ingredients in shrimp (*Litopenaeus vannamei*) diets at 2.5 and 5.0% dry weight. There was significantly higher crude protein in shrimp muscles with the highest hydrolysate levels (5%). No effect on growth or survival was observed, indicating that they could be utilized to partially replace sardine fishmeal in aquafeed. The authors concluded that hydrolysates from jumbo squid by-products can be potentially utilized in fishmeal for the aquafeeds industry.

Squid hydrolysates can also be used as organic fertilizers, which are naturally low in nitrogen (N) and rely on microbial activity to mineralize organic N into plant-available forms. Hydrolysates, can improve their quality because N is released more slowly from organic sources over longer periods of time as compared to synthetic fertilizers (Fetter *et al*., 2013). Organic fertilizers have the potential to increase soil fertility and soil organic matter content (SOM) on the long term (Booze-Daniels and Schmidt, 1997; Nardi *et al*., 2004). The conversion of squid by-products into organic fertilizer may present a solution to problems with high disposal costs for squid processors. The high N content (75% dw) associated with squid by-products, suggests their potential to develop a marketable product from waste (Fetter *et al*., 2013).

Peña-Cortés *et al.* (2010) studied the effect of the hydrolysate obtained from squid waste using alcalase as a proteolytic enzyme (55ºC, pH 7.5). The hydrolysate was applied through irrigation because it appeared to have a negative effect on plants vegetative tissues when applied directly. The use of hydrolyzed waste irrigation solutions caused an increment in the foliar diameter of treated plants, increasing whith higher digestion time and hydrolysate concentration. Seed yields were also significantly different in treated plants.

Fetter *et al.* (2013) prepared granular and liquid squid fertilizer

from head, fin, viscera, mantles and tentacles to evaluate their effectiveness compared with synthetic fertilizer on soil fertility and turfgrass quality. Organic fertilizers are often presumed to increase soil PO_4 concentrations (Wright *et al*., 2008), which can increase the risk of environmental pollution (Ginting *et al*., 2003). However, this was not the case in the present study. An important parameter is microbial activity, closely related to soil fertility through mineralization of organic forms of nutrients from SOM and dead microbial biomass into plant-available inorganic forms of nutrients (Frankenberger and Dick, 1983). Both liquid formulations produced significantly higher microbial activity than the granular products, probably as a result of the decreased time and lower energy required for microbes to break them down. Also, squid hydrolysates produced higher microbial activity values compared with the synthetic formulations (Fetter *et al*., 2013).

Regarding turf quality, it displayed uniformity and density along with high clipping production, which reflects sufficient N availability (Turgeon, 2012). When quality was examined over the entire study period, there were no significant differences among the four products (synthetic and squid hydrolysates, both liquid and granular). Thus, the use of squid-based products offer a turf quality comparable to synthetic fertilizers. Overall data suggest that squid hydrolysate can be effective as an organic fertilizer applied to turfgrass, either in liquid or granular state. It consistently provided high-quality, and uniform turf when compared with synthetic fertilizers applied at the same level (Fetter *et al*., 2013).

Thermal instability

Thermal stability is essentially the resistance of the protein molecule to unfolding as a result of any thermal treatment (Bernal *et al*., 1987). Myofibrillar proteins in the muscles of several species form aggregates between 40-60ºC, but in giant squid unfolding begins at 30-32ºC, followed by protein association at 45-50ºC (Kristinsson and Rasco, 2000; Tornberg, 2005; Tolano-Villaverde *et al*., 2013). This behavior is unexpected, because giant squid dwells in warm waters and their proteins should have higher thermal stability.

This instability could be related to the low content of sulfhydryl groups present in the myosin molecule, which have been reported at ~0.9mol/10^5g of protein, whereas most fish species contain around ~4.8mol/10^5g of protein. Sulfhydryl groups confer stability, so lower values would make the molecule less stable (Murrieta-Martínez *et al*., 2015). Myosin is the main component of the myofibrillar fraction, which makes up the majority of muscular proteins. Nevertheless, the instability may also be related to a high autolytic activity. Squid mantle muscle contains endogenous metalloproteinases that selectively degrade myosin molecules into heavy meromyosin (HMM) and light meromyosin (LMM) (Yoshioka *et al*., 2005).

However, even when marine proteins are known for their thermal instability, squid proteins in particular have shown a high stability under freezing, which can compensate the behavior previously reported.

High solubility

Functional properties of seafood muscle are highly related to protein solubility (Valencia-Pérez *et al*., 2008). All publications on the solubility of giant squid proteins (Borderias and Montero, 1985; Sato *et al*., 1991; Ando *et al*., 1999, 2001; Enzinas-Arzate *et al*., 2014) have concluded that muscular proteins in squid are highly soluble and, thus, conventional washing processes used to produce surimi from fish are not a viable alternative for obtaining protein concentrates from this species (Sánchez-Alonso *et al*., 2007). Even when most proteases and all compounds producing an undesirable odor and bitter taste are removed, a large portion of the myofibrillar proteins are solubilized and washed away, reducing the total protein yield (Palafox *et al*., 2009).

This behavior has been reported by Rocha-Estrada *et al.* (2010), who evaluated several properties from squid mantle and fin proteins under operational variables, particularly pH and ionic strength. They found that solubility was a requisite for useful protein properties. For example, a homogenate with highly soluble protein is expected to have a high foaming capacity. The SDS-PAGE pattern showed the presence of the myosin heavy chain (MHC) in the sarcoplasmic fraction, indicating high solubility of squid myofibril proteins even at low ionic strength. This phenomenon is of particular interest for processes that involve washing with water, as in surimi production. If squid muscle is washed with water to isolate myofibril proteins, the MHC fibers could be lost by solubilization.

Galvez-Rongel *et al.* (2014) compared acidic (AcPC), alkaline (AkPC), isoelectric (IPC) and neutral (NPC) protein concentrates and reported that during protein fractionation at low ionic strength (I= 0.05), the soluble protein fraction was 4.98 ±0.09%, 7.7 ±0.2% and 5.83 ±0.84% for AcPC, IPC and AkPC, respectively. However, even though most of the sarcoplasmic fraction was removed, NPC still contained a high amount of soluble protein with values of 20.5 ±1.5%. This confirms the high solubility of squid mantle protein, previously documented in other studies (Cortes-Ruiz *et al*., 2008; Dihort-Garcia *et al*., 2011).

Encinas-Arzate *et al.* (2014) evaluated washing with different ionic strengths (I= 0.0, 0.1 and 0.3) and reported high solubility of myofibrillar proteins from squid muscle at low ionic strengths. Similar results were reported by Gomez-Guillen *et al.* (1996), who studied the solubility of squid mantle protein (*D. gigas*) as a function of temperature and ionic strength. These researchers detected the presence of electrophoretic bands corresponding to actin, tropomyosin and low molecular weight proteins at 0.05M NaCl. They concluded that the removal of sarcoplasmic proteins by increasing ionic strength also gradually removed myofibrillar proteins because of their high solubility.

Stability under freezing

In cephalopods, the myofibrillar proteins are highly resistant to freeze-induced denaturation (Moral *et al*., 1983). Gómez-Guillén *et al.* (2003) determined changes in the functional and chemical properties of squid muscle proteins during 10 days of chilled (2ºC) and 30 days of frozen (-20ºC) storage. Functionality, apparent viscosity and extractability of proteins were not affected; even thermal behavior remained relatively stable. The SDS-PAGE profile showed MHC degradation and its further disappearance, but paramyosin and actin remained stable and were unaffected during the entire study, confirming the freezing stability previously reported.

This property, as stated above, may prove useful for the development of value-added products such as protein concentrates, imitation of restructured fish fillets and emulsified-gel products where preserving the gelling capacity of the muscle is essential even after the freeze-thaw processes. Without the use of cryoprotectants, most muscles lose functionality when frozen because of protein denaturation and/or aggregation (García-Sánchez *et al*., 2015), but this is not the case for giant squid muscle. Additionally, as raw material, giant squid would be an ideal target for these types of products due to its high post-process yield, low fat content, white muscle, scale-

less, boneless and good organoleptic characteristics (Campo-Deaño *et al*., 2009).

García-Sánchez *et al.* (2015) noted that the gelling capacity of the jumbo squid muscle was maintained even after frozen storage without the use of cryoprotectants. They evaluated the effect of freezing (-20ºC) and thawing (4ºC) on protein denaturation and gelling capacity the *D. gigas* mantle muscle. In a differential scanning calorimetry study, they observed similar patterns in T_{max} and enthalpy and no denaturation in any of the thermograms, suggesting a high stability of squid muscle proteins. Increased surface hydrophbicity (SoANS) values suggested that proteins had unfolded due to freezing, but increases in enthalpy suggested protein aggregation. They hypothesized that the interactions formed among proteins were reversible. Finally, the quality of gels as determined by a folding test, water holding capacity (WHC) and texture profile analysis (TPA) were not affected by any treatment. With this evidence, they concluded that freezing for up to 30 days does not affect squid muscle proteins or their gelling capacity, and squid muscle should be prioritized over other muscle types as a raw material for value-added products.

This resistance to freezing can be explained by the presence of paramyosin (Iguchi *et al*., 1981), a protein present only in marine species that decreases the rate of protein denaturation. Paramyosin is found in greater amounts in squid, representing ~14% of the myofibrillar fraction. This could explain the freeze resistance reported in squid proteins.

However, gels made from giant squid (*D. gigas*) have lower gel strength than the ones made from other fish species (Sánchez-Alonso *et al*., 2007). Additionally, application of the traditional surimi process to squid results in a concentrate with reduced functional-technological quality and low yield (Matsumoto, 1958). The acidic/alkaline solubilization process developed by Hultin and Kelleher (1999, 2000) is a better option to obtain protein concentrates from this species. Under acidic conditions (pH= 3), the concentrates display high gel strength, elasticity, and cohesiveness (Cortés-Ruiz *et al*., 2008) while alkaline dissolution (pH= 10) promotes autolysis of the myosin heavy chain and low water retention, resulting in better protein solubility and similar protein recovery as under acidic conditions. On the other hand, the gel-forming capacity was improved over traditional methods (Dihort-García *et al*., 2011).

Another option is the use of differential ionic strength process, during the preparation of protein concentrates to discard sarcoplasmic proteins. However, myofibrillar proteins can support low levels of sarcoplasmic proteins without affecting the resulting gel properties. Encinas-Arzate *et al.* (2014) used NaCl solutions of different ionic strengths (I= 0.0, 0.1, and 0.3) to obtain three protein concentrates. They found that hardness increased when I increased, as well as S-S bond formation, during thermal gelation. This indicates that removal of sarcoplasmic proteins as a function of I results in better quality gels.

Jumbo squid proteins can also be an important protein source incorporated into food products, either as a raw material or as protein supplement (Palafox *et al*., 2009). An example of this is the use of squid protein as a flour additive. Similar studies have been carried out by Ramírez-Suarez *et al.* (2012), who added lyophilized jumbo squid fin (JSF) and mantle (JSM) to commercial wheat flour and evaluated the effects on the resulting dough and bread properties. The addition of JSF and JSM during dough production increased resistance to mixing, likely as a consequence of the strengthening of the protein network by the animal protein. However, stronger dough does not necessarily produce a larger bread loaf; a balance between dough strength and extensibility is required (Wrigley *et al*., 2005). The most relevant characteristic was the bread loaf protein content, which was significantly different from the control. The control samples contained 92.8 ±6.0g·kg^{-1} protein, while the samples with squid muscle, either JSF or JSM at 50g/kg, had higher contents of ~105.8 ±3.4g·kg^{-1}, which represents an important nutritional advantage. Furthermore, the sensory analysis showed acceptable results for this type of bread when there was an addition of 25g·kg^{-1} of JSF or JSM (protein content was still higher than the control, 98.8 ±1.0g·kg^{-1}), being a good option for a value-added product (Ramírez-Suarez *et al*., 2012). Matrel Foods S.A.C. has already developed a bread enriched with squid flour as the base, but they only worked with the mantle region**.** This new product is distinguished by its high protein content (86% *vs* 8% in common bread), level of unsaturated fat, and source of ω3 and ω6 fatty acids (Anonymous, 2007).

Squid flour (called CPP-Lamolina) has been produced since 2001 by the Universidad Nacional Agraria La Molina, Peru, and it is used in several enriched foods for human consumption. This concentrate comes from the mantle region and has a high protein content of ~85%, whereas similar products such as milk powder and eggs only contain up to 26% and 12.5%, respectively. It is also shelf-stable and contains ω3 fatty acids. CPP-Lamolina can be incorporated as an ingredient without impacting the appearance, smell or flavor of food products and is currently being added to products such as enriched bread, noodles, chocolate coated flakes, and wheat flour (Roldán-Acero, 2007).

Conclusions

Value-added products have received great attention in recent years. One of the most prominent uses of squid proteins is the production of protein concentrates from the mantle, fins or tentacles. Thus, there are many uses for this organism, like a protein-rich additive for bread making or the obtention of protein hydrolysates for aquaculture feed and for the elaboration of organic fertilizers. Other possibilities (not reviewed in this work) include the characterization of fatty acids from the viscera, chitosan from the pen, and pigment recovery. This review presented the major characteristics of squid proteins and some applications for them. More uses of this resource must be considered, and the mantle, fins, head and viscera should be used for different purposes.

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CARACTERÍSTICAS DE LAS PROTEÍNAS DE CALAMAR Y SU POTENCIAL APLICACIÓN INDUSTRIAL

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RESUMEN

El aumento global de la población ha provocado un aumento en la demanda de alimentos y los productos marinos no son la excepción. Esto ha llevado a una sobreexplotación de las especies de consumo habitual, por lo que han sido propuestas nuevas especies para satisfacer las demandas actuales. Una de estas especies es el calamar gigante, cuyas características le otorgan un gran potencial. Este recurso pesquero es uno de los más importantes de México, pero apenas un 10-20% de la captura se consume a nivel nacional. El resto se exporta a países asiáticos con poco valor agregado. En este sentido, el uso integral de este recurso debe ser considerado, ya que el manto, aletas, cabeza y vísceras se pueden utilizar para diferentes propósitos. En esta revisión, se resumen las principales características de las proteínas del calamar y su aplicación como ingredientes alimentarios, ya sea en la acuicultura o en otras aplicaciones prácticas, así como lo que ha hecho o se está siendo en la industria.

CARACTERÍSTICAS DAS PROTEÍNAS DE LULA E SUA POTENCIAL APLICAÇÃO INDUSTRIAL

Claudia Lizeth Murrieta-Martínez, Víctor Manuel Ocaño-Higuera, Guadalupe Miroslava Suárez-Jiménez e Enrique Márquez Ríos

RESUMO

O aumento global da população tem provocado um aumento pela procura de alimentos, e os produtos do mar não são a exceção. Isto tem levado a uma superexploração das espécies de consumo habitual, pelo qual tem sido apontadas novas espécies para satisfazer as demandas atuais. Uma destas espécies é a lula gigante, cujas características lhe outorgam um grande potencial. Este recurso pesqueiro é um dos mais importantes do México, mas apenas entre 10 e 20% da captura é consumida em nível nacional. O restante é exportado, com pouco valor agregado, para países asiáticos. Neste sentido, o uso integral deste recurso deve ser considerado, já que o manto, aletas, cabeça e vísceras podem ser utilizados para diferentes propósitos. Nesta revisão, são resumidas as principais características das proteínas do calamar e suas aplicações como ingredientes alimentários, seja na aquicultura ou em outras aplicações práticas, como o que tem sido feito ou se está sendo feito na indústria.

Edible protein films: Sources and behavior

C. L. Murrieta-Martínez., H. Soto-Valdez., R. Pacheco-Aguilar., W. Torres-Arreola., F. Rodríguez-Felix., E. Márquez Ríos (2018). *Packaging Technology and Science.* DOI: 10.1002/pts.2360

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REVIEW ARTICLE

Edible protein films: Sources and behavior

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Edible and biodegradable films are made of renewable resources, offer an alternative to synthetic plastics for packaging, and, unlike these, can be placed between phases in direct contact with food. Although there are different sources, such as lipids and carbohydrates, proteins are popular due to their abundance and their nutritional qualities. Protein for film elaboration can be extracted from cereals (gluten, prolamins), milk (casein, whey), muscle (myofibrillar), and the connective tissue (gelatin) of marine or terrain species. Requirements may vary depending on their future application; therefore, it cannot be said that 1 source or the other is better, because film properties will also differ due to their method of obtention. Therefore, it is necessary to know what kind of film is needed in order to choose the most suitable one.

KEYWORDS

biopolymer, edible film, protein film

1 | INTRODUCTION

Currently, the food industries are looking for ways to reduce the amount of food packaging. Different options had been analyzed; one of these is to use biodegradable materials, which are an environmentally friendly alternative to synthetic polymers.[1] Packaging material has numerous functions, the most general of these, to protect food from its environment, through which it gains or loses moisture or aroma, takes up oxygen (leading to oxidative rancidity), or becomes contaminated with microorganisms, thus ensuring its quality and shelf life.[2]An alternative to plastic films is represented by biopolymer films, which are made of renewable resources, usually obtained from natural raw materials such as starch, cellulose, and proteins.[1] This type of material has taken on more importance lately, not only because of their potential biodegradability and renewability but also because their manufacture may sometimes provide a use for by-products or even waste products of the agricultural and food industry, elimination of which is generally problematic.[3]

Research activity in biodegradable films has been especially intense over the past 10 years. Although, edible films are not meant to replace synthetic packaging films totally; they have the potential to provide a replacement and/or fortification of the natural layers at the product surfaces to prevent moisture losses, gas aromas, and solute movements out of the food, while selectively allow to control the exchange of important gases, such as oxygen, carbon dioxide, and ethylene, which are involved in food product respiration. Furthermore, the materials that are used for this purpose can completely coat the food or can be used as a continuous layer between food components.[4]

By definition, edible polymer films comprise a thin layer of edible material prepared separately and then applied to food surface, whereas a coating is formed directly onto the food surface and could be placed between food components, used as a food wrap, or as a pouch to contain foods.[5] Their purpose is to inhibit the migration of moisture, oxygen, carbonic dioxide, aromas, and lipids, to transport ingredients or bioactive compounds (eg, antioxidants, antimicrobials, and flavor), and/or improve mechanical integrity or handling characteristics of the food.[6,7] However, for packaging materials that will be in contact with food, additional requirements are needed.

These include acceptable sensory properties, adequate biochemical, physicochemical, and microbial stability, the absence of toxics, and safety.[8] These requirements are fulfilled by natural polymers or polymers derived from natural monomers, because their biodegradability and environmental compatibility are ensured.[9] However, their mechanical properties and permeability are generally poorer than synthetic films, particularly the hydrophilic nature of edible polymers limits their ability to provide desired edible film functions to specific applications. For this reason, the relative humidity, which greatly influences the majority of properties, must be taken into account when considering its applications.[4] Due to this, their use as moisture barriers usually requires the formation of composite films that contain hydrophobic materials, such as edible fatty acids and waxes.[3]

Materials utilized to produce edible films can be divided into 3 main categories based on their origin and production: category 1) polymers directly extracted/removed from biomass (polysaccharides and proteins); category 2) polymers produced by classical chemical synthesis using renewable biobased monomers (polylactic acid), and category 3) polymers produced by microorganisms or genetically modified bacteria. From category 1, polysaccharides like wheat and derivatives as chitosan/chitin, as well as proteins, from animal (muscular and connective tissues, whey) and plants (prolamins) have been extensively studied.[10] Proteins have been used extensively for developing biodegradable films because of their relative abundance, film-forming ability, and their uniqueness in terms of the film quality obtained, as well as for their high nutritional value.[11] Thus, the aim of this review was to compare the characteristics of different protein films to provide useful information regarding their behavior.

2 | PROPERTIES OF BIOPOLYMER-BASED FILMS

Biopolymer-based films are prepared from solutions comprising 3 main components: the biopolymer, the plasticizer, and the solvent. The properties of the formed film are affected by the intrinsic properties of film components and extrinsic processing factors. As a rule, protein films provide mechanical stability and polysaccharides are employed to control oxygen and the transmission of other gases, while fats are utilized to reduce water transmission. However, it must be borne in mind that only protein films provide a source of nitrogen during their degradation, thus acting as fertilizer, which is a benefit that is not available from nonprotein-based films.[12]

It has been reported that the inherent properties of proteins make them excellent starting materials for films. Proteins have a unique structure (based on 20 different monomers) that provide a wider range of functional properties, being their high intermolecular binding potential that can form bonds at different positions.[11] Also, due to the distribution charge, polar and nonpolar amino acids along the protein chain create a chemical potential resulting in interactive forces that produce a cohesive protein film matrix.[13] Moreover, proteins contain a great variety of functional groups which make it possible to alter them enzymatically, chemically, or physically, varying the properties of the materials obtained in order to adjust them to the specific needs of each application.[3]

In general, the formation of protein-based films can be described in 3 main steps: first, low-energy intermolecular bonds, which stabilize polymers in the native state; second, polymer chains are arranged and oriented, and third, the formation of a 3-dimensional network is stabilized by new interactions and bonds.[14] This is achieved by 2 types of processes: (1) wet ("casting"), consists in dissolving proteins in suitable solvents to make a film-forming solution to which desired additives are added, functional compounds or fillers are used (plasticizers, cross-linkers, antimicrobials, microparticles/nanoparticles, etc.), and pouring it onto a leveled surface to allow solvent evaporation. (2) dry, in the presence of plasticizers, at low moisture levels, high temperatures and with pressure or shear forces, proteins acquire a viscoelastic behavior; there are 2 kinds of dry process to obtain packaging materials: thermo-pressing/thermoforming and extrusion. Each process results in different modifications in the protein network that affect the final film matrix structure.[3]

Aside from the previously mentioned information in reference to packaging materials, films in this particular case, there are some properties that must be taken into account, the main ones being the following:

2.1 | Barrier properties

These properties are crucial for predicting the product's shelf-life and/or packaging, because a specific requirement will be related with product needs and final application. Water vapor and oxygen are the main permeant agents studied in packaging applications because they can transfer from the intern/extern environment through the polymer, which results in a continuous change in the product's quality and shelf-life.[15] Because biopolymer-based films generally present relatively inclination to high water vapor permeability (WVP), the solubility and diffusivity of water molecules are important factors to control permeability in polymeric matrix.[16] This property is very important for the packaged product, because physical and chemical deterioration is related with humidity-content equilibrium in order to maintain or extend its shelf-life. Water vapor permeability is quantified by WVP coefficients, which indicate how much water vapor passes per area unit and time of packaging material (kg mm^{-2} s^{-1} Pa^{-1}). For fresh foods, it is crucial to avoid dehydration, while for those such as bread, water permeation is crucial.[15]

2.2 | Thermic behavior

Thermal analysis allows to comprehend the material behavior under cooling/heating rates, or in an inert/reduction/oxidation atmosphere,[16] which makes possible to predict the behavior of the protein package through different processing stages such as freezing or even cooking. In the latter, proteins undergo denaturation, dissociate, and realign. These changes allow protein molecules to combine and cross-link by means of specific linkages.[17] In packaging materials, crucial information is given by the glass transition temperature (T_g), which occurs over the temperature range at which a glassy material enters the rubbery domain, resulting in a drop of moduli of Young.[18] Then, polymers with high T_g values are either difficult to blow into films or, tend to be brittle for their use as a wrap. On the other hand, low T_g values result in low melting temperatures (usually), which complicates to make them into sheets and films without the tendency of self-adhesion. Moreover, these films may lack adequate strength, water vapor barrier, and/or make them suitable for wraps or laminate coatings.[19]

2.3 | Mechanical properties

The polymer's architecture plays an important role in its mechanical behavior. In this regard, tensile tests are carried out to evaluate tension force (MPa), elongation percentage at breaking (%), and elastic modulus (GPa). These values permit us to obtain information on flexibility, hardness, and elongation to predict package behavior during handling and storage.[20]

However, even though the properties noted must be considered, 1 factor to take into account is that it is expected for different biopolymers to possess different properties. For practical purposes and based on their source, we will divide polymers into cereal proteins, milk proteins, and meat proteins, the latter divided into beef, poultry, and fish. The main characteristics of the films obtained are described later.

2.4 | Cereal proteins

The great availability of raw materials derived from crops render prolamins, storage proteins in cereals, a good source for producing films at a low cost. Reported as excellent oxygen barriers, prolamins are suitable for food packaging materials, in that food degradation is often oxygen dependent.[21] The most common source of prolamins is wheat gluten (WG), a mixture of proteins separated into glutenins and gliadins on the basis of their extractability in aqueous ethanol[22]; these are present in nearly equal quantities [21]. An alternative classification divides them into 3 groups: high molecular weight prolamins (HMW subunits of glutenin); S-poor prolamins (ω-gliadins), and S-rich prolamins. The latter group includes gliadins (α, β, and γ) and low molecular weight (LMW) glutenins.[23] From these fractions, glutenin films are stronger and have better barrier properties than those of gliadins or whole gluten. On the other hand, gliadin films present better optical properties but are not water resistant.[24] This behavior is due to that gliadins are single polypeptide chains associated by hydrogen bonding and hydrophobic interactions, as well as intramolecular disulfide bonds.[25] On the other hand, glutenins comprise a diverse number of protein molecules of HMW (95–145 kDa) and LMW (~44 kDa), capable of forming extensive networks in intermolecular disulfide bonds. The hydrogen bond between the repeat regions of HMW has also been found responsible for the elasticity of gluten: the larger the subunits involved in cross-linking, the greater the contribution to the matrix's elastic properties.[26]

2.5 | Wheat

WG proteins are insoluble in water and require a complex solvent system with basic or acidic conditions in the presence of alcohol. The isoelectric point is at approximately 7.5; however, WG dispersions at pH 5 to 6 result in films of uneven thickness and containing big particles of coagulated protein. Even so, WG-based materials characterize for being homogeneous, transparent, mechanically strong, and relatively water-insoluble.[21] However, some amino acid sequences in gliadin and glutenin proteins are responsible for celiac toxicity and wheat allergies;[27,28] this a major limitation for their use in edible materials. Even so, their use as packaging material for groceries bag is an option.[29] A recent work has also proposed the use of WG biopolymers to monitor carbon dioxide in food packaging, because the dielectric properties get modified by carbon dioxide due to structural changes in amino groups (which act as receptors). In intelligent packaging systems, the aim is monitoring the headspace.[30] However, unless WG-based materials are limited to non-edible packaging materials, new sources of prolamins are needed.

2.5.1 | Prolamins from sources other than wheat

Apart from WG, other prolamins employed for film barrier elaboration are those obtained from corn, sorghum, and oats, these being zein, kafirin, and avenin, respectively. Both zein and gluten display promising material properties, and kafirin, the prolamin of sorghum, is largely homologous to zein; thus, the same is expected. This information is of relevance in semi-arid parts such as southern Africa, where sorghum is a staple crop. There is limited evidence that some persons may be allergic to the precursors of zein,[31] and there is good evidence that kafirin is non allergenic[32]; this is the same for avenin. This affords them a plus, in that even resembling gluten renders them a more appropriate food packaging or coating material [19] and individuals with celiac disease could consume these foods.

In the studies reported by Gillgren and Stading [33], the mechanical and barrier properties of avenin, kafirin, and zein films were studied. With regard to their thermodynamic properties, glass transition (T_g) values ranged between 31°C and 44°C for avenin, 28°C and 49°C for kafirin, and between 31°C and 51°C for zein, depending on the amount of plasticizer (PEG/glycerol/LA for zein and kafirin and EtOH/glycerol for avenin). An increase in plasticizer content decreased T_g values, resulting in more extensible films. The glass transition temperature (T_g) refers to the point at which the polymer becomes viscoelastic (a property that allows it to be extruded); however, the majority of organic polymers denature before reaching this point. Low T_g values may permit it to be processed at the industrial level (casting is not an option because a continuous process is required).

With respect to their mechanical properties, avenin films were generally quite weak, but rather extensible at low plasticizer contents. When content demonstrated to be up to 44%, avenin films could not be elongated as much as those of zein and kafirin. It is also remarkable that the most plasticized kafirin could be elongated 30% more than the most plasticized avenin film, while zein had 3 times the extensibility of kafirin at the same plasticizer content. In this regard, kafirin and zein were stronger than avenin.[33]

According to barrier properties, kafirin exhibited lower WVP values (~2 to ~3 g mm/m^2 h kPa) than avenin (~5 to ~8 g mm/m^2 h kPa) or zein (~3 to ~4 g mm/m^2 h kPa) films. Something unexpected was that the less plasticized avenin films (23% glycerol) had higher WVP compared with those with 34% of glycerol. The results suggest that kafirin films are more resistant to water vapor. On the other hand, oxygen permeability (OP) was higher in avenin films (~200 to ~230 cm^3 μm/m^2 d kPa), while kafirin and zein shared approximately the same OP characteristics. Like WVP, OP increased with plasticizer content, which can be explained by that permeability generally increases with the enhanced motion of the polymer segments.[10]

Taylor and others[28] coincided with Gillgren and Stading[34] in that, due to a remarkable degree of homology, zein and kafirin can be considered together. However, it is necessary to take into account that whatever the source, the extraction method affects the composition of the prolamins, and there is also considerable interbatch variation even when the same process is employed. This appears to be mainly due to differences in molecular mass, and may be due to processing conditions or differences in feedstock.[35] Similarly, the functionality of

commercially processed WG is also highly variable due to denaturation during the drying process.[36] It is noteworthy that zein and kafirin are more hydrophobic than wheat prolamins.[37]

2.6 | Milk proteins

Films from milk proteins have gained importance because they have proven to be flavorless, flexible, and transparent. In addition, milk proteins also possess excellent nutritional value. It has been reported that producing films from total milk protein is difficult due to the presence of lactose, in that this crystallizes during film drying, resulting in a non-homogeneous film that adheres to casting surfaces.[38] Therefore, in the majority of cases, milk protein films are prepared by utilizing casein or whey protein individually.

2.6.1 | Casein

Casein is a predominant protein found in milk. Casein micelles form colloids composed of 4 different proteins (αS1-, αS1-, β-, and κ-caseins) and colloidal calcium phosphate with sizes normally distributed between 50 and 400 nm.[38] Casein is inexpensive, readily available, non-toxic, and highly stable; thus, it is used extensively in the food industry as a nutritional supplement and emulsifier, being also widely studied for chemical, biological, and nutritional applications.[39]

Casein coatings have shown to delay loss of firmness with minimal loss in weight and juice level, during the storage of kinnows under environmental conditions.[40] Casein films have also demonstrated high TS, rendering them suitable as film coatings for tablets; additionally, they have shown to provide sustained release properties [40]. Wagh and others[41] reported that at least 20% glycerol and 75% sorbitol (w/w of biopolymer) are required to produce flexible, brittle-free, and transparent casein films, and these authors compared them with whey protein concentrate (WPC) films.

Regarding tensile strength (TS), tensile strain, and elastic modulus (EM) of casein and WPC films, values ranged from 0.7 to 4.6 MPa, 19.2% to 66.6%, and 2.1 to 0.9 MPa, respectively. Maximal TS and EM were obtained at the lowest concentration of plasticizers. It was observed that the plasticizer concentration, to a greater extent than the biopolymer type, exerted an influence on the tensile properties. Excess plasticization increases free volume in the film network and weakens the intermolecular forces among adjacent polymer chains.

In terms of WVP and oxygen barriers, casein films had low values, ranging from 3.9 to 9.8 g mm/m^2 h kPa, depending on the plasticizer concentration, while WPC ranged from 6.8 to 10.4 g mm/m^2 h kPa. Meanwhile, oxygen (OP) was approximately 856 cc/m^2 per day, nearly 2 times lower than that obtained for WPC films, rendering casein films better at preventing oxidation, which has been confirmed in cheddar cheese samples in storage. On the other hand, OP differences may be related with differences in structure.

Although casein films possess good qualities, in recent years whey fraction proteins have been receiving attention, mainly due to the tendency to supply added value to by-products.

2.6.2 | Whey fraction

Whey is a by-product of cheese manufacturing that contains approximately 7% of dry matter, which includes 13% proteins, 75% lactose, 8% minerals, approximately 3% organic acids, and <1% fat. However, only 50% of the whey produced is treated and transformed into different food and feed products (the remaining 50% is discarded). One half of this amount is utilized in liquid form, 30% as powdered cheese-whey, 15% as lactose and its by-products, and the remaining amount, as WPCs or isolates.[42]

Whey protein isolates (WPI) and concentrates (WPC) are mainly composed of β-lactoglobulin, α-lactoalbumin, and bovine serum albumin, which are an important source of essential branched amino acids. WPI and WPC differentiate in protein concentration, 90% and 76.6%, respectively, and due to that WPC contain 6.8% of milk fat [45–1995]. Both are rich in sulphur-containing amino acids, cysteine, and methionine.[43]

The film-forming properties of whey proteins have been exploited to produce transparent, flexible, colorless, and odorless films[44] and coatings. Hence, their application in packaging has resulted as very promising, in particular after a denaturation process.[45] Native whey proteins are globular, with free thiol and hydrophobic groups buried inside the molecule, while heat denaturation induces protein unfolding and the exposure of internal functional groups.[46] Due to these differences in structure, denatured whey solutions have the ability to form dense and strong films, whereas films made of native whey exhibit higher permeability to gases, such as oxygen and water vapor.[47]

Films have been obtained from both WPC and WPI.

Ramos and others[48] carried out a comparison between WPC and WPI films. In this case, 3 levels of plasticizer (40%, 50%, and 60% of glycerol) were evaluated. No differences in appearance were observed when different plasticizer levels were utilized; however, WPC films exhibited a slightly yellowish color. In this study, moisture content, density (ρ^s), and water activity (a_w) values were obtained, where WPI exhibited significantly lower values of moisture content and a_w, as well as higher values of ρ^s than WPC films. Likewise, WPI films exhibited lower WVP values, from 8.25 to 11.92 g mm m^{-2} d^{-1} kPa^{-1}, compared with WPC films, which ranged from 10.81 to 14.04 g mm m^{-2} d^{-1} kPa $^{-1}$ for a given glycerol content. This was attributed to a higher lactose content in WPC, due to the relatively LMW of this compound which can exert a plasticizing effect, with consequent increases in permeability.

In regard to tensile properties, WPI films demonstrated higher TS, from ~33 to ~60 MPa, and elongation values from ~52% to ~62%, in comparison with WPC, whose TS ranged from ~29 to ~50 MPa and elongation, from ~15% to ~42% for the same glycerol content. Hence, WPI films are stronger and more flexible. For both films, when the content of glycerol increased, TS decreased, thus leading to weaker films. According to Gurgel-Adeodato,[10] a rising glycerol content increases film elasticity and elongation because it constrains the establishment of hydrogen bonds among the protein chains, thus increasing intermolecular spacing, therefore, chain mobility. However, in this work, such an increase only took place up to a certain level of plasticizer: the threshold was 50% (w/w); above this value, an increase did not produce any change, probably as a result of film matrix saturation.

In addition, in terms of the essentially protective function, whey protein edible films and coatings can be utilized in the food industry to develop single-dose, premeasured pouches of food ingredients. Phupoksakul et al[49] developed pouches of polylactic acid and WPI

for baby formula, obtaining a delayed lipid oxidation and good storage stability. Then, application of the classical extrusion process to whey protein films will allow easy sharpening into pouches, for example, for milk powder and other dry foods. However, further research must be done to address its use for commercial applications.

2.7 | Muscle proteins

According to Dangaran and Tomasula,[13] there are 3 types of meat proteins as follows: sarcoplasmic, stromal, and myofibrillar. Enzymes, myoglobulin, and cytoplasmic proteins are examples of sarcoplasmic proteins. Stromal proteins include collagen and elastin. Myofibrillar proteins include myosin, actin, tropomyosin, and troponins. However, only stromal and myofibrillar proteins are utilized for making edible films and coatings (the sarcoplasmic fraction is mainly composed of enzymes).

The main myofibrillar proteins found in muscle (mammalian or fish) are myosin (500 kDa) and actin. These proteins are obtained after removing other components, such as blood, lipids, myoglobin, and collagen, through a series of washing treatments. Fibrous proteins (myosin, F-actin) can form films with good mechanical properties, while globular proteins such as G-actin need to be unfolded first.[50]

On the other hand, within the stromal fraction, gelatin is the most important protein, and it is derived by means of partial collagen hydrolysis.[51] It is extracted from skin and bones as well as from the connective tissue of animals.[52] Gelatin is widely used for producing edible films/packaging due to its thermo-reversible properties and a melting point close to that of the human body temperature. This latter attribute is particularly significant in edible applications.[53]

2.7.1 | Cattle (bovine)

For cattle, one of the main by-products is gelatin, obtained from the hide and bones. According to the report of the Gelatin Manufacturers of Europe, most commercial gelatin (95%) derives from this source (and also porcine). However, frequent occurrences of bovine spongiform encephalopathy and foot/mouth diseases have been problems for human health; thus, the by-products of mammalians are limited in usefulness in the processing of functional food, cosmetic, and pharmaceutical products.[54]

At any rate, the amount of gelatin employed in the worldwide food industry is increasing annually, and new sources are needed.[55] Even though the by-products of poultry and fish are rarely used as a resource of gelatin, it is time to consider them seriously.

2.7.2 | Poultry

The majority of gelatins available are made from the resources of mammals; however, gelatin derived from porcine skin is not acceptable to Judaism and Islam due to sociocultural issues; thus, there is a need for alternative extraction sources.[56] The poultry industry has become a promising option, where one of the main by-products are chicken feet, which have become an environmental concern due to the increase of chicken consumption. Chicken feet consist of 85% protein, mainly collagen, and 2.7% fat. It has been reported that gelatin films possess good mechanical properties depending on gelatin composition and conditioning; then, gelatin extracted from chicken feet may be a suitable material for developing edible films.[57]

Recently, gelatin from chicken-foot skins and tendons has been extracted for film elaboration, in order to obtain a product with adequate characteristics for consumption.[58] While it presents low brightness values (~19.92), it compensates with high strength values (bloom) ~294.78 g, higher than values usually reported for bovine gelatin.[59]

Because the quality of a gelatin can be measured by gel strength or bloom value, classified as: high bloom (200–300 g), medium bloom (100–200 g), and low bloom (50–100 g).[60] Chicken-foot skin and gelatins from tendons can be classified as high quality, which results in higher melting and gelation points and shorter gelation time in the final products, as well as requiring lower amounts of gelatin. It is remarkable that brittleness was not present even at 40% of compression, making a suitable base material for preparing edible films.

Film elaboration has been performed by Lee et al.[57] A mixture of glycerol and sorbitol was needed as plasticizer in that the films containing only glycerol or sorbitol were too weak or rigid. This mixture also had a remarkable effect on their TS and elongation values (E). More glycerol resulted in higher E values, while adding sorbitol increased the TS. Similarly, Song and others[61] also reported that the TS of chicken-feather protein film increased compared with the film incorporated with glycerol or sorbitol alone, after adding a blend; in this case, a ratio of 3:2 (w/w) glycerol-sorbitol was considered optimal. Regarding WVP values, these depend on the concentration and the type of the plasticizer, with $3.49 \pm 0.46 \times 10^{-9}$ g m/m^2 s Pa the value obtained for this ratio. On the other hand, TS and E were 7.13 ± 0.36 MPa and 21.78 ± 1.66%, respectively, for the same glycerol-sorbitol ratio. Therefore, chicken-foot gelatin as a precursor for edible films could be a good option to take seriously into account; however, more investigation is needed.

2.7.3 | Marine species

It is a fact that edible gelatin films prepared from bovine and porcine skins exhibit stronger mechanical properties than fish-skin gelatin, due to the higher concentration of proline and hydroxyproline.[56] However, in recent years, edible films prepared from fish gelatin have begun to receive increasing attention.[62] This is mainly due to bovine and porcine diseases, but also to the current increase of fishery waste. The latter is due to the 3 following factors: (1) greater elaboration and processing of fishery products, thereby, generating larger quantities of waste; (2) greater concentration of waste because of the implantation of new larger rather than smaller industries; and (3) fewer and larger fish auctions.[63] The same applies for myofibrillar proteins, especially for fish and fish by-products with low commercial value.[12]

2.7.4 | Stromal fraction

From the stromal fraction, the main gelatins employed to develop edible films comprise those obtained from skins.[64-66] However, at present the properties of edible films based on gelatin from fish scales have been investigated, although scarcely.

Characterization of edible films from tilapia-scale gelatin with pH values ranging from 3 to 9 was carried by Weng and others.[67] pH 5 was considered optimal, because those films presented highest TS

values (~55 MPa), similar to those of edible films based on bovine- or porcine-skin gelatin.[68] Additionally, higher elongation at break (EAB) (~36%) was observed, suggesting that scale gelatin possesses excellent film-forming properties. Regarding appearance, high brightness values of (L* ~90) were reported, and a* and b* values were quite close to those of the white standard, confirming a nearly colorless film visually. UltraViolet (UV) light (280 nm) transmission of the films ranged from 40.33% to 43.59%, higher than that of other fish gelatin films[69] and myofibrillar protein films.[70] Weng and others[62] established that α-helix structures, rather than molecular weight distribution and T_g, were significantly involved in formation of the films; this was based on the results of electrophoretic, differential scanning colorimetry, and Fourier-transform infrared analysis of the films.

Aside from pH, the extraction temperature also influences the film properties, as observed in mackerel-scale gelatin films, where temperatures of 70°C, 80°C, and 90°C were utilized.[71] In this case, 70°C was considered as the optimal extraction temperature because highest TS (~35 MPa) and EAB (~46%) were obtained. Lowest WVP values (~0.98 × 10^{-10} gm^{-1} Pa^{-1} s^{-1}) were also observed at this temperature, being much lower compared with mammalian- or fish-gelatin films. This behavior could be due to a higher level of hydrophobic amino acids (a total of 653 residues per 1000 residues) than that of mammalian- (pork skin, 651 residues), cold-water fish- (haddock skin, 647 residues), and warm-water fish- (catfish skin, 649 residues) gelatins.[72] The transmission of UV light ranged from ~15% to ~20%, suggesting the potential preventive effect in lipid oxidation delay induced by UV light.[71]

Skin, unlike scale, gelatins have been widely studied. Rawdkuen and others[53] worked with gelatin from catfish and compared the film obtained to one of bovine-bone gelatin. Catfish-gelatin films were transparent (~3.34 ± 0.020) and with TS and EAB values of 40.74 MPa and 34.14%, respectively, higher than those of bovine-gelatin films (32.56 MPa and 26.63%). WVP values for catfish-gelatin films were 0.91 × 10^{-10} gm^{-1} s^{-1} Pa^{-1}, in comparison with 0.81 × 10^{-10} gm^{-1} s^{-1} Pa^{-1} for bovine-gelatin films.

Differential scanning colorimetry demonstrated that fish gelatin had a transition temperature (T_t) of 89.5°C, higher than that of bovine-gelatin films (T_t = 88.42°C). T_t indicates the precise temperature that causes a disruption of protein interaction during film preparation. The higher value obtained may be due to the greater amino acid amount (proline and hydroxyproline) of catfish gelatin (211 residues/1000 residues), showing a direct correlation with thermal stability.[67] On the other hand, film solubility was 41%, less than that of bovine, while, SEM revealed a smooth and continuous surface without grainy and porous structures, meaning that an ordered matrix was formed.

Giant squid gelatin has also been investigated. In this respect, Giménez and others[73] reported solubility in water of 90% that could be related with the low hydroxylysine content, resulting in a low cross-linking degree through covalent links. In comparison, mammalian-gelatin films are less water soluble due to a higher hydroxylysine content, as reported by Bertan and others,[74] with water solubility values around 30% for films based on bovine-hide gelatin. In regard to WVP, values of ~1.89 10^{-8} g mm h^{-1} cm^{-2} Pa^{-1} were obtained; however, taking the amino acid composition into account, higher values were expected because squid gelatin possesses fewer hydrophilic residues compared with fish gelatins.

In terms of film appearance, low L* values (~33) were obtained when compared with skin-derived gelatin films obtained from fish species. Squid-gelatin films demonstrated a considerably lower yellow component (b*). Also, opacity values were higher than those observed for films made from tuna[75] or cod skin,[76] attributed to a higher amount of LMW components. This lack of coloring is of special interest for use as a coating in the food industry. Finally, although squid gelatin exhibited lower thermal stability and viscoelastic behavior, when compared with studies using flat-fish skin-derived gelatins,[77] it continued to exhibit better gelling ability and viscoelastic behavior than other fish skin-derived gelatins, such as those extracted from cod or hake.[78]

2.7.5 | Myofibrillar fraction

In the case of myofibrillar fraction, the films obtained are usually rigid due to the presence of strong covalent bonding, especially disulphide bond.[79] Additionally, they show a poor water vapor barrier property, owing to the hydrophobic nature of amino acids in protein molecules, and to the significant amounts of hydrophilic plasticizers added, such as glycerol and sorbitol, which are required to impart adequate film flexibility.[80]

An example of this film type is the obtained by Zavareze and others[12] from whitemouth-croaker muscle myofibrillar protein (MMP). Even more so, the authors also employed residue protein isolate (RPI). Aside from the films differences, the apparent viscosity of film-forming solutions for MMP demonstrated higher values. The film water solubility values were 26.55% and 35.73% for MMP and RPI, respectively. Films in this study had lower solubility compared with films made with soluble proteins from the threadfin bream (*Nemipterus hexodon*), which exhibited values ranging from 55.61% to 79.22%.[81] The low dissolution rate could be due to cohesion of the matrix. With respect to color parameters, L* values decreased with increasing protein concentrations, which were of ~91 for MMP and ~78 for RPI, which was visually darker. TS was similar in both films, with values of ~5 MPa, similar to those reported by García and Sobral[82] in Nile tilapia muscle protein films. However, the authors reported that an increment in protein concentration increased the TS, which was not the case for whitemouth croaker. EAB was lower for MMP with ~173%, while for RPI this was ~227%, and it demonstrated an inverse relationship with the films' thickness. WVP values were 4.10 and 3.25 g mm/m^2 d kPa for MMP and RPI, respectively. Lower values in RPI could be due to the presence of sarcoplasmic proteins, which are responsible for reducing the capacity of myofibrillar proteins to absorb water. Finally, MMP films with low fish-protein concentrations presented better film characteristics, reduced thickness, and opacity, as well as higher elongation than RPI films.

Oujifard and others[83] studied protein films from red tilapia, adding cryoprotectants. All the films were similar in thickness but were higher in this regard than the control, due to the lower degree of compaction in the film matrix. Cryoprotectants more likely impeded the association or interaction of protein chains, thereby yielding the weaker network. WVP values showed no differences among the films (~1.24 × 10^{-10} gs^{-1} m^{-1} Pa^{-1}); the distinct cryoprotectants had no effect. The control film had the lowest WVP value. Because sucrose and sorbitol are highly hydrophilic, they were able to absorb more water from the

environment. As a result, films containing cryoprotectants had higher WVP values. Thermal degradation of films began at approximately 181°C for the control, and at 188 and 179°C for sucrose-added and sorbitol-added films, respectively. These results were in agreement with those of Tongnuanchan and others,[84] who reported that the initial temperature for red tilapia-protein isolate films was 170°C. The film with the addition of sorbitol was more susceptible to heat, possibly due to the weaker film network attributable to strong interaction with protein molecules; this coincided with the higher EAB observed. TS and EAB of films were affected by the addition of cryoprotectants. TS values ranged from 2.12 to 2.97 MPa, lower than that of the control (3.12 MPa). Cryoprotectants most likely impeded the interaction of protein chains, thereby yielding the weaker network. Artharn and others[85] also reported this behavior on a film from fish mince. EAB values ranged from 94.74% to 122.14% and increased when cryoprotectants were added.

Aside from film properties, the film's stability during storage is also an important factor. Mantle-muscle films from *Todarodes pacificus* were characterized, and their stability during storage (30 days) was evaluated by Leerahawong and others.[86] An increase in yellowness could be appreciated, as well as in transparency. These changes could be due to the orientation of polymers in the film matrix during storage. SDS-PAGE showed that myosin heavy chain decreased while the remaining amount of the protein appeared to increase during storage. The WVP value was ~10.51 × 10^{-11} gm^{-1} s^{-1} Pa^{-1} and remained relatively constant during the study. TS values increased significantly during the first 10 days (from ~2 to ~3 MPa), while no changes were observed in EAB. Anker and others[87] described that glycerol can migrate out of the film matrix due to its high mobility, and the film becomes more fragile with storage time (increase in TS and decrease in EAB). However, in this case, only TS increased, while there was no change in EAB. Moreover, migration of the plasticizer was not reported. It was concluded that the increase in the TS of plasticized films during storage was caused by protein cross-linking due to the Maillard reaction. Regardless of the species, myofibrillar proteins from marine sources can be employed for film-forming solutions, because they possess among other properties, excellent UV barrier properties.

In the end, biodegradable materials, especially those derived from replenishable, natural resources, are gradually replacing synthetic polymers. However, functional properties equal to those of synthetic ones are still far from reach (Table 1).

2.8 | Tendencies and perspectives

Changes in industrial procedures, such as the introduction of combined techniques for the obtention of medium-moisture and high-moisture food products and research of the application of emergent stress factors such as high pressures and the development of convenient food products with a longer shelf-life, have promoted the development of novel and/or improved packaging materials. One of these are edible films, which can be employed to control moisture, gases, and lipid migration, and they can additionally be supporters of additives and nutrients, also responding to demands for more natural products and lower contamination of the environment.[92]

On the other hand, the ultimate functionality of edible films is related with their bioactive (antioxidant, antimicrobial, and antibrowning activities) and functional properties (barrier to oxygen, carbon

TABLE 1 Biopolymer and common commercial films, properties reported

Film	Plasticizer	Opacity (A.nm)	Mechanical properties (TS in MPa)	Thermal properties	Water Vapor Permeability	References
Cereal						
Prolamins						
Wheat gluten						
Gliadins	Gly 35%	~34	%E= ~390 TS= ~7	NR	~7 x 10^{11} [(g m)/(m^2 s Pa)]	25
Glutenins	Gly 35%	~101	%E= ~250 TS= ~1	NR	~4 x 10^{11} [(g m)/(m^2 s Pa)]	
Other sources						
Zein	Gly 40%	NR	%E= ~118 TS= ~4	Tg= ~30 °C	~4 (g mm/m^2 h kPa)	33
Kafirin	Gly 40%	NR	%E= ~24 TS= ~1	Tg= ~30 °C	~8 (g mm/m^2 h kPa)	
Avenin	Gly 40%	NR	%E= ~40 TS= ~4	Tg= ~28 °C	~3 (g mm/m^2 h kPa)	
Milk						
Casein	Gly 50%	NR	%E= ~65 TS= ~2.5	NR	~7 (g mm/m^2 h kPa)	38
Whey fraction						
WPI	Gly 40%	NR	%E= ~33 TS= ~0.9	Tg= ~50 °C	~8 (g mm/m^2 d kPa)	48
WPC	Gly 40%	NR	%E= ~18 TS= ~0.7	Tg= ~43 °C	~10 (g mm/m^2 d kPa)	
Muscle						
Poultry	40%					
Stromal	(3:2, gly:sor)	~3.4	%E= ~21 TS= ~7	NR	~3 x 10^{-9} (g m/m^2 s Pa)	57
Marine species						
Stromal	Gly 25%	NR	%E= ~34 TS= ~40	Tg= 61 °C	~0.9 x 10^{-10} (g/m s Pa)	53
Myofibrillar	Gly 30%	~12	%E= ~173 TS= ~5	NR	~2 (g mm/m^2 d kPa)	12
High density polyethylene (HDPE)	NR	NR	%E= ~600 TS= ~54	~ -80 °C	~6 (g/m^2 d)	88-91
Low density polyethylene (LDPE)	NR	NR	%E= ~300 TS= ~27	~ -125 °C	~18 (g/m^2 d)	
Polypropylene (PP)	NR	NR	%E= ~150 TS= ~151	~ -10 °C	~8 (g/m^2 d)	
Polyethylene terephthalate (PET)	NR	NR	%E= 70 TS= 79	~ 76 °C	~21 (g/m^2 d)	

dioxide, and UV light). Natural additives from plant extracts, their isolated active compounds, plant-based products, and vitamins can provide antioxidant, antimicrobial, and/or antibrowning activity on edible films and coatings. However, compatibility with biopolymer, stability during processing and storage, and the conditions of the bioactivity of the natural additives continue to require evaluation.[93]

Finally, the future of protein bioplastics will depend on the adaptation of their processing, because most of the papers related to this topic are on laboratory or pilot scale. Therefore, the material production at industry level must be developed.[3]

3 | CONCLUSIONS

Among the previously mentioned sources, crops and the fishery industry account for the major source of proteins from co-products, and although cattle and bovine co-products are also numerous, they are not considered, due to diseases and cultural beliefs. Regarding their mechanical behavior, it is remarkable that the lowest TS is afforded by milk and myofibrillar proteins, with values <10 MPa, due to the high quantity of S-S linkages which confer rigidity on the films, while the other protein sources range between 28 and 55 MPa. On the other hand, cereal proteins are better oxygen barriers, while gelatins work as UV barriers, the latter also expected from myofibrillar proteins, due to the high amount of aromatic amino acids present in their structure. In the case of WVP, best source and behavior will vary according to the needs of the product, inferring that a low WVP film may not always be the best material. Finally, manufacturers will choose the most appropriate protein source depending on the nature and requirements of the product, the degree and nature of the protection needed, the distribution method, the shelf-life, and the environmental impact.

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CAPITULO 2

Effect of different polyalcohols as plasticizers on the functional properties of squid protein film (*Dosidicus gigas*)

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Article

Effect of Different Polyalcohols as Plasticizers on the Functional Properties of Squid Protein Film (*Dosidicus Gigas*)

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Abstract: Conventional plastic materials accumulation has led to a constant search to develop friendly packaging, edible coatings from biopolymers are an example. Since different proteins have different behavior and plastizicer compatibility, in this work, the effect of different polyalcohols (glycerol, sorbitol, maltitol, mannitol, and xylitol) as plasticizers on squid protein films behavior was studied. The results show that except for mannitol, transparent, and flexible films can be obtained. None of them showed transmission to light on the ultraviolet (UV) spectrum. However, only glycerol and sorbitol were sufficiently flexible to evaluate their mechanical properties, in which glycerol had a more elastic behavior with an elongation at a break of 920% and tensile strength (TS) of 0.94 MPa, while sorbitol exhibited a more plastic behavior with an elongation at break of 511% and a TS of 4.41 MPa. Water-vapor transmission rate was higher in glycerol, with 194.41 $g \cdot m^{-2} d^{-1}$, while sorbitol had 44.27 $g \cdot m^{-2} d^{-1}$ but presented blooming. This could be due to low interaction between sorbitol and the protein matrix, correlating with the film-solubility results. Amide I band of the Fourier transform infrared (FT-IR) spectra demonstrated higher denaturation and loss of alpha helical structure in glycerol film, followed by maltitol/sorbitol, xylitol, mannitol, and the control film. This in accordance with thermogravimetric analysis (TGA) results. The results of this study prove that only glycerol and sorbitol are suitable to obtain a see-through flexible film.

Keywords: edible films; packaging materials; biopolymers

1. Introduction

An increasing concern in the last years due to the excessive waste caused by synthetic packaging materials, resulting in a major environmental problem, has led to researchers to develop environmentally friendly packaging based on biopolymers. Research activity in biodegradable films has been especially intense over the past 10 years. Edible films can be applied directly on the product surface to prevent moisture losses, gas aromas, and solute movements out of the food; even, selectively

control the exchange of gases like oxygen, carbon dioxide, and ethylene, involved in food product respiration [1]. Biopolymers derived from polysaccharides, proteins, and lipids are widely used, among which proteins are the most popular due to their mechanical and gas-barrier properties, as well as to their abundance [2]. Moreover, the great variety of functional groups that proteins contain renders it possible to alter them (enzymatically, chemically, or physically) to obtain tailored materials (such as films) for specific needs [3]. Recently, proteins from the waste seafood industry have been taking importance, due to the necessity of both diminish the amount of residues and give a more integral uses to the fishery resources.

Many fisheries generate a lot of waste, varying from 50% to 75% of the total production [4]. With a global marine production of 79.3 million t, the waste from the seafood industry can be estimated in at least 39.6 million t [5]. An inappropriate cold chain of seafood products in underdeveloped countries like Mexico, can worsen this values. One of the most important fisheries in Mexico is the jumbo squid; therefore, it is important to propose how to manage the generated waste. For this, several options can be possible; however, the use of myofibrillar proteins to made films have been considered recently [6,7].

Myofibrillar proteins can be employed for film formation if the forming solution is adjusted to a higher or lower pH than that of the isoelectric point, to obtain complete solubilization [8]. However, in order to provide biopolymers with workability, plasticizer agents are needed, with the primary role of improving flexibility. In terms of biopolymer films, their addition leads to a decrease of intermolecular forces along polymer chains, and can help improve handling and integrity, avoiding pores and cracks in the polymeric matrix [9]. Nevertheless, adverse effects such as a decrease in cohesion, which affects mainly mechanical properties, are also a possibility. Thus, a better workability of films based on biopolymers will depend on an equilibrium between the cross-linking degree of the polymer matrix and the addition of plasticizers. Therefore, different plasticizers will result in different film properties and behavior [10].

Polyols are usually used, with glycerol the most polyol studied and incorporated into hydrocolloid films [11], which may be related to its molecular weight, since it is associated with its efficacy: the smaller the molecule, the greater the plasticization effect upon the polymer matrix. Nonetheless, small molecules diffuse to the film surface, especially during extended storage, resulting in film brittleness. Moreover, due to hydroxyl groups, the hydrophilicity of the plasticized films could be incremented [12].

It must be noted that the use of a biopolymer film for a certain purpose will depend on several features including cost, availability, functional attributes, mechanical properties (strength and flexibility), optical quality (gloss and opacity), barrier requisites (water vapor, O_2 and CO_2 permeability), structure resistance to water, and sensorial acceptance, among others. The aforementioned features will also be affected by the plasticizer used.

Even though squid protein films have previously been elaborated/synthesized, only glycerol was used as plasticizer agent [6,7]. Therefore, it is necessary to establish the effect on film behavior and how properties are affected when different plasticizers are used. In this work, the effect of glycerol, sorbitol, maltitol, D-mannitol, and xylitol (Figure 1) on the optical and mechanical properties of jumbo-squid protein film were studied. The purpose was to observe changes in the films' behavior and to determine the most appropriate according to the material's final application.

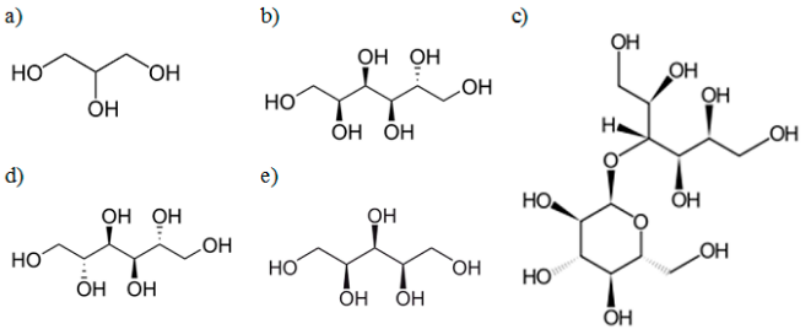

Figure 1. Chemical structure of the plasticizers used: glycerol (**a**); sorbitol (**b**); maltitol (**c**); mannitol (**d**) and xylitol (**e**).

2. Materials and Methods

2.1. Raw Material

Frozen (−20 °C) jumbo squid (*Dosidicus gigas*) was commercially obtained at a local fish market (Álvarez Fish Market, Hermosillo, Sonora, Mexico). The mantles were placed in plastic bags and stored at the Laboratory of Conservation and Processing of Marine Products, University of Sonora, Mexico, and were stored at −20 °C until their utilization.

2.2. Muscle Protein Extraction

An acid protein concentrate was obtained following Cortes-Ruiz et al. [13] methodology. First, the muscle was homogenized in a ratio of 1:5 (mantle:water) with cold distilled water by employing a tissue homogenizer. Then, pH was adjusted at 3 (HCl 3M) and the solution left for 30 min/4 °C under stirring. The homogenate mantle was then centrifuged at 15,000× *g* for 20 min at 4 °C using a refrigerated centrifuge (Sorvall stratos, Biofugue, Thermo Fisher Scientific, Waltham, MA, USA). The soluble fraction was collected, and pH adjusted to 5.5 (NaOH 10 M). The solution was centrifuged again, the supernatant discarded, and the precipitate (protein concentrate) collected.

2.3. Film Elaboration

Film-forming solutions (15% protein concentrate *w*/*w*) were prepared as described by Blanco-Pascual et al. [6] with minor modifications. The pH of the solution was adjusted to 3 ± 0.05 with 3 M HCl before it was stirred gently during 12 h at 5 °C. Plasticizer was added as follows: glycerol 40%, sorbitol 30%, maltitol 20%, mannitol 20%, and xylitol 20%. A film without plasticizer was made as a control; however, due to its brittle nature, only the attenuated total reflectance (ATR-FTIR, Perkin Elmer, Model Spectrum GX, Washington DC, USA) test could be performed. The plasticizer percentage, and plasticizer-protein ratios were established according to the literature reported, as well as, the preliminary trials. The ratios that produced the more flexible, and transparent films were selected. Aliquots were then cast into plates and left for 23 h at 5 °C and 85% relative humidity (RH) prior to drying (to ensure all samples have the same humidity) in an oven at 50 °C/23 h. All films were left in a glass chamber at 25 °C/2 days prior to analysis. Film thickness was measured using a micrometer (Quick mini micrometer Mitutoyo 700-118-20, Kawasaki, Japan); the average of five measurements was taken as the thickness value.

2.4. Light Transmittance and Transparency

Films were cut into strips (4 cm × 1 cm) and placed on the inside wall of a plastic cuvette (1 cm). The ultraviolet (UV) and the visible light barrier of the films were measured at between 200 and 800 nm. Transparency was calculated by the following Equation:

$$\text{Transparency} = A_{600}/x \quad (1)$$

where A_{600} is absorbance at 600 nm and x is the film thickness (mm) [14].

2.5. Tensile Strength and Elongation Percentage

Tensile strength and percentage of elongation at break were evaluated using a texture analyzer TA-XTplus (Food Technology Corp., Sterling, WV, USA) with a load cell of 5 kg based on the ASTM D-882-91 standard method (1996) [15]. Films were cut into strips (2.5 cm × 8.5 cm) and conditioned in a glass chamber at 25 °C/2 d/65% RH. The glass chamber conditions were selected during the preliminary stages of this work in order to diminish the brittle nature of the films. Before testing, strip thickness was measured at 5 points (in the film area). Force and distance were recorded during the extension at 2 mm/s up to the breaking point. Tensile strength and percentage of elongation were calculated as follows:

$$TS = F_m/A \quad (2)$$

$$E = (d_r/d_o) \times 100 \quad (3)$$

where TS is the tensile strength (MPa), F_m is the maximal force (N), A is the area of film cross-section (thickness × width: m^2), E is the elongation (%), d_o is the distance onset of separation (cm), and d_r is the distance of rupture (cm).

2.6. Water Solubility

Water solubility was determined using film circumferences of 4 cm in diameter, which were placed in containers with 50 mL distilled water at 25 °C/24 h. The solution was filtered through filter paper (Whatman #1), and the remaining undissolved film was desiccated at 100 °C/24 h. The weight of solubilized dry matter was calculated by subtracting the weight of the insolubilized dry matter from the initial weight of the dry matter and expressed as a percentage of the total weight [6,16].

2.7. Water Vapor Transmission Rate (WVTR)

WVTR was measured following standard test methods for the water-vapor transmission of materials (ASTM E96/E96M-05 [17]). The films were sealed onto circular test cups containing anhydrous calcium chloride (0% RH). Then, they were placed in a glass chamber at 45% RH and maintained at 25 °C. Weight changes were recorded daily for 10 days. *WVTR* was calculated with the following equation:

$$WVTR = \Delta m/\Delta t A \quad (4)$$

where $\Delta m/\Delta t A$ is weight gain per unit-of-time ($g \cdot m^{-2} d^{-1}$), and A is the area of the exposed film surface (m^2). Five replicates were tested for each sample.

2.8. Thermogravimetric Analysis (TGA)

The thermal stability of the films was measured in a thermogravimetric analyzer (Pyris 1 TGA, Shelton, CT, USA). The analyses were performed with an initial weight of 8 mg. Samples were set in aluminum pans and evaluated in a temperature ranging of 25–800 °C under an N_2 atmosphere. Weight loss as a function of temperature (TG) and the differential of the TG curves (DTG) were analyzed [18].

2.9. Fourier Transform Infrared Spectroscopy (FT-IR)

Possible interactions of functional groups due to different plasticizer interactions were analyzed by means of total attenuated reflectance mode. All spectra were recorded within the range between 4000 and 600 cm^{-1}, with a 4-cm^{-1} resolution [19].

2.10. Statistical Analysis

All of the experiments were run at least in triplicate. Data were subjected to analysis of variance (ANOVA), and mean comparisons were carried out by Tukey test, using software JMP ver. 8 for Windows (SAS Institute, Inc., Cary, NC, USA). The statistically significant difference was defined as $p < 0.05$. Data were presented as mean ± standard deviation.

3. Results and Discussion

3.1. Films Obtained

Five films were obtained and were referred as Gly, Sor, Man, Mal, and Xyl according to the plasticizer utilized. As shown in Figure 2, all films were similar (transparent and flexible) except for mannitol. A control film (without plasticizer) was also obtained; however, it was too brittle; therefore, only FT-IR analysis was carried out on the control film. Film thickness was controlled by the amount of film forming solution placed, ranging from 0.17 to 0.22 mm.

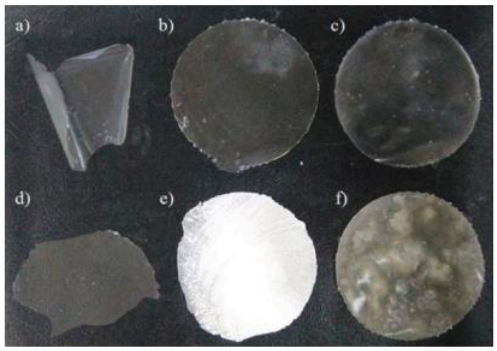

Figure 2. Protein films of jumbo squid (*Dosidicus gigas*), control (squid protein only) (**a**); glycerol (**b**); sorbitol (**c**); mannitol (**d**); maltitol (**e**) and xylitol (**f**).

3.2. Fourier Transform Infrared Spectroscopy (FT-IR)

Infrared spectroscopy is a valuable tool for the investigation of protein structure, molecular mechanism of protein reactions, as well as folding and unfolding [20]. In this case, it allows a better understanding of the films characterization. The spectra of the films are presented in Figure 3. The control film presents bands corresponding to amide A (~3300 cm^{-1}, N–H stretching) related to free water and OH signals as reported by Blanco-Pascual et al. [7], amide I (~1600 cm^{-1}, C=O stretching), amide II (~1500 cm^{-1}, out-of-phase combination of N–H bending and C–N stretching), and amide III (~1200 cm^{-1}, in phase combination of N–H bending and C–N stretching), as reported by Arfat et al. [16] were observed in all of the films. For plasticizers alone, expected spectra for alcohols were obtained, with major signals corresponding to the O-H bond at ~3000–3500 cm^{-1}, and the C–O bond at 1000–1100 cm^{-1} [8].

Amide A band, related to water in the film, appeared less defined (less round and spikier) in control and Man films, which could be related to lack of plasticizer in the control and the incapability to absorb water from the environment of mannitol. Amide-I band form refers to the presence of alpha helix (band with a little shoulder, and a position between 1648 and 1657 cm^{-1}), or beta sheet (large splitting of the band, and positions between 1623–1641 and 1674–1695 cm^{-1}) [20]. Generally, higher wave numbers (amide I) refer to a higher denaturation degree and the loss of alpha helical structure [6]. In this regard, Gly showed the highest loss of helical structure, followed by Mal/Sor, Xyl, Man, and last, the control film. This behavior could be attributed to a better protein-plasticizer interaction, since all are alcohols and bind by hydrogen bonds, which are the main stabilizing forces in secondary structures, especially alpha helix.

Regarding film vs. plasticizer patterns (data not shown), the band related to plasticizer film interactions due to C–O bonds situated around at ~1000 cm^{-1} was found in all of the films; however, it was not found in the control film, which was expected [8,16]. An increase in width in the ~3200 cm^{-1} band suggested protein-plasticizer interaction. The exception to this behavior was the Sor film, in which a weaker protein-plasticizer interaction was assumed, as seen below in the solubility test results, and also due to plasticizer blooming on the film surface during the *WVTR* evaluation. Finally, it must be noted that the amide III band (NH stretching vibration) was not present in plasticized films, while it was present in the control film. This latter behavior was reported by Limpan et al. [8] in myofibrillar films, in which the addition of the plasticizer caused the disappearance of the band, suggesting a major change in the protein-matrix arrangement.

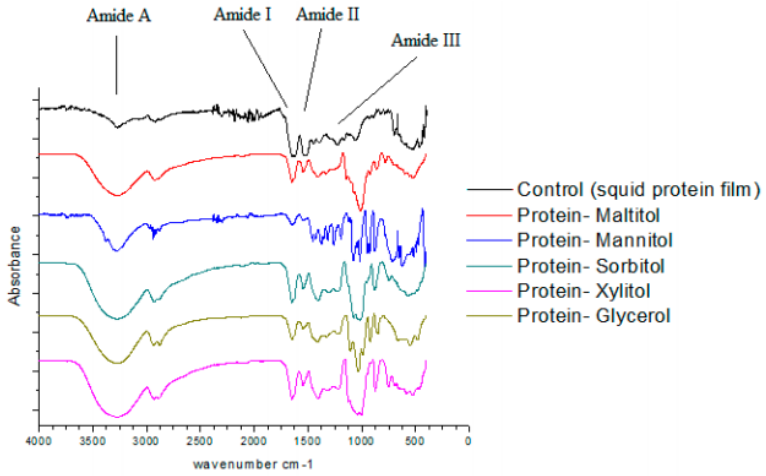

Figure 3. FT-IR spectra of the protein films of jumbo squid (*Dosidicus gigas*), control (only protein) and with the addition of different plasticizers.

3.3. Thermogravimetric Analysis (TGA)

Different polymers decompose over different ranges of temperature, releasing some volatiles and leaving some residues. Thermogravimetric analysis is a useful technique for recording weight loss or weight retained of a test sample, which may then be used to stablish the thermal stability of the material. The thermal stability curves are depicted in Figure 4. Two weight loss stages were observed for all samples. The first was observed immediately after the temperature increase, ending at ~100 °C and associated with water elimination from the sample [18]. The second weight loss, which occurred between 200 and 300 °C, was due to sample degradation (except for the glycerol, which started at ~150 °C), namely, progressive deamination, decarboxylation, and depolymerization arising from the breaking of polypeptide bonds [21]. This range is to be expected for myofibrillar proteins according to Rocha et al. [22].

Temperature derivative curves were obtained to clearly appreciate the film-degradation temperature. Gly film had the lowest value, while the control film had the highest, which was presumable due to the lack of plasticizer, thus a higher degree of protein-protein interactions. Man was also expected to show a high temperature and was followed by Sor. This is in accordance with the behavior later observed in solubility and mechanical tests, where Gly demonstrated higher interaction with the protein matrix in comparison with the other plasticizers, observed on the strong films obtained with Sor. The results are also in accordance with the temperature stability of the plasticizers alone, as reported in the literature [23] with Gly as most thermolabile and mannitol, the least, with Xyl, Sor, and Mal in-between. Thus, the thermal stability of plasticizer and the interaction of protein-plasticizer determines the film-degradation temperature, which will decide its future application.

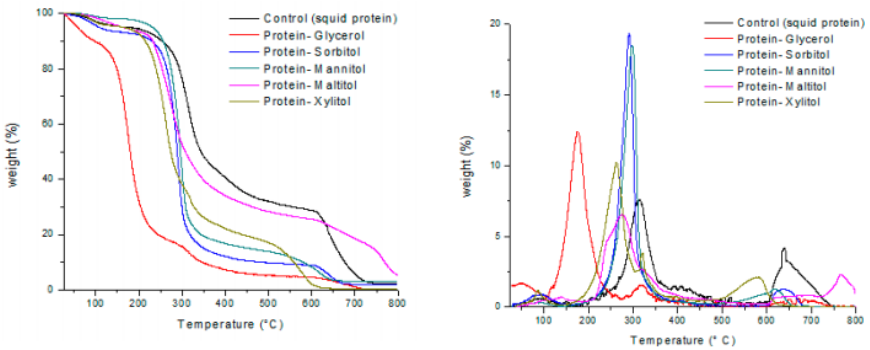

Figure 4. (**a**) TGA thermograms (TG) and (**b**) temperature derivative (DTG) thermograms of the protein films of jumbo squid (*Dosidicus gigas*) with different plasticizers.

3.4. Light Transmittance and Transparency

Film transparency is an important feature of packaging materials. A polymer film with a light transmission rate higher to 90% (600 nm) is transparent to the eye [24] The chemical structure as well as the molecular weight of the material are related to the color and transparency of a polymer, which are a consequence of its morphology. For a food-coating or packaging application, these characteristics are very important in that high clarity is often desirable. Light transmittance as well as transparency are presented in Table 1. In all of these cases, the films demonstrated low transmission to light in the UV spectrum, rendering them suitable for preventing food-component oxidation. This behavior was assumed to be related to the presence of aromatic amino acids. Contrariwise, synthetic materials such as PVC films [2] usually have elevated values ranging from 12.06 at 200 nm, to 91.85 at 800 nm, higher than those obtained in this work.

Table 1. Light transmittance and the transparency of protein films from jumbo squid (*Dosidicus gigas*) with different plasticizers.

Film	Light Transmittance (%)								Transparency
	200	250	350	400	500	600	700	800	
Gly	0.0 ± 0.0^{b}	0.3 ± 0.2^{ab}	51.9 ± 3.3^{a}	61.8 ± 3.7^{a}	66.1 ± 2.9^{a}	71.0 ± 3.9^{a}	71.9 ± 3.9^{a}	72.4 ± 4.0^{a}	0.5^{c}
Sor	0.0 ± 0.0^{b}	1.0 ± 0.7^{a}	22.7 ± 2.4^{b}	26.8 ± 3.4^{b}	29.6 ± 3.7^{b}	30.9 ± 3.9^{b}	31.8 ± 4.0^{b}	32.3 ± 4.1^{b}	0.7^{b}
Mal	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	44.5 ± 16.1^{a}	52.4 ± 17.4^{a}	57.5 ± 18.1^{a}	59.2 ± 18.3^{a}	59.6 ± 18.5^{a}	60.1 ± 18.5^{a}	0.5^{c}
Man	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.8 ± 0.2^{c}	0.9 ± 0.2^{c}	1.2 ± 0.3^{c}	1.4 ± 0.4^{c}	1.6 ± 0.5^{c}	1.7 ± 0.6^{c}	9.8^{a}
Xyl	0.3 ± 0.1^{a}	0.6 ± 0.2^{ab}	36.6 ± 5.0^{ab}	44.0 ± 6.3^{ab}	49.9 ± 6.9^{ab}	52.3 ± 7.1^{ab}	54.0 ± 7.1^{ab}	55.0 ± 7.0^{ab}	0.1^{d}

The values are the means of three determinations ± the standard deviation. Different letters in the same column show significant differences ($p < 0.05$). The means of ^{a}, ^{b} and ^{ab}: The letters are for comparison in the column, which means that for example, in the 200 column, Mal is different from Xyl; and in the 250 column Gly is similar to both, Sor and Mal, however Sor and Mal are different from each other.

Similar behavior was obtained for transparency: squid protein films presented values lower than 1 (except Man), meaning that they are more transparent than films such as PVC that have a value of 3.95, since higher value means lower transparency of the film. On the other hand, when compared with other protein films, those with sorbitol as plasticizer were similar to those reported by Blanco-Pascual et al. [7], who found a value of 0.6 when glycerol was used as plasticizer. This could be attributed to these authors mixing sorbitol and glycerol (in equal proportions at 0.4 g/1 g of total protein concentrate), while we employed them separately. On the other hand, Arfat et al. [16] obtained transparency values of ~3.6 for films made of yellow stripe fish with glycerol as plasticizer, similar to tilapia films with ~3.3 [25], which means their films were less transparent than those obtained in the present study. This may be related to the whiteness of squid muscle, while yellow stripe and tilapia are reddish in color.

According to the results, D-mannitol is not a viable option for the elaboration of a see-through film. This is likely due to its non-hygroscopic nature; it does not attract moisture (little formation of hydrogen bonds, as seen previously on FT-IR) from the air until it exceeds 90% RH, which explains the appearance of the films: dehydrated, and with the formation of something resembling filaments. The remaining four plasticizer agents do not exhibit any drawback to this point; thus, the rest of the tests were performed without utilizing mannitol.

3.5. Film Solubility in Water

Since the film could protect food from water, film solubility comprises an important property. In addition, certain types of applications may require films to be soluble, so in order to choose and appropriate application, evaluation of this value must be considered. As shown in Figure 5, only Gly demonstrated a significant difference ($p < 0.05$) in solubility. Sor and Xyl films showed a blooming effect and high solubility values. This could be due to lower protein-plasticizer interaction, perhaps related with the similar orientation of OH groups in their structure (mainly toward one side of the molecule, unbalanced), which could impede them from forming a structured network with the protein.

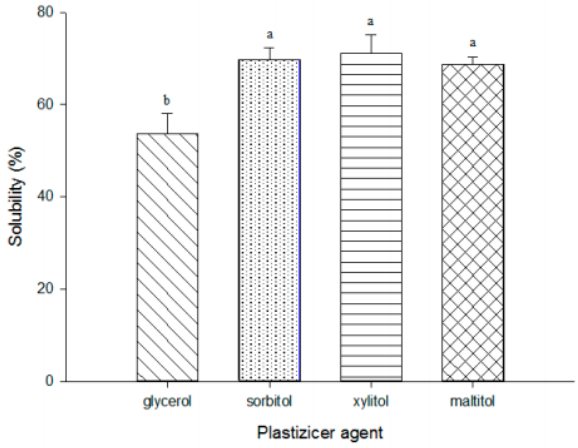

Figure 5. Water solubility of protein films from jumbo squid (*Dosidicus gigas*) with different plasticizers. The values are the means of three determinations ± the standard deviation. Different letters show significant differences ($p < 0.05$).

A slightly higher solubility value was observed in the Xyl film. To discard protein solubility as a possible cause, protein content in the filtered water (data not shown) was measured and revealed no significant differences with Sor and Mal; therefore, it was assumed that the plasticizer was migrating out of the film. This could be related to a higher protein-protein interaction resulting in less interaction with the plasticizer. Thus, its migration may be related with Xyl film behavior, it was the only one to become brittle across time, even when flexible at the beginning.

Similar results have been reported in studies conducted with proteins of marine origin. In this respect, Blanco-Pascual et al. [6], working with myofibrillar protein from jumbo squid (*Dosidicus gigas*), obtained a solubility of ~42%, lower in comparison to the results of this research. However, these authors worked with a mixture of glycerol/sorbitol. In a research with muscle protein from yellow stripe (*Selaroides leptolepis*) and glycerol as plasticizer, a solubility of ~36% was found [16], while in whitemouth-croaker (*Mocropogonias furnieri*) films, also with glycerol, a value of ~26% was reported [26]. These differences may be due to the different species from which protein was extracted, but the type and percentage of plasticizer added must also be accounted for, since it will significantly influence the results (as seen in this work).

3.6. Mechanical Properties

The chemical structure of the film is directly related to its mechanical properties, which are of importance since they reflect the durability and ability of the material to be used, said as a wrapping or coating. Usually *TS*, elongation at break, and Young's modulus are measured. Supposedly, elongation values could determine the film application; however, high *TS* is always required [27]. The use of xylitol and maltitol as plasticizer resulted in the formation of brittle films that yielded no results during the tests; thus, only Gly and Sor films were analyzed (Figure 6). The data show that Gly films were more elastic, while Sor films were stronger, in agreement with Young's-modulus values (data not shown), which were higher for Sor (stiffer than gly). These results agree with those reported by Blanco-Pascual et al. [7] for an acidic protein film from jumbo squid with glycerol as plasticizer, which had TS values of 0.9 MPa. Similarly, Arfat et al. [16], working with yellow-stripe muscle to obtain protein films, reported values of 10.2 and 7.97 MPa for 30% and 50% of glycerol, respectively. On the other hand, Zavareze et al. [26] found a TS of 5.76 MPa for protein films from whitemouth croaker, a similar result to that obtained in the present investigation using sorbitol as plasticizer; nevertheless, those authors reported 102.6 as elongation-at-break percentage, which is fairly lower than our results. Something similar occurs with materials such as hydroxy propyl methyl cellulose (HPMC), for which Mahadevaiah and Singh [27] reported *TS* values of 10.01 MPa. However, squid protein films have higher elongation than HPMC films, which barely reached 38%.

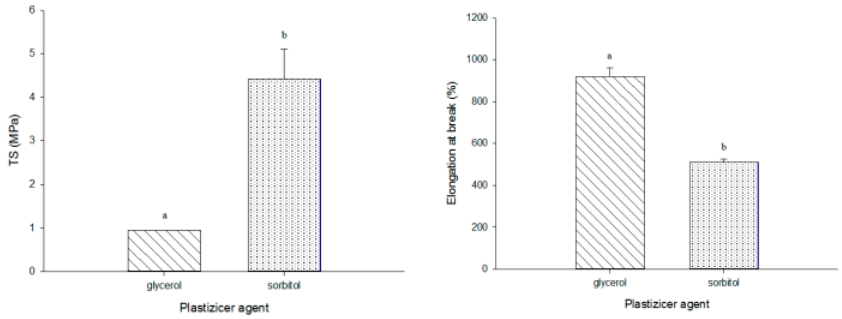

Figure 6. (**a**) Tensile strength and (**b**) elongation at break of protein films from jumbo squid (*Dosidicus gigas*) with glycerol and sorbitol as plasticizers. The values are the means of 12 determinations ± the standard deviation. Different letters show significant differences ($p < 0.05$).

The films produced in the present work showed more elastic behavior for Gly and more plastic for Sor, which is somehow in accordance with the solubility results. In addition, as shown below by the *WVTR* test, blooming was present in Sor films, suggesting its migration to the surface. This could be attributed to higher protein-protein interaction for Sor films, with migration out of the matrix, resulting in a stronger film with plastic characteristics.

3.7. Water-Vapor Transmission Rate (WVTR)

One of the most important functions of a packaging is to act as a barrier that separates and protects the product from exposure to the environment. Water can enhance the rate of reactions as browning, lipid oxidation and enzyme activity, even the rate of micro-organism growth and cause texture changes. Thus, controlling water permeability is of great importance in the development of edible films and in the selection of their future uses [28] Protein films are associated with high water-vapor permeability, caused by the high content of hydrophilic groups in their structure, as well as significant amounts of hydrophilic plasticizers [8,29]. *WVTR* refers to the rate of water vapor permeating through the film. The results (Figure 7) reveal that Sor films were less permeable than Gly films; however, at the end of the experiment, blooming of the plasticizer could be observed on the film surface, while Gly films did

not exhibit this effect. This, as mentioned above, could be due to the low interaction between sorbitol and the protein matrix, which correlates with the film solubility results, and could be explained by observing the plasticizers structures (Figure 1). Glycerol is a small molecule (3C) with three OH groups oriented in the same direction, which makes diffusion between protein chains easier. Contrariwise, sorbitol is bigger (6C) and has its OH groups oriented in different directions (mostly to one side), which is why it may be more difficult for it to diffuse, leading to a tighter protein structure, consequently rendering the Sor film less permeable.

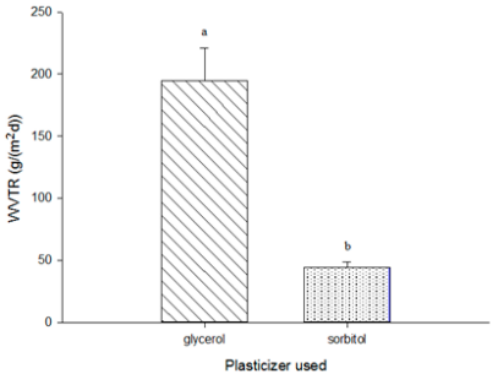

Figure 7. Water-vapor transmission rate of protein films from jumbo squid (*Dosidicus gigas*) with glycerol and sorbitol as plasticizers. The values are the means of five determinations ± the standard deviation. Different letters show significant differences ($p < 0.05$).

Other studies on biopolymer films have reported similar values to those obtained in this work. Potato- peel waste films with glycerol as plasticizer have reported values of 113.36 $g \cdot m^{-2}d^{-1}$ (at RH 57%) [30]. In another work, commercial polylactide acid films (PLA), as well as those coated with sodium alginate-chitosan, were used (also with glycerol), obtaining values of 53 and 106 $g \cdot m^{-2}d^{-1}$, respectively (at RH 75%) [31]. Protein and polysaccharide films contain hydrophilic groups, thus higher *WVTR* is to be expected in comparison to commercial films such as PLA. However, when compared with other protein films, the values differ considerably. Schmid [32] reported a *WVTR* of 450 $g \cdot m^{-2}d^{-1}$ (at RH 50%) for a whey protein- isolate film. This behavior could be related either to different test conditions, different protein sources or to the type and quantity of the plasticizer, which was glycerol at 66%. Nonetheless, in this research, when sorbitol was used as plasticizer, the value decreased considerably. The results suggest that, when low *WVTR* are required, sorbitol could be employed as instead of glycerol; and, even though the values are still far from the level required for dry food (0.136 $g \cdot m^{-2}d^{-1}$), they are comparable to polyamide films (40 $g \cdot m^{-2}d^{-1}$ at RH 75%), commonly used in food packaging [33].

4. Conclusions

In this work, the capacity of jumbo squid protein to form flexible films with different polyols as plasticizer agents has been proven. However, glycerol and sorbitol were more adequate plasticizers than maltitol, xylitol, and mannitol, which were discarded as the experiment progressed. The protein-plasticizer interaction in the films added with sorbitol and xylitol were weaker compared to the remainder of the plasticizers. This behavior was confirmed by the migration of the plasticizers to the surface of the film during the solubility test and a connection with the OH-group orientation on their structure structureould be hypothesized, however this cannot be proven with the analysis done in this work. On the other hand, mannitol did not show compatibility with the protein, resulting in a film with dry and filamentous appearance. Therefore, only glycerol and sorbitol are suitable to obtain a see through flexible film of myofibrillar protein from jumbo squid.

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CAPÍTULO 3

Development, characterization and stability of blend films based on squid protein (*Dosidicus gigas*) and whey protein concentrate

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Development, characterization and stability of blend films based on squid protein (*Dosidicus gigas*) and whey protein concentrate

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Abstract

Proteins are highly studied due to their abundance and good film forming capacity, and the continuous search for performance improvement has led among other things to mix different proteins in a single film. However, due to their nature, aging of the material caused by molecular changes is expected within short periods of time, resulting in modifications in their properties. In this work, squid myofibrillar proteins and whey protein were used to develop a protein film and evaluation of functional properties during a 3 months storage was carried out. Transparent, flexible films were obtained, and water vapor transmission rates reduced to 18.9 $g.m^{-2}$ d^{-1} in sorbitol films (44.2 $g.m^{-2}$ d^{-1} if only squid protein is used) with blooming reduction. Moreover, FT-IR spectra shows a change from α-helix to β-sheets in Amide I band, which suggest important changes in the film matrix. During storage, UV barrier of films remained stable, and transparency had an increment over time. Plasticizer leaking was observed during storage, which was in accordance with FT-IR spectra, in which Amide A band showed diminishing in hydrogen bonds, related to higher degree of protein-protein interactions, and thus less interaction with the plasticizer.

Keywords

squid protein, whey protein, blend, biopolymer film, stability

1. Introduction

Consumers demand for environment friendly packaging has increased in recent years, and thus has increased the necessity to look for new materials, among them edible biofilms. Raw materials from agricultural origin (renewable) have been proposed as eligible sources, being proteins the most popular, due to their nutritional value and the possibility to make a tailored material, since their functional groups could be modified by means of enzymatic, chemicals or physical methods, varying and adjusting their performance and functional properties (Gómez-Estaca et al., 2016).

In recent years fishery industry has gained importance as a source for raw material, with a waste generation up to 75% of the total production, from which gelatin and myofibrillar proteins are the most usual for film development (Mekpiroon et al., 2016). However, myofibrillar proteins produce rigid films, mainly due to the presence of covalent bonds, but also due to ionic bonds (myofibrillar proteins main component, myosin, is a highly charged protein) and hydrophobic interactions. Moreover, the hydrophilic nature of amino acids and plasticizer agents makes them prone to have poor water vapor barriers (Prodpran et al., 2007). Giant squid (*D. gigas*) has previously been used as raw material to obtain protein films (Blanco-Pascual et al., 2014; Murrieta-Martínez et al., 2019) and the typical behavior reported for such materials was observed: high solubility, and poor water vapor barriers. Improvement of such properties can be achieved by mixing different protein sources. In this work addition of whey protein was suggested. Whey is a by-product of the cheese elaboration process, in which only ~60% of the total production is processed and the rest is discarded (Guimarães et al., 2010). Films and coatings have been successfully developed from whey proteins, from isolates (WPI) and concentrates (WPC), with positive results, resulting in transparent and flexible films with lack of odor or color (Ramos et al., 2013).

Due to their composition, it is expected that molecular changes and reorganization take place over time. These changes are caused by the intrinsic instability of their components, and can affect their functionality (Osés et al., 2009). There are few studies regarding protein films stability, but it has been reported that fish muscle protein-based films tend to show yellow discoloration during extended storage, mainly attributed to nonenzymatic browning or Maillard reaction (Artharn et al., 2009; Limpan et al., 2010; Tongnuanchan et al., 2011a).

While for whey protein films it is been reported that even though there is an increment in stress at break values, barrier properties can remain unaffected up to 120 days (Anker et al., 2001).

It must be mentioned that compatibility should not be a problem, since both proteins have isoelectric points near 5, and both can be solubilized in either alkaline or acid conditions, as well as undergo thermal treatments, which in whey protein case are highly desirable for their functionality. Thus, the purpose of this work was to obtain a film using giant squid myofibrillar proteins and whey proteins to improve the performance of squid protein films and evaluate their stability during storage.

2. Materials and methods

2.1 Raw material

Jumbo squid (*Dosidicus gigas*) was obtained frozen (-20°C) from a local fish market (Álvarez Fish Market, Hermosillo, Sonora, Mexico). The mantles were stored at -20°C in plastic bags until their utilization at the Laboratory of Conservation and Processing of Marine Products, University of Sonora, Mexico. Whey protein concentrate (80 % of protein, dry basis) was obtained from Fabpsa, Industrias Alimentarias Fabp, S.A. de C.V. (CDMX, México)

2.2 Film elaboration

Squid myofibrillar protein solutions (15% protein concentrate w/w) were prepared following the methodology reported by Blanco-Pascual et al. (2013) with minor modifications. The solution pH was adjusted at 3 ± 0.05 using HCl and was stirred gently during 12 h at 5 °C. Whey protein concentrate solution (10 % w/w) was made as reported by Schmid et al. (2012). Nine film forming solutions were made, varying on WPC concentration (5, 10 and 15 %) and plasticizer added (glycerol 40 %, sorbitol 30% and a mixture of glycerol-sorbitol (1:1)). Aliquots were then cast into plates and left for 23 h at 5 °C and 85 % relative humidity (RH) before drying at 50 °C/23 h in an oven. All films were stored in a glass chamber at 25 °C/2 days prior to analysis. Films will be referred according to Table 1.

Table 1. Films acronyms according to the percentage of whey protein concentrate added and plasticizer used.

Plasticizer	Whey Protein %		
	5	**10**	**15**
Glycerol	G5	G10	G15
Sorbitol	S5	S10	S15
Gly:Sor	GS5	GS10	GS15

2.3 Fourier Transform InfraRed spectroscopy (FT-IR)

Possible interactions of functional groups due to different plasticizer interactions were analyzed by means of total attenuated reflectance mode in an infrared spectrometer (Frontier FT-IR, Perkin Elmer, MA). All spectra were recorded within the range between 4000 and 600 cm^{-1}, with a 4-cm^{-1} resolution (Sockalingam & Abdullah, 2015).

2.4 Thermogravimetric Analysis (TGA)

Thermal stability of the films was measured in a thermogravimetric analyzer (Pyris 1 TGA, Shelton, CT). An initial weight of 8 mg was used to perform the analyses. Samples were set into aluminum pans and evaluated from 25-800°C under an N_2 atmosphere. Weight loss as a function of temperature (TG) and the differential of the TG curves (DTG) were analyzed (Nascimento, Calado, & Carvalho, 2012).

2.5 Tensile strength and elongation percentage

Tensile strength and percentage of elongation at break evaluation was performed in a texture analyzer TA-XT2 (Food Technology Corp., Sterling, Virginia) with a load cell of 5 kg according to the ASTM D-882-91 standard method (1996). Strips (6 x 1 cm) were cut from the film and kept in a glass chamber at 25 °C/2 d/65 % RH. Strip thickness was measured at 5 points (in the film area). Extension was at 2 mm/s up to the breaking point while force and distance were recorded. Tensile strength and percentage of elongation were calculated with the equations:

$$TS = F_m/A$$

$$E = (d_r/d_o) *100$$

Where TS is the tensile strength (MPa), F_m is the maximal force (N), A is the area of the film cross-section (thickness x width: m^2), E is the elongation (%), d_o is the distance onset of separation (cm), and d_r is the distance of rupture (cm).

2.6 Light transmittance and transparency

Strips (4 x 1 cm) were cut from the films and placed on the inside wall of a plastic cuvette (1 cm). Ultraviolet (UV) and visible light barrier of the films were measured between 200 and 800 nm. Transparency was calculated by the following equation:

$$Transparency = A_{600}/x$$

Where A_{600} is absorbance at 600 nm and x is the film thickness (mm) (Shiku, Hamaguchi, & Tanaka, 2003).

2.7 Water solubility

Water solubility was determined in containers with 50 mL distilled water where film circumferences of 4 cm in diameter were placed and kept at 25 °C/24 h. The solution was vacuum filtered using filter paper (Whatman #1), and the undissolved film dessicated at 100°C/24 h. Solubilized dry matter weight was calculated by substrating the insolubilized dry matter weight from the initial dry matter weight and expressed as a percentage of the total weight (Blanco-Pascual et al., 2013; Arfat et al., 2014).

2.8 Water Vapor Transmission Rate (WVTR)

WVTR was measured by the standard test method (ASTM E96/E96M-05). Films were sealed onto test cups containing anhydrous calcium chloride (0% RH) and placed in a glass chamber at 45% RH maintained at 25°C. Weight changes were recorded for 10 days and WVTR calculated with the following equation:

$$WVTR = \Delta_m/\Delta tA$$

Where $\Delta_m/\Delta t$ is weight gain per unit-of-time (g/day), and A is the area of the exposed film surface (m^2). At least three replicates were tested for each sample.

2.9 Film stability during storage

The obtained films were stored in wax paper envelopes within plastic containers under refrigeration (4 °C) in a refrigerator Torrey (VRD-42) equipped with a force air cascade system, for a period of 3 months. Every month all the previously described evaluations were performed. It must be noted that the refrigerator was opened constantly, since it was not exclusive for this study purposes.

2.10 Statistical Analysis

Experiments were run at least in triplicate. Data were subjected to analysis of variance (ANOVA), mean comparisons were carried out by Tukey test, using the software JMP ver. 8 for Windows (SAS Institute, Inc., NC, USA). Statistically significant difference was defined as $p \leq 0.05$ and data presented as mean ± standard deviation.

3. Results and discussion

Nine films were obtained with thickness ranging from 0.12 to 0.37 mm, depending on the percentage of WPC added. Appearance was that of translucent transparent films, being soft and flexible when handling. However, sorbitol films were stiffer, while glycerol and glycerol-sorbitol films were difficult to tell apart. Also, differences could not be observed at glance regarding the WPC % added.

3.1 FT-IR

Infrared spectroscopy is a technique that allows to have a general idea of the chemical structure of the materials conforming the film. The spectra of the films obtained are shown in Figure 1. The characteristic band for each plasticizer can be seen around 1000 cm^{-1}, also amide A band at 3269 cm^{-1}, they showed slight differences between plasticizers used and WPC percentage. It can be observed that when sorbitol and higher WPC concentrations were used, Amide A bands were less round, which may indicate a higher degree of crosslinking between proteins, as reported by Ramos et al. (2013) when comparing WPI and WPC films. Also, a lower width of the Amide A band was associated with a higher number of chains closer to each other promoted by more frequent hydrogen bonding, thus fewer free -OH groups available.

Moreover, when comparing Amide I band with that of a previous work in which only squid protein was used (Murrieta-Martínez et al., 2019), the band shape and wavenumber (a strong band near 1630 cm^{-1} and a weaker band near 1685 cm^{-1}) suggest a change from α-helix to β-sheets, (Barth, 2007). These results are agreement with the work of Ramos et al. (2013), in which deconvolution of Amide I band of WPC films showed eight bands that were observed within the range 1618-1683 cm^{-1}, characteristic of amide groups involved in extended β-sheets structure. This is in accordance with Amide III band, these changes could indicate a modification in secondary structure, which is mainly stabilized by hydrogen bonds.

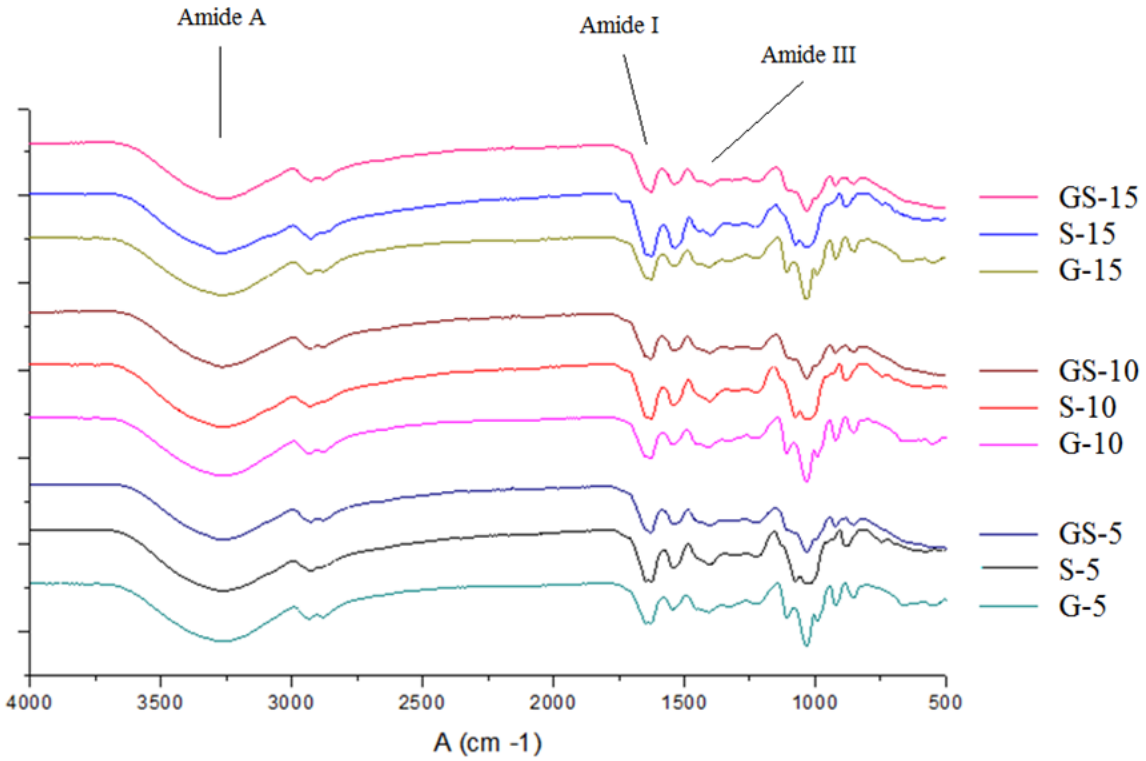

Figure 1. FT-IR spectra of the protein films of jumbo squid (*Dosidicus gigas*) with addition of whey protein concentrate and glycerol and sorbitol as plasticizers.

3.2 TGA

This technique was used in order to obtain the thermal stability of the material and the temperature at which each component is decomposed. Figure 2 shows the thermograms (a)

and temperature derivative (b) of the films. Water elimination can be observed at ~100 °C, followed by a transition from 200 to 350 °C, which according to Rocha et al. (2017) correspond to myofibrillar protein, allowing a quick look on whether there is an effect on the protein matrix. However, in our study two protein sources were used, which means whey proteins should be there too, this could be explained by their reported degradation temperatures, 319 and 313 °C, respectively. Also, in the temperature derivative of sorbitol films a single transition can be seen for both proteins, accompanied by the water and plasticizer. However, in glycerol films, myofibrillar and whey proteins are separated, with temperatures of 204-215 °C and 328-333 °C, which may correspond to myofibrillar and whey proteins respectively. Ramos et al. (2013) reported that WPC films (using glycerol) have a degradation temperature range of 280-500 °C, which is in accordance to this work results. Results are in accordance to a previous work (Murrieta-Martínez et al., 2019) in which sorbitol appeared to interact less with the protein matrix in comparison with glycerol, thus allowing a better interaction between proteins, and a higher temperature stability. Regarding WPC percentage effect, it could barely be noticed in this test.

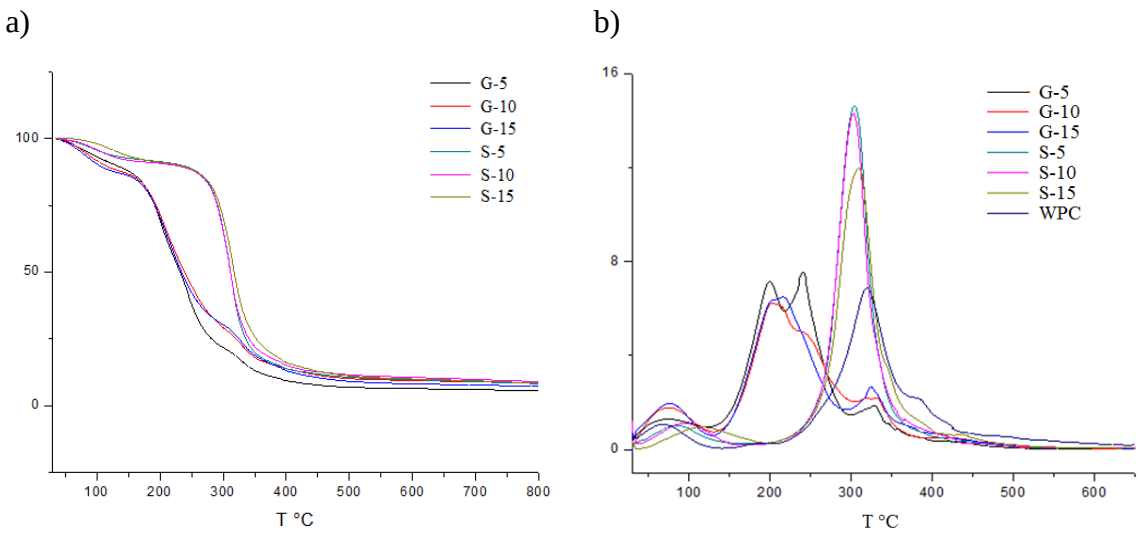

Figure 2. (**a**) TGA thermograms (TG) and (**b**) temperature derivative (DTG) thermograms of the protein films of jumbo squid (*Dosidicus gigas*) with glycerol and sorbitol as plasticizers and addition of whey protein concentrate.

3.3 Light transmittance and transparency

Properties like color, light transmission and transparency are of great importance regarding consumer acceptation, see-through films are usually preferred. Results are shown in Table 2.

As can be seen plasticizer-whey protein interaction was significative ($p \leq 0.05$), with higher transparency values when glycerol and whey in 5 or 15% were used, while in sorbitol films transparency diminished, this may be due to a higher interaction degree between proteins, as solubility and TGA results suggest. Regarding transmission, all films proved to be good UV barriers with transmission beginning at 350nm. In comparison with a previous work (Murrieta-Martínez et al., 2019), light transmission diminished when WPC was added. At 350 nm, when transmission begins, G films had values of 50, while the G-WPC films in this work have 31; the same occurs for S-WPC films values that went down to 4. This could be due to the addition of another protein source, which will increase the number of aromatic groups present in the film, to which is attributed UV light absorption and thus a minor light transmission. Blanco-Pascual et al. (2014) work with giant squid films also reported transmission starting at 350nm, and transparency values of 0.6, similar to of G-15 and GS-15 in this work, but still less transparent than some of the films obtained. For whey protein films good UV light barriers have also been reported, with transmission also beginning at 350nm, but less transparent films, with values of 1.29 (Ramos et al., 2013), which is in accordance with the diminishing in transparency values when higher WPC percentage is used

Table 2. Transparency of protein films from jumbo squid (*Dosidicus gigas*) with glycerol and sorbitol as plasticizers and addition of whey protein concentrate.

Film	WPC%	Transparency
Glycerol	5	0.52 ± 0.05^{ab}
	10	0.39 ± 0.07^{a}
	15	0.67 ± 0.07^{b}
Sorbitol	5	1.83 ± 0.16^{de}
	10	1.91 ± 0.18^{e}
	15	1.7 ± 0.16^{d}
Gly:Sor	5	0.5 ± 0.08^{ab}
	10	1.34 ± 0.04^{c}
	15	0.67 ± 0.02^{b}

The values are the means of three determinations ± the standard deviation. Different letters show significant differences ($p \leq 0.05$).

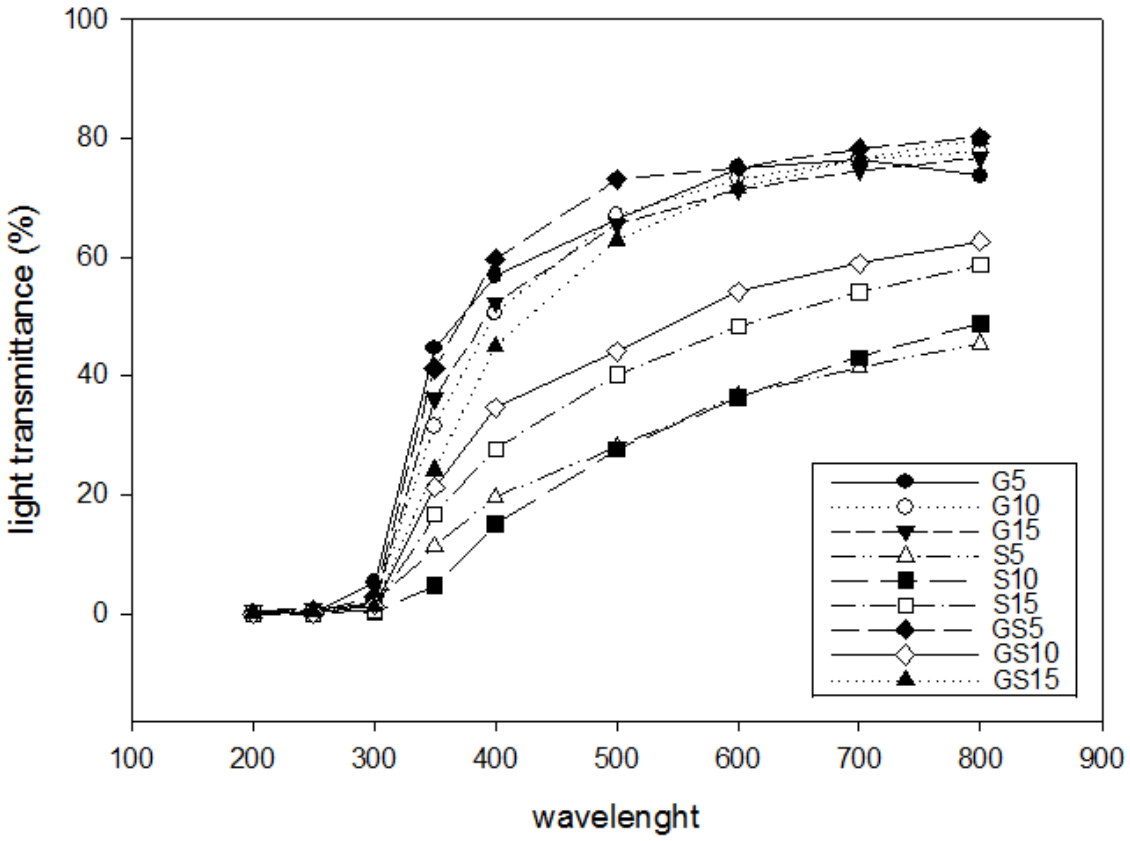

Figure 3. Light transmittance of protein films from jumbo squid (*Dosidicus gigas*) with glycerol and sorbitol as plasticizers and addition of whey protein concentrate.

3.4 Film Solubility in Water

Film solubility was performed due to possible film applications in the future but also because values are affected depending of the packaging of the protein matrix, which is supposed to be tightly packed due to whey protein addition. Values are shown on Table 3, with the highest value for G-5 and the lowest for S-15. However, solubility values are higher to those of only squid protein films (53 %), which is logic due to addition of another protein source, (hydrophilic nature). Still, comparing with Ramos et al., (2013) who reported a solubility value of 78 % for a WPC protein film with glycerol as plasticizer, G-15 film value is the closer, and it is the less soluble of the glycerol films, thus WPC percentage had a positive effect on film solubility, and could be appreciated more clearly in sorbitol.

Table 3. Water solubility percentage of protein films from jumbo squid (*Dosidicus gigas*) with glycerol and sorbitol as plasticizers and addition of whey protein concentrate.

Plasticizer	WPC %		
	5	10	15
Glycerol	83.9 ± 0.34^{bA}	80.4 ± 0.57^{bB}	74.2 ± 0.95^{bC}
Sorbitol	77.3 ± 3.94^{aA}	72.6 ± 0.26^{aB}	68.9 ± 1.12^{aC}
Gly:Sor	81.3 ± 0.91^{bA}	80 ± 1.06^{bB}	74.3 ± 0.99^{bC}

The values are the means of three determinations ± the standard deviation. Different letters show significant differences ($p \leq 0.05$). Lower case for plasticizer and capital case for WPC %.

3.5 Water vapor transmission rate (WVTR)

Water permeability values are an important factor when developing protein films, which are known for being poor barriers due to the hydrophilic nature of their amino acids, and a way to improve this behavior is adding another polymer to the formulation. Table 4 shows the values of water transmission rate, in which a positive effect of WPC addition can be seen, with lower values when WPC percentage was higher.

Table 4. Water vapor transmission rate of protein films from jumbo squid (*Dosidicus gigas*) with glycerol and sorbitol as plasticizers and addition of whey protein concentrate.

Plasticizer	WPC %		
	5	10	15
Glycerol	191.1 ± 14.1^{de}	211.4 ± 5.2^{e}	177.7 ± 9.6^{d}
Sorbitol	30.6 ± 4.7^{a}	39.4 ± 2.7^{a}	18.9 ± 1.3^{a}
Gly:Sor	150.3 ± 8.3^{c}	150.9 ± 6.5^{c}	85.0 ± 3.12^{b}

The values are the means of three determinations ± the standard deviation. Different letters show significant differences ($p \leq 0.05$).

A considerable reduction could be achieved in sorbitol films, in comparison with those made only with myofibrillar protein (Murrieta-Martínez et al., 2019, with transmission rates going from 44.2 $g.m^{-2}d^{-1}$ down to 18.9 $g.m^{-2}d^{-1}$. The reduction of WVTR values may be due to a

more tightly packed protein matrix, as reported by Ramos et al. (2013), who reported that reduction of the interstitial spacing between molecules lead to a more compact matrix in WPI films in comparison with WPC, and had a lower diffusion rates. This may also be related to blooming diminishing in sorbitol films, due to lower amounts of plasticizer migrating out of the matrix.

3.6 Stability study

FT-IR spectra at 0 and 3 months are shown in Figure 4. After storage Amide A band, related to water presence in the film, appears to be less round and more spiky, which may indicate a reduction in hydrogen bonding, leading to water release (solubility of all films incremented over time) and thus a possible development of protein-protein cross linking. A band intensity decrease can also be appreciated, and as reported by Blanco-Pascual et al. (2014) may be caused by less α and β structures, related to an increment of aggregation over storage time. Regarding Amide III band, a change of shape can be seen for all films after storage, and it may indicate a modification of secondary structure, mainly stabilized by hydrogen bonds, which supposedly diminished due to cross linking (Amide A).

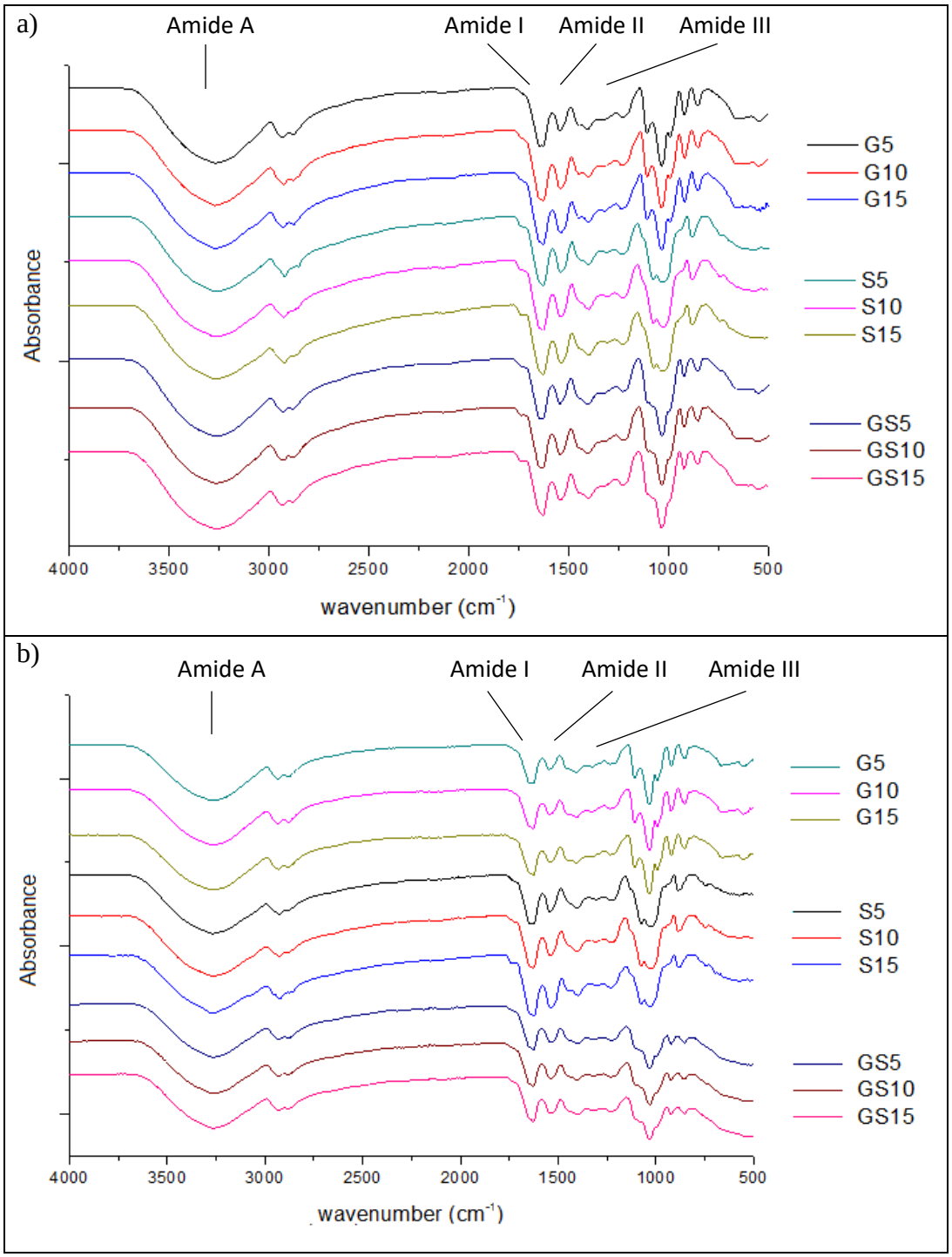

Figure 4. FT-IR comparison of protein films from jumbo squid (*Dosidicus gigas*) proteins with glycerol and sorbitol as plasticizers and addition of whey protein concentrate: a) 0 and b) 3 months of storage.

While optical properties are important from the consumer point of view, they are also related to the chemical structure of the material. Transparency is shown in Figure 5, it must be noted that films in this study were more transparent in comparison to those of *Todadores pacificus* and *Dosidicus gigas*, which may be due to whey protein addition to the formulation, because whey proteins are known for producing highly transparent films (Ket on et al., 2016). Within the nine films obtained, G films showed to be the most transparent, at either 0 or 3 months, followed by GS and S the least. Leerahawong et al. (2012) reported a significant increment during storage for *Todadores pacificus* films, which was also observed in this study, especially in S films. Blanco-Pascual et al. (2014) also reported this behavior, with transparency values diminishing approximately at half and becoming stable after four months. This behavior has been attributed to orientation of polymers in the film matrix during storage (Artharn et al., 2009).

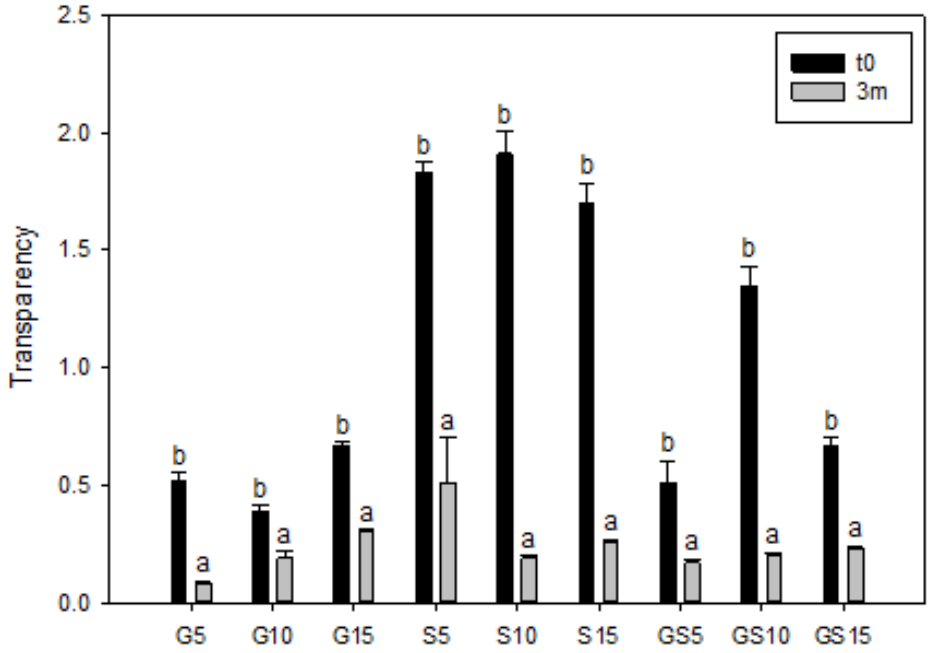

Figure 5. Transparency comparison of protein films from jumbo squid (*Dosidicus gigas*) proteins with glycerol and sorbitol as plasticizers and addition of whey protein concentrate, 0 vs 3 months of storage. The values are the means of three determinations ± the standard deviation. Different letters show significant differences ($p \leq 0.05$).

Regarding UV barrier property all films presented a stable behavior, improving during storage (Table 5). A similar behavior was reported by Blanco-Pascual et al. (2014) in *Dosidicus gigas* films, who reported a slightly more effective UV barrier, but only at higher wavelengths after four months storage. An increment in yellowish color of the films, due to Maillard reaction was also reported and could be observed in these materials.

Table 5. Transmittance values of protein films from jumbo squid (*Dosidicus gigas*) with glycerol and sorbitol as plasticizers and addition of whey protein concentrate, 0 vs 3 months storage.

Film	Storage (months)	Wavelenght (nm)		
		200	300	400
G5	0	0.13 ± 0.12^{b}	5.21 ± 0.30^{b}	56.92 ± 0.82^{a}
	3	0.03 ± 0.0^{a}	2.75 ± 1.6^{a}	61.12 ± 7.5^{a}
G10	0	0.06 ± 0.06^{a}	1.67 ± 0.20^{a}	50.59 ± 0.57^{a}
	3	0.18 ± 0.1^{b}	2.88 ± 0.5^{b}	48.22 ± 6.1^{a}
G15	0	0.41 ± 0.06^{b}	1.38 ± 0.27^{b}	52.30 ± 0.97^{b}
	3	0.06 ± 0.1^{a}	0.34 ± 0.4^{a}	24.76 ± 3.6^{a}
S5	0	0.02 ± 0.01^{a}	1.75 ± 0.0^{b}	19.65 ± 0.51^{a}
	3	0.08 ± 0.0^{b}	0.49 ± 0.2^{a}	22.12 ± 15.4^{a}
S10	0	0.03 ± 0.03^{a}	0.23 ± 0.58^{a}	15.08 ± 0.02^{a}
	3	0.21 ± 0.2^{a}	1.30 ± 0.8^{b}	40.85 ± 12.2^{b}
S15	0	0.03 ± 0.0^{a}	0.62 ± 0.06^{a}	27.75 ± 1.88^{b}
	3	0.20 ± 0.2^{a}	0.49 ± 0.6^{a}	14.06 ± 7.5^{a}
GS5	0	0.00 ± 0.01^{a}	2.75 ± 0.0^{b}	59.66 ± 0.61^{a}
	3	0.17 ± 0.2^{a}	1.98 ± 0.6^{a}	52.13 ± 11.2^{a}
GS10	0	0.05 ± 0.0^{a}	1.45 ± 0.0^{b}	34.70 ± 0.0^{b}
	3	0.29 ± 0.5^{a}	0.64 ± 0.4^{a}	30.53 ± 1.9^{a}
GS15	0	0.15 ± 0.02^{a}	1.25 ± 0.10^{b}	44.96 ± 0.02^{b}
	3	0.13 ± 0.1^{a}	0.47 ± 0.3^{a}	25.64 ± 3.7^{a}

The values are the means of three determinations ± the standard deviation. Different letters show significant differences ($p \leq 0.05$) in time of storage.

Film solubility in water is a valuable attribute to take into account when deciding the material final use and is affected by the matrix protein arrangement. Water solubility values of the films are reported in Figure 6. There is an increment in solubility during storage in almost all the films. Similar results were obtained by Blanco-Pascual et al. (2014) in a *Dosidicus gigas* film, attributing this behavior to a certain degree of matrix disruption during storage, with

plasticizer release leading to a more rigid polymer structure, partial hydrolyzation of proteins and aggregation. This correlates with FT-IR results, especially in the bands Amide A and Amide III which indicate the presence of OH and secondary structure integrity respectively. Thus, diminishing of OH bonds leads to protein aggregation and plasticizer leaking out of the matrix, resulting in an increment in blooming.

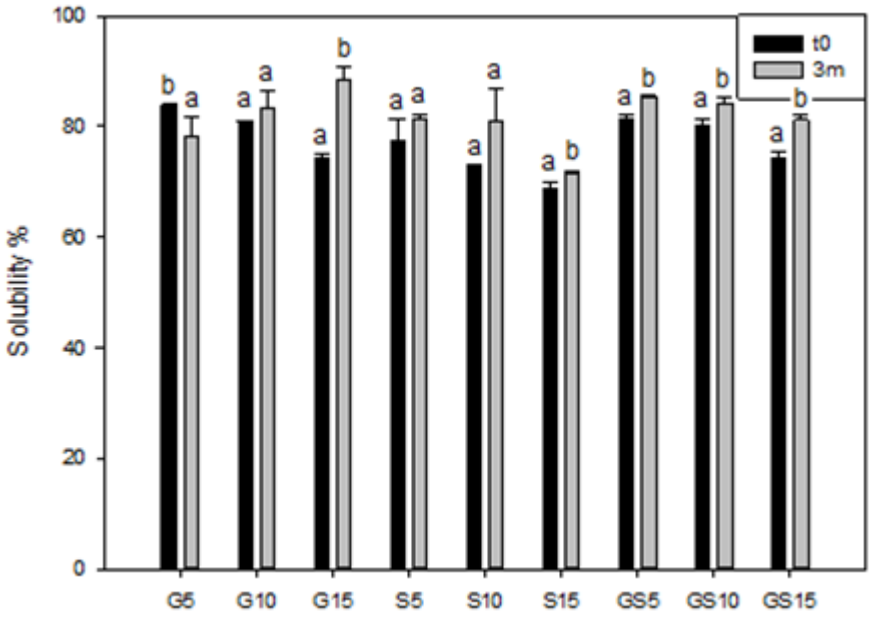

Figure 6. Water solubility of protein films from jumbo squid (*Dosidicus gigas*) proteins with glycerol and sorbitol as plasticizers and addition of whey protein concentrate, 0 vs 3 months storage. The values are the means of three determinations ± the standard deviation. Different letters show significant differences ($p \leq 0.05$).

4. Conclusion

Overall positive results had been obtained from the composite films in this work, especially regarding to water vapor transmission rate, which values were lowered and blooming diminished in sorbitol films. Also, FT-IR spectra showed a change of secondary structure from alfa helix to beta sheets (characteristic of WPC), given a more packed matrix when mixing both proteins. Stability results are in accordance to those reported for other squid protein films, with some variations attributed to the addition of whey protein into the mixture. UV barrier properties proved to be stable and transparency got higher during storage. However, changes in the matrix structure, most likely increment in protein-protein

aggregation, resulted in higher film solubility, an important property regarding their future application, with film integrity being adequate even after 3 months storage.

Therefore, myofibrillar and whey proteins as a mix are good materials to elaborate edible films with good functional properties.

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CONCLUSIONES

El llevar a cabo el presente trabajó permitió establecer la capacidad de las proteínas miofibrilares de calamar gigante (*D. gigas*) para formar un biomaterial en forma de películas, al emplear distintos polialcoholes como agentes plastificantes, así como al mezclarse con otra proteína, concentrado proteico de suero de leche.

Los resultados mostraron mayor compatibilidad al emplear glicerol y sorbitol, obteniéndose un material flexible y transparente, en tanto que el resto de los polialcoholes generaron películas frágiles, además de presentar un color amarillento y poca transparencia. Por otra parte, al adicionar suero de leche, y empleando los polialcoholes seleccionados, se obtuvieron materiales con una matriz proteica más compacta, la cual mostró cambios estructurales importantes que resultaron favorables en lo que a las propiedades funcionales del material respecta, que era lo que se pretendía, disminuyendo además el blooming.

Finalmente, durante la evaluación de estabilidad de los materiales, se observó que incluso al haber pasado tres meses en refrigeración, las propiedades funcionales en general seguían sin cambios importantes, habiendo incluso mejoras en lo referente a propiedades ópticas.

Los resultados del estudio en general sugieren que el uso de proteína de calamar adicionada con suero de leche resulta una mezcla adecuada para la obtención de películas con buenas propiedades funcionales, observándose una mejora en las mismas en comparación con el uso de solo un tipo de proteína. Además se observó buena estabilidad de los materiales obtenidos.

RECOMENDACIONES

Sería interesante una vez hecha la mezcla clamar-suero, probar con otros agentes plastificantes, ya sea otros polialcoholes o incluso la adición de ácidos grasos, los cuales además podrían tener efecto en la disminución del carácter hidrofílico característico en las películas de proteína.

Se tuvo la oportunidad de evaluar la capacidad antioxidante de las películas obtenidas, investigar mas a fondo dicha área podría ser muy beneficioso, pues no hay muchos trabajos al respecto y podría ampliar el campo de aplicación.

Finalmente, se considera que evaluar el material ya sobre un alimento es definitivamente el siguiente paso, para comprobar si realmente será capaz de desempeñarse como sugieren las propiedades encontradas en este estudio.

ANEXO (CALCULOS DE WVP – WVTR)