Centro de Investigación en Alimentación y Desarrollo, A.C.

PROPIEDADES VISCOELÁSTICAS, ESTRUCTURALES Y DIFUSIONALES DE MATRICES COVALENTES DE ARABINOXILANOS FERULADOS DE PERICARPIO DE TRIGO

POR

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TESIS APROBADA POR LA

COORDINACIÓN DE TECNOLOGÍA DE ALIMENTOS DE ORIGEN ANIMAL

COMO REQUISITO PARCIAL PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS

Hermosillo, Sonora Diciembre de 2011

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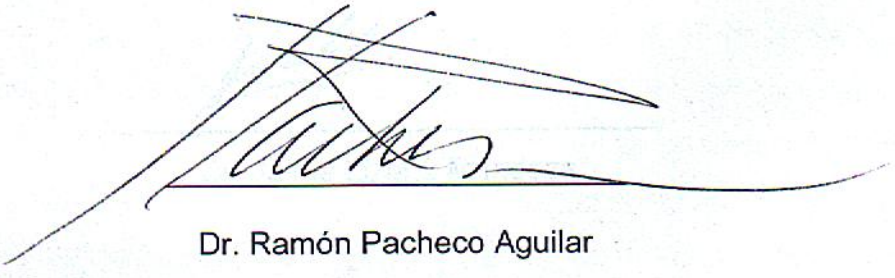

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APROBACIÓN

Los miembros del comité asignados para revisar la tesis de Claudia María Berlanga Reyes, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias.

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AGRADECIMIENTOS

Un agradecimiento al gran aporte de personas e instituciones que han facilitado las cosas para que este trabajo llegara a su feliz término. Por ello, es para mí un verdadero placer utilizar este espacio para ser justo y consecuente con ellas, expresándoles mis agradecimientos.

Al Consejo Nacional de Ciencia y Tecnología (CONACYT), por el importante apoyo económico brindado durante el tiempo que permanecí en el programa de doctorado.

Al Centro de Investigación en Alimentación y Desarrollo A.C., por abrirme sus puertas y darme la oportunidad de realizar mis estudios de doctorado y por la sabiduría transmitida de mis asesores. Muchísimas gracias de todo corazón.

Debo agradecer de manera muy especial y sincera a mi asesora, la Dra. Elizabeth Carvajal Millán, por aceptarme para realizar esta tesis doctoral bajo su dirección como también su apoyo y confianza en el mismo. Las ideas u opiniones siempre han sido enmarcadas por su orientación, lo cual ha sido clave del buen trabajo que hemos realizado juntas. Como también el que se me hayan facilitado los medios suficientes para llevar a cabo todas las actividades propuestas durante el desarrollo de la tesis.

A mis asesores la Dra. Alma Rosa Islas Rubio, el Dr. Agustín Rascón Chu y el Dr. Jaime Lizardi Mendoza, por todo el apoyo y tiempo dedicado durante mis estudios.

A la Q.B. Karla G. Martínez Robinson, M.C. Alma Rosa Toledo Guillén y Q.B. Alma C. Campa Mada (y a su maravillosa familia), por su gran apoyo técnico en el laboratorio de Biopolímeros para la realización de este trabajo.

Para mis compañeros de grupo por su apoyo y haber hecho agradable mi estancia en el proceso. Este ha sido un largo camino en el que algunas veces la fijación por lograr tus objetivos te hace olvidar la importancia del contacto humano.

A todos mis amigos que forman parte importante para mí y que conocí durante el transcurso del doctorado.

DEDICATORIAS

A Dios toda mi gratitud y amor. Él ha sido siempre mi compañero de viaje; mis planes y proyectos en sus manos deposité; siempre puso a las personas idóneas en mi camino; no hubo obstáculo que no lograra vencer; hasta a los rayos del sol ardiente de mi ya querida ciudad de Hermosillo logré adaptarme y aceptarlos con cariño.

Para mi familia el agradecimiento más profundo y sentido ya que sin su apoyo, colaboración e inspiración habría sido imposible llevar a cabo esta meta. A mis padres Ruth e Ignacio, por su ejemplo de lucha, honestidad y amor. A mis hermanos David Ignacio, Flor María y José Martín por su gran apoyo y cariño. A mi hija Sofia Valentina, el amor de mi vida..... por ellos y para ellos!

A mis tíos Fernando e Ivonne y primos Adda y Fer, por representar un gran apoyo y estímulo de superación.

A mi cuñado Jorge Grijalva: por sus valiosas aportaciones y apoyo.

A Jorge Emiliano e Isabella Grijalva: por su alegría, puntadas y cariño; quienes matizan mi vida de entusiasmo y bellos colores.

A Ana Paula, la más pequeña sobrinita que llegó hace 4 meses a inundar aún más de felicidad a nuestra familia.

A mi cuñada Vero por su cariño y apoyo, siempre escuchándole palabras llenas de esperanza y ánimo

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RESUMEN

Los arabinoxilanos (AX) son polisacáridos que constituyen una parte estructural de la pared celular de los cereales. Estos polisacáridos están formados por una cadena lineal de xilosas con ramificaciones de arabinosa, a las cuales puede estar esterificado el ácido ferúlico. La presencia de ácido ferúlico confiere a los AX la capacidad de formar geles covalentes mediante la oxidación por enzimas como la lacasa. Esta oxidación resulta en la formación de dímeros y trímeros de ácido ferúlico que permiten unir las cadenas de polisacárido entre sí formando un gel. Los geles de AX son neutros, sin color ni olor y debido a su naturaleza covalente, no son afectados por cambios de temperatura, fuerza iónica o pH, lo cual les confiere un gran potencial de aplicación como agentes de encapsulación y liberación controlada. El objetivo de esta investigación fue estudiar las propiedades viscoelásticas, estructurales y difusionales de matrices covalentes de AX ferulados de pericarpio de trigo, como una alternativa de uso para este subproducto. Se encontró que variando el tiempo de exposición del pericarpio de trigo a una hidrólisis alcalina es posible extraer AX con características fisico-químicas y capacidad gelificante distintas, lo cual a su vez resulta en la formación de geles de AX con características reológicas y estructurales diferentes. Al aumentar el tiempo de hidrólisis de los AX de 0.5 a 2.0 h, la relación A/X, el contenido de ácido ferúlico, la viscosidad intrínseca y la masa viscosimétrica del polisacárido disminuyeron de forma significativa de 0.83 a 0.76, de 0.009 a 0.006 μg/mg, de 206 a 184 kDa y de 74 a 66 kDa, respectivamente. Los AX obtenidos mediante tiempos de extracción de 1-2 horas formaron geles de AX al 5 y 6% (p/v) mientras que los AX extraídos con 0.5 h de hidrólisis fueron poco solubles en agua, lo cual no permitió llevar a cabo la gelificación. Se encontró que el aumento en la concentración de polisacárido permite formar geles más firmes y con menor tamaño de poro. Sin embargo, el tiempo de extracción de los AX reduce la firmeza de los geles

formados, los cuales presentan mayores tamaños de poro. En general, los valores de firmeza y tamaño de poro en los geles de AX formados variaron de 40 a 119 nm y de 1.7 a 5, respectivamente. Para la última etapa de esta investigación, correspondiente al estudio de los geles de AX como matrices para la liberación controlada de proteínas, se utilizaron solamente AX extraídos después de 1 h de hidrólisis por formar geles con mejores características reológicas y estructurales. Se utilizaron además solamente geles al 5% (p/v) en AX debido a que la alta viscosidad de la solución al 6% (p/v) en AX, lo cual dificulta la incorporación de las proteínas modelo. Los geles al 5% (p/v) en AX así formados presentaron una elasticidad de 177 Pa, un tamaño de poro de 57 nm y un contenido de dímeros y trímero de ácido ferúlico de 0.008 y 0.003 µg/mg polisacárido, respectivamente. Estos geles son capaces de atrapar proteínas de distinto peso molecular (5-97 kDa) y en distintas proporciones en peso proteína/AX (0.06-1.0). Se estudió la distribución de las proteínas atrapadas en la red entrecruzada de AX mediante microscopía confocal de barrido láser. Se encontró la formación de agregados de proteína en geles de AX con proporciones en peso proteína/polisacárido de 0.25 a 1.0. Los agregados se presentan distribuidos de forma heterogénea indicando que la proteína y el polisacárido están localizados en fases distintas. Este comportamiento fue similar para las distintas proteínas estudiadas (insulina, ovoalbúmina, seroalbúmina de bovino). Por último, en los geles de AX conteniendo proteína que no presentaron separación de fases (proteína/AX de 0.06 y 0.12) se estudió la liberación de las proteínas modelo atrapadas. Para insulina, el coeficiente de difusión aparente (Dm) aumentó significativamente de 2.64×10^{-7} a 3.20×10^{-7} cm^2/s al incrementar la proporción en peso proteína/AX de 0.06 y 0.12, respectivamente. Para las mismas proporciones proteína/AX no se encontraron diferencias significativas en Dm al utilizar ovoalbúmina y seroalbúmina de bovino como proteínas modelo. Los resultados indican que los geles de AX son capaces de atrapar proteínas de distintos tamaños y en

distintas proporciones formando sistemas homogéneos que podrían ser utilizados para la liberación controlada de estas moléculas.

CAPÍTULO I

INTRODUCCIÓN GENERAL

La industria productora de harina de trigo genera diariamente grandes cantidades de pericarpio como subproducto, el cual es utilizado como alimento para ganado con un precio de mercado sumamente bajo. El pericarpio de trigo contiene polisacáridos no almidonados, principalmente arabinoxilanos ferulados, por lo que la extracción de este polisacárido permitiría dar un uso alternativo a este subproducto. Dado que a nivel internacional las fuentes comerciales de arabinoxilanos ferulados son escasas, la extracción de arabinoxilanos ferulados a partir de pericarpio de trigo, podría tener un impacto socio-económico importante en nuestro país. Los arabinoxilanos ferulados son capaces de formar geles covalentes resistentes a cambios de pH, temperatura y fuerza iónica y no son degradados durante su paso por el sistema gastrointestinal. Existen reportes previos sobre las características físico-químicas y funcionales de los arabinoxilanos ferulados extraídos del pericarpio de trigo (Rattan *et al*., 1994; Izydorczyk y Biliaderis, 1995). Sin embargo, el estudio del pericarpio de trigo generado por la industria harinera como posible fuente de arabinoxilanos ferulados con capacidad gelificante, no había sido abordado anteriormente. En este trabajo de investigación se estudiaron las propiedades viscoelásticas, estructurales y difusionales de matrices covalentes de arabinoxilanos ferulados recuperados de pericarpio de trigo.

Como resultado de este trabajo de tesis se han obtenido los siguientes productos:

Artículos en Revistas Arbitradas e Indizadas

Berlanga-Reyes, C.M., Carvajal-Millan, E., Lizardi-Mendoza, J., Islas-Rubio, A.R., Rascón-Chu, A. (2011). Enzymatic Cross-Linking of Alkali Extracted Arabinoxylans: Gel Rheological and Structural Characteristics. International Journal of Molecular Sciences, 12 (9): 5853-5861.

C. Berlanga-Reyes[1], E. Carvajal-Millan[1]*, Kevin B. Hicks[3], Madhav P. Yadav[3], Agustín Rascón-Chu[2] , Jaime Lizardi-Mendoza[1], Alma R. Islas-Rubio[2]. Arabinoxylan Gels as Delivery Devices of Proteins: Defining Mass Ratio Compatibility by Confocal Laser Scanning Microscopy. En preparación Carbohydrate Polymers

Capítulos de Libro

Berlanga Reyes C., Carvajal Millán E.*, Niño Medina G., Rascón Chu A., Ramírez Wong B., Magaña Barajas E. (2011). Low-Value Maize and Wheat By-Products as a Source of Ferulated Arabinoxylans. In: Waste Water-Treatment and Reutilization. García- Einschlag, F.S. (Ed). InTech: Croatia. ISBN: 978-953-307-249-4. Pp. 341-352.

Participación en Congresos

Arabinoxylans gels for insulin delivery: defining mass ratio compatibility by confocal laser scanning microscopy. C. Berlanga-Reyes, E. Carvajal-Millan, Jaime Lizardi-Mendoza, Alma R. Islas-Rubio, Agustín Rascón-Chu, Kevin B. Hicks, Yadav Madhav. XIX International Materials Research Congress, August, 2011.

Participación en proyecto de investigación

Este trabajo de tesis se desarrolló en el marco de un proyecto de investigación financiado por CONACYT: Caracterización de las propiedades estructurales, fisico-químicas y funcionales de arabinoxilanos ferulados de pericarpio y endospermo de trigo (CB-2009-01,134301, Responsable Dra. Elizabeth Carvajal, CIAD).

CAPITULO II

1. REVISIÓN BIBLIOGRÁFICA

1.1 Pericarpio de Trigo

El trigo es uno de los productos agrícolas más importantes en el Estado de Sonora, ya que constituye la base de diversos productos alimenticios típicos en la dieta del sonorense, especialmente la tortilla de harina de trigo. El trigo tiene un alto contenido de fibra, especialmente el pericarpio, llamado también salvado de trigo. El pericarpio de trigo es un subproducto generado por la industria productora de harina de trigo (aproximadamente 80 toneladas diarias). Este subproducto es actualmente utilizado como alimento para ganado a un costo muy bajo. En los últimos años se han buscado alternativas para el uso de este subproducto. Investigaciones recientes se han centrado en la composición del pericarpio de trigo con la intención de proponer usos alternativos para esta biomasa. El pericarpio está formado principalmente por biopolímeros como son celulosa (25-35%), hemicelulosa (40-50%) y lignina (7-10%) (Ishii, 1997). Los componentes de la pared celular son: proteína 17%, almidón 16.7%, lípidos 4.2%, ceniza 5.9% y fibra 58.4%. El 22.6% de esta fibra son arabinoxilanos (AX) (Maes y Delcour, 2002).

1.2. Los Arabinoxilanos

1.2.1 Estructura Química y Propiedades Funcionales

Los arabinoxilanos (AX) son polisacáridos neutros no almidonosos de los granos de cereales. Aunque los AX han sido de interés durante muchos años en el área de investigación en cereales, su estudio tomó mayor interés en los años 90, principalmente en relación a sus propiedades funcionales. Los AX están constituidos por un esqueleto lineal de unidades β-(1-4)-D-xilanopiranosas a las cuales se encuentran unidos sustituyentes de α-L-arabinofuranosas en O-2 y/o O-3 (Fincher y Stone, 1986). Algunos de los residuos de arabinosas tienen esterificados residuos de ácido ferúlico en (O)-5 (ácido 3-metoxi, 4 hidroxicinámico), por lo que son llamados arabinoxilanos ferulados (Smith y Hartley, 1983) (Figura 1). Dependiendo de su peso molecular, los arabinoxilanos pueden ser solubles (PM<1000 kDa) o insolubles (PM>1000 kDa) en agua (Izydorczyk y Biliaderis, 1995). Los AX solubles en agua están presentes en el endospermo de los cereales mientras que los AX insolubles en agua se encuentran principalmente en el pericarpio del grano. Debido a su alto peso molecular, la extracción acuosa de los AX insolubles en agua implica una hidrólisis parcial de la molécula. La cantidad de AX insolubles en agua presentes en el pericarpio de los cereales es alta (30-40% p/p), comparada con la cantidad de AX solubles en agua contenida en el endospermo (1-3% p/p) (Saulnier *et al*., 1995, Rouau *et al.,* 2003; Carvajal-Millán *et al.,* 2005a, 2007).

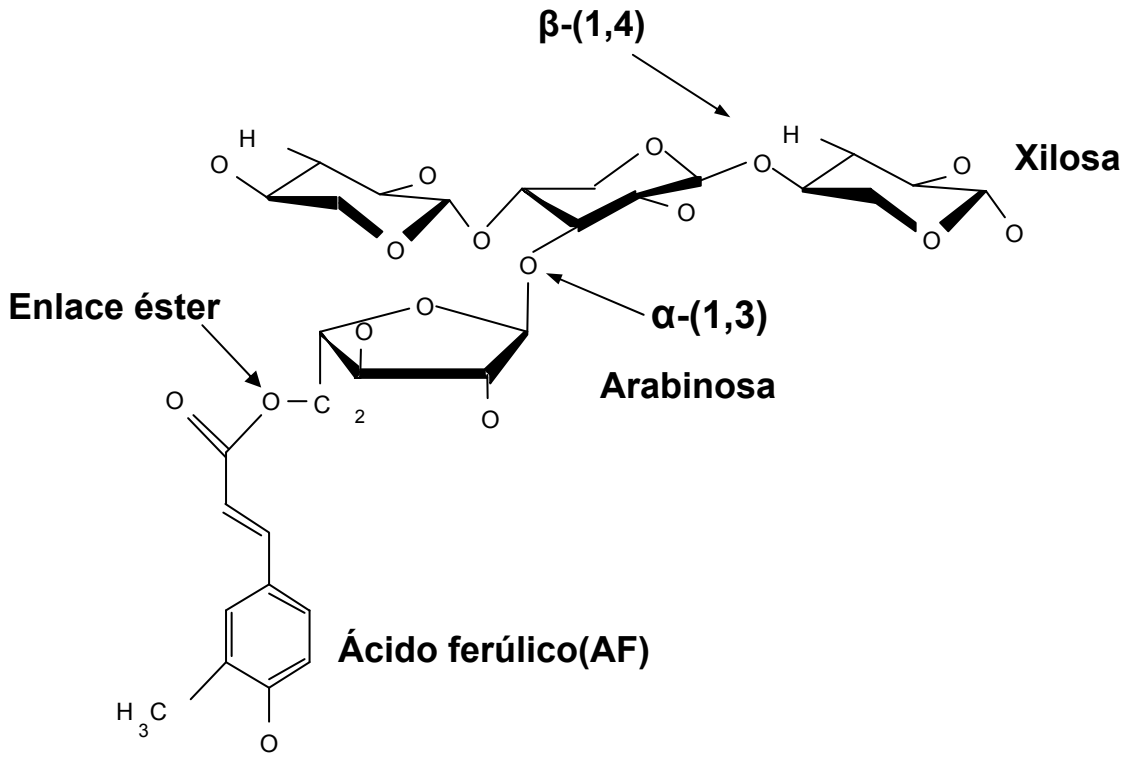

Figura 1. Estructura química de los AX

Las características estructurales de los AX influyen de forma directa en las propiedades fisicoquímicas de los mismos. En los AX la sustitución de arabinosas a lo largo de la cadena de xilosas le proporciona al polímero cierta flexibilidad. Sin embargo, la agregación de las cadenas está limitada por impedimento estérico (Izydorczyk y Biliaderis, 1995). Debido a su conformación no rígida, los AX suelen formar soluciones acuosas con una alta viscosidad intrínseca, en comparación con soluciones acuosas de otros polímeros como es el dextrán. El peso molecular de los AX de cereales varía de acuerdo al método de estimación y la solubilidad en agua de este polisacárido. Se ha reportado que para AX extraíbles en agua provenientes de trigo, el PM estimado por cromatografía de permeación en gel varía de 70 a 1000 KDa, mientras que para

AX de cebada puede ser de 5000 kDa y para AX de trigo no extraíbles en agua de hasta 850 kDa. Por otro lado, factores estructurales como el grado de sustitución y su distribución modifican el comportamiento de la solubilidad de los AX. Por ejemplo, estudios anteriores han demostrado que una proporción A/X menor a 0.43 da lugar a la agregación y precipitación de los AX. Este fenómeno es atribuido a interacciones entre las regiones no sustituidas de la cadena de xilosa, como sucede con la estructura de la celulosa y otros polímeros poco solubles en agua (Dervilly-Pinel *et al*., 2001; Saulnier *et al*., 2007a, b). Los AX de pericarpio de trigo presentan una relación A/X de 0.80, lo cual permite una mayor solubilidad en agua (Izydorczyk y Biliaderis, 1995).

Una de las propiedades fisicoquímicas más importante para los AX es la viscosidad intrínseca [η], la cual es una medida del volumen hidrodinámico ocupado por un polímero. La [η] depende del peso molecular y la conformación del polímero en el solvente. Para AX, la [η] presenta valores que varían entre 100 y 400 mL/g, dependiendo de las características estructurales del polisacárido. La [η] de los AX no extraíbles en agua generalmente son menores a la de los AX extraíbles en agua, debido a la reducción del tamaño de la molécula durante el proceso de extracción con tratamiento químico, lo cual no sucede con los AX solubles en agua provenientes de endospermo (Carvajal-Millán *et al.,* 2007; Dervilly-Pinel *et al.,* 2001).

1.2.2 Capacidad de Gelificación de los Arabinoxilanos

Los AX ferulados pueden formar geles bajo la acción oxidante de tipo enzimático (lacasa, peroxidasa/H_2O_2) sobre el ácido ferúlico, conduciendo a la formación de radicales libres. Estos radicales libres son posteriormente acoplados, resultando en la formación de dímeros de ácido ferúlico (di-AF), los cuales unen las cadenas de polisacárido formando un gel tridimensional acuoso

(Geissman y Neukom, 1973; Hoseney y Faubion, 1981; Izydorczyk y Biliaderis, 1995; Figueroa-Espinoza y Rouau, 1998) (Figura 2).

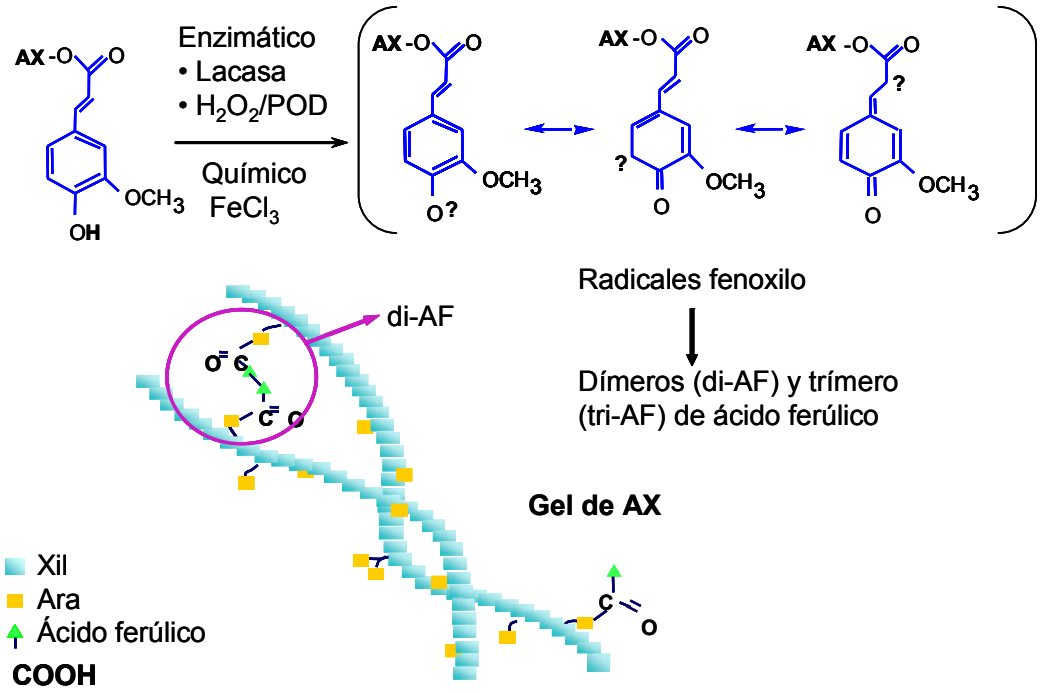

Figura 2. Gelificación de los arabinoxilanos ferulados

Hasta el momento han sido identificados cinco formas isoméricas de di-AF en geles de arabinoxilanos (5-5', 8-5' benzo, 8-O-4', 8-5' y 8-8'). Las formas 8-5' y 8O-4' son generalmente más abundantes en geles de arabinoxilanos de trigo (Figueroa-Espinoza y Rouau, 1998; Schooneveld-Bergmans *et al*., 1999; Vansteenkiste *et al*., 2004, Carvajal-Millán *et al*., 2005a-c, 2006) (Figura 3). Sin embargo, en geles de AX de maíz el di-AF 5-5' ha sido reportado como el mayoritario (Carvajal-Millán *et al*., 2007; Niño-Medina *et al*., 2009; Berlanga-Reyes *et al*., 2009a). Hasta el momento se ha encontrado la formación de un

sólo trímero de ácido ferúlico (tri-AF) (ácido 4-O-8', 5'-5''-dehidrotriferulico) en geles de AX de trigo y de maíz (Carvajal-Millán *et al*., 2005a, 2007) inducidos por una lacasa (Figura 3).

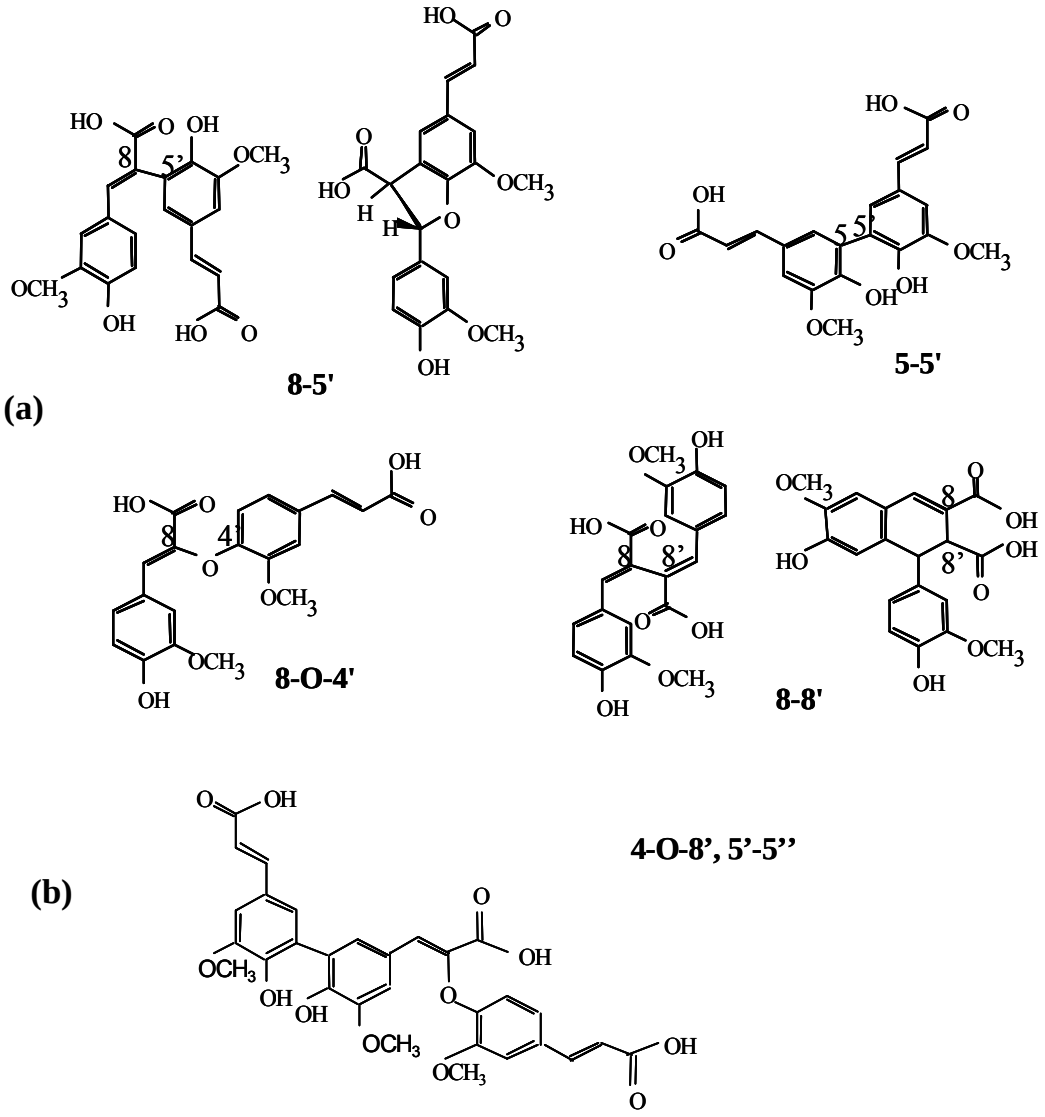

Figura 3. Estructura química de los dímeros (di-AF) (a) y trímero (tri-AF) (b) reportados en geles de arabinoxilanos

Dado que, en general, más del 90% del contenido inicial de ácido ferúlico presente en los AX es oxidado al formarse el gel y sólo el 30% es recuperado en forma de los di y tri-AF hasta el momento conocidos, algunos autores señalan la formación de estructuras de di y tri-AF distintas a las hasta el momento identificadas, o bien a oligómeros superiores de ácido ferúlico (tetrámeros, pentámeros) (Vansteenkiste *et al*., 2004; Carvajal-Millan *et al*., 2005a, 2006, 2007). Bunzel *et al*. (2005, 2006) han aislado e identificado dos nuevos trímeros de ácido ferúlico (ácido 8-5(no-cíclico)/5-5-dehidrotriferúlico y ácido 5-5-dehidrotriferulico) y, por primera vez, dos tetrámeros de ácido ferúlico (ácido 4-O-8/5-5/8-O-4-dehidrotetraferúlico y ácido 4-O-8/5-5/8-5(no-cíclico)-dehidrotetraferúlico) en pericarpio de maíz. Estas nuevas estructuras no han sido aún estudiadas en geles de AX pero, dado que en la naturaleza su función es unir estos polisacáridos para dar soporte estructural a la pared celular, es muy probable que se formen durante el proceso de gelificación de este polisacárido. Además de la participación de las estructuras di-AF y el tri-AF anteriormente mencionados, algunos autores señalan la contribución de interacciones físicas entre las regiones lineales de las cadenas de AX en el establecimiento de la estructura tridimensional del gel (Vansteenkiste *et al*., 2004; Carvajal-Millan *et al*., 2005b). La presencia de cadenas laterales de xilosa han sido detectadas en AX de trigo en variedades europeas (Adams *et al*., 2003; 2005), las cuales pueden afectar el acercamiento de las cadenas de AX y el establecimiento de la estructura del gel. Esta estructura estaría entonces determinada tanto por los enlaces covalentes de ácido ferúlico (di, tri-AF) como por las interacciones físicas entre las regiones lineales de xilosa, lo cual a su vez puede afectar a los parámetros estructurales y las propiedades funcionales del gel formado. Anteriormente se reportó una relación directa entre los parámetros estructurales del gel de AX (tamaño de poro, densidad de puntos de unión, distancia entre dos puntos de unión) y sus propiedades funcionales (capacidad de retención de agua al equilibrio, capacidad para liberar

biomoléculas de manera controlada) (Carvajal-Millán *et al*., 2005b). Sin embargo, no ha sido posible, hasta el momento, cuantificar la contribución relativa de los enlaces covalentes y de las interacciones físicas a la formación del gel de AX.

1.2.3 Los Geles de Arabinoxilanos como Matrices de Liberación Controlada

Los geles de AX presentan características interesantes: son neutros, sin color ni olor y, debido a la presencia de enlaces covalentes, son poco afectados por cambios de temperatura, fuerza iónica o pH (Izydorczyk y Biliaderis, 1995). Estas características y la estructura macroporosa de los geles de arabinoxilanos con un tamaño de poro entre 40 y 400 nm, le confieren a los AX un alto potencial de aplicación en el transporte de biomoléculas. Algunos autores han reportado que los geles de AX podrían ser utilizados como matrices para la liberación controlada de proteínas en el colon (Vansteenkisten *et al*., 2004; Carvajal-Millán *et al*., 2005b, 2006; Berlanga-Reyes *et al*., 2009b). Lo anterior se debe a que los AX son fibra dietética y resisten el ataque de las enzimas digestivas, y a su vez protegen las proteínas contenidas en el gel durante su paso por el sistema gastrointestinal. Una vez en el colon, el gel de AX sería degradado por las enzimas de la microflora, liberando la proteína para ser absorbida. Investigaciones realizadas utilizando AX de endospermo de trigo reportan dos métodos para introducir la proteína en el gel de AX, uno de ellos es gelificando la mezcla AX/proteína y el otro es cargando por difusión con la proteína al gel de AX formado. En ambos casos la estructura de la proteína no se ve afectada y la molécula puede ser posteriormente liberada de manera controlada (Carvajal-Millán *et al*., 2005, 2006). Además, los geles de AX de trigo pueden proteger las proteínas de la acción de las enzimas digestivas hasta en un 90% (Vansteenkisten *et al*., 2004). Utilizando seroalbúmina de

bovino y ovoalbúmina como proteínas modelo Vansteenkisten *et al*. (2004) y Carvajal-Millán *et al*. (2006) reportan que el gel de AX es capaz de atrapar una cantidad de proteína hasta 100 veces mayor que la del polisacárido (relación 1/100 AX/proteína p/p). Sin embargo, en la actualidad, el uso de AX como agentes de encapsulación y liberación controlada de biomoléculas no es común ya que las fuentes comerciales de este polisacárido no son abundantes y su precio es elevado. Lo anterior se debe, por un lado, al alto costo de los procesos de extracción utilizados, y por otro, a que generalmente se extraen solamente los AX solubles en agua contenidos en cantidades pequeñas en el endospermo de cereales como el trigo, el centeno y la cebada.

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CAPITULO III

Low-Value Maize and Wheat By-Products as a Source of Ferulated Arabinoxylans

Chapter 16

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In: Waste Water-Treatment and Reutilization. García- Einschlag, F.S. (Ed). InTech: Croatia. ISBN: 978-953-307-249-4. Pp. 341-352.

1. Introduction

The major polymers in the cell walls are cellulose (25-35%), hemicelluloses (40-50%) and lignin (7-10%). Both cellulose and hemicelluloses function as structural supporting materials in the cell walls; cellulose has a high tensile strength and gives rigidity to the walls, whereas hemicelluloses impart elasticity to the structure by cross-linking cellulose micro fibrils (Ishii, 1997). Xylans occur

as the most common hemicelluloses, and after cellulose they are the second most abundant polysaccharides in the plant kingdom.

Arabinoxylans (AX) are hemicelluloses built up of pentose sugars, mostly arabinose and xylose residues, and are therefore often referred to as pentosans (Izydorczyk & Biliaderis, 1995). AX consist of backbone chains of β(1,4) linked-linked D-xylopyranosyl units to which α-L arabinofuranosyl substituents are attached through O-2 and/or O-3 (Fincher & Stone, 1974). Some of the arabinose residues are ester linked on (O)-5 to ferulic acid (FA) (3-methoxy, 4 hydroxy cinnamic acid) (Smith & Hartley, 1983). AX are mayor dietary fiber components of many cereals like wheat, rye, corn, barley, oat, rice and sorghum (Fincher & Stone, 1974). AX are classified into water-extractable (WEAX) and water-unextractable AX (WUAX). The WUAX present a combination of no covalent interactions and covalent bonds with other cell walls components, such as proteins, cellulose and lignin (Andrewartha *et al*., 1979).

AX can be isolated by water and by alkali extraction (Cui *et al*., 2001). The extractability of these polysaccharides is based on the conformational aggregation, the covalent ester bonds between ferulic acid and other components such as lignin, the degree, and substitution patter of arabinoses at side chain, and nature of physical entanglement. Once extracted AX form highly viscous solutions with gelling capacity by covalent cross-linking through dimerization of ferulic acid substituents under oxidative conditions (e.g., use of enzymatic free radical generating agents as laccase and peroxidase H_2O_2) (Geissman *et al*., 1973; Figueroa Espinoza *et al*., 1998). Diferulic acids (di-FA) and triferulic acid (tri-FA) (Vansteenkiste *et al.,* 2004; Carvajal-Millan *et al.,* 2005a) have been identified as covalently cross-linked structures in AX gels.

AX gels present interesting properties like neutral taste and odor, high water absorption capacity and absence of pH or electrolyte susceptibility (Izydorczyk & Biliaderis, 1995). Interest on AX and AX gels has increased in the last years and new information on their sources and applications are being reported.

Recuperation of AX from cereal by-products of the food industry has been reported (Niño-Medina (2009a), b; Carvajal-Millan *et al*., 2007) and would offer new advantages for future industrial applications of this biomolecule.

Maize and wheat are important sources of food in Mexico. They are used to obtain different food products such as cereal breakfasts, bread, tortilla, among others. During processing, maize and wheat generate high amounts of low-value by products. In the past, Mesoamerican Indians learned that wood ashes facilitated maize cooking, the removal of the hard outer covering, and improved the quality of the resulting material. We now know that this process also releases the bound niacin in the maize into a readily available form. Thus, the population did not suffer the ravages of what we now call pellagra. In Mexico, this alkali cooking, called 'nixtamalization' (from the Nahuatl nixtli=ashes and tamalli=dough) is widely used to improve the maize nutritional value.

Maize nixtamalization is important in Mexico as half of the total volume of consumed food is maize, which provides approximately 50 % of the energy intake, this proportion being even greater for lower income groups. Nixtamalization consists of cooking maize grains in a lime solution, soaking for 2-8 hours and washing them by hand to remove the pericarp. The product obtained is then ground to obtain nixtamal (dough or masa) used to prepare a variety of products, tortilla being the most popular one. The nixtamalization process degrades and solubilizes maize cell wall components and this facilitates pericarp removal. As a matter of fact, the 'nejayote' (maize nixtamalization waste water) contains, in general, more than 60% of non-starch polysaccharides. These alkali-soluble non-cellulosic cell wall polysaccharides present in maize pericarp (mainly arabinoxylan) show interesting functional properties as thickeners, stabilizers, emulsifiers and film and gel formers.

The nejayote obtained from nixtamalization is highly alkaline waste water, with high chemical and biological oxygen demands and is considered an environmental pollutant. A typical maize nixtamalization facility processing 50 kg

of maize every day uses over 75 liters of water per day and generates nearly the equivalent amount of alkaline waste water in 24 hours. Thus, alternatives of nejayote residues utilization in Mexico are needed. Niño-Medina (2009a) recently reported that nejayote can be a novel source of AX. During the milling process of maize and wheat the starchy endosperm is isolated with the minimum contamination by peripheral layers of the grain (i.e. aleurone layer and bran).

Maize and wheat bran are by-products of the commercial flour industry in Mexico. Because of the high volume of maize and wheat bran produced in Mexico, these residues are becoming into potential sources of added-value biomolecules as AX for the food industry. Maize bran contains heteroxylans (approximately 50%), cellulose (approximately 20%) and phenolic acids (approximately 4%, mainly ferulic and diferulic acid) (Saulnier *et al*. 1995a). Starch (9-23%), proteins (10-13%), oil (2-3%) and ash (2%) are also present in maize bran (Hespell, 1998). The heteroxylans portion of maize bran can be extracted with alkaline (Whistler, 1993; Saulnier *et al*. 1995b; Carvajal-Millan *et al*., 2007) or acid solutions (Saulnier *et al*., 1995a) to produce to produce water-soluble AX. Wheat bran contains approximately 19% of water-insoluble AX, which can be extracted with alkaline or acid solutions to produce water-soluble AX (Hashimoto *et al*., 1987).

This chapter includes some of the most recent findings on physico-chemical and functional properties of water-soluble ferulated arabinoxylans from three cereal by-products: nejayote (nixtamalization waste water), maize bran and wheat bran.

2. Experimental

2.1 Materials and Methods

Nejayote, maize bran and wheat bran were kindly provided by commercial milling industries in Northern Mexico. All chemical products were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.2 Arabinoxylans Extraction

AX from nejayote (FAXN) and AX from maize bran (FAXMB) presented in this study were previously extracted and characterized (Carvajal-Millán *et al.* 2007; Niño-Medina (2009a)).

AX from wheat bran (FAXWB) were extracted as follows. Wheat bran was ground to a 20-mesh particle size using a M20 Universal Mill (IKA®, Werke Staufen, Germany). Wheat bran (500 g) was treated with ethanol (2500 ml) for 12 h at 25 °C to remove lipophilic components.

The ethanol treated bran was then filtered and subjected to starch gelatinization and enzymes inactivation (boiling for 30 min in 3500 ml of water). After boiling, wheat bran was recovered by filtration and treated with 2500 ml of NaOH 0.5 N solution at 25 °C in darkness for 1 h under shake (100 rpm). Residual bran was then eliminated by filtration and the filtrate was centrifuged (12,096*g*, 20 °C, 15 min).

Supernatant was acidified to pH 4 with HCl 3N. Acidified liquid was centrifuged (12,096*g*, 20 °C, 15 min) and supernatant was then recuperated and precipitated in 65 % (v/v) ethanol for 4 h at 4°C. Precipitate was recovered and dried by solvent exchange (80 % (v/v) ethanol, absolute ethanol and acetone) to give FAXWB.

2.3 Chemical Composition of FAXWB

Sugar composition was determined according to Carvajal-Millan *et al*. (2007) after FAXWB hydrolysis with 2 N trifluoroacetic acid at 120 °C for 2 h. The reaction was stopped on ice, the extract was evaporated under air at 40 °C and rinsed twice with 200 µL of water and resuspended in 500 □L of water.

All samples were filtered through 0.45 µm (Whatman) and analyzed by high performance liquid chromatography (HPLC) using a Supelcogel Pb column (300 × 7.8 mm; Supelco, Inc., Bellefont, PA) eluted with 5mM H_2SO_4 (filtered 0.2 □m, Whatman) at 0.6 mL/min and 50 °C. A refractive index detector Star 9040 (Varian, St. Helens, Australia) and a Star Chromatography Workstation system control version 5.50 were used. The internal standard was inositol.

Ferulic acid was quantified by high performance liquid chromatography (HPLC) after deesterification step as described by Vansteenksite *et al*., (2004). An Alltima C_{18} column (250 × 4.6 mm) (Alltech associates, Inc. Deerfield, IL) and a photodiode array detector Waters 996 (Millipore Co., Milford, MA) were used. Detection was by UV absorbance at 320 nm.

Ash content was determined according to the AACC methods (AACC, 1998). Protein was determined by using the Bradford method (Bradford, 1976).

2.4 Intrinsic Viscosity of FAXWB

Specific viscosity, ŋsp was measured by registering FAXWB solutions flow time in an Ubbelohde capillary viscometer at 25 ± 0.1 °C, immersed in a temperature controlled bath. FAXWB solutions were prepared at different concentrations, dissolving dried polysaccharide in water for 10 h with stirring at room temperature. FAXWB solutions and water were filtered using 0.45 µm membrane filters before viscosity measurements.

The intrinsic viscosity ([ŋ]) was estimated from relative viscosity measurements, ŋrel, of FAXWB solutions by extrapolation of Kraemer and Mead and Fouss curves to “zero” concentration.

2.5 Viscosimetric Molecular weight of FAXWB

The viscosimetric molecular weight (Mv) was calculated from the Mark–Houwink relationship, Mv = ([*η*]/k)1/α.

2.6 FAXN, FAXMB and FAXMB gelation

FAXN, FAXMB and FAXWB solutions (4, 3.5 and 5.0 % w/v) were prepared in 0.05 M citrate phosphate buffer pH 5. Laccase (1.675 nkat per mg polysaccharide) was used as gelling agent. Gels were allowed to form at 25 °C.

2.7 Small Deformation Measurements

The formation of FAXN, FAXMB and FAXWB gels was followed using a strain-controlled rheometer (AR-1500ex, TA Instruments, U.S.A.) in oscillatory mode as follows. Cold (4 °C) solutions of FAXN, FAXMB and FAXWB at 4, 3.5 and 5.0%, respectively (w/v) were mixed with laccase (1.675 nkat per mg polysaccharide) and immediately placed in the cone and plate geometry (5.0 cm in diameter, 0.04 rad in cone angle) maintained at 4 °C.

Exposed edges of the sample were covered with silicone fluid to prevent evaporation during measurements. Gelation kinetic was started by a sudden increase in temperature from 4 °C to 25 °C and monitored at 25 °C over time by following the storage (G') and loss (G'') modulus.

All measurements were carried out at a frequency of 0.25 Hz and 5 % strain (linearity range of viscoelastic behavior). Frequency sweep (0.1 to 50 Hz) was carried out at the end of the network formation at 5 % strain and 25 °C.

2.8 Large Deformation Measurements

The hardness of FAXN, FAXMB and FAXWB gels was analyzed with a TA.XT2 Texture Analyzer (RHEO Stable Micro Systems, Haslemere, England) equipped with a XTRAD software version 3.7.

The gels were deformed by compression at a constant speed of 1.0 mm/s to a distance of 4 mm from the gel surface using a cylindrical plunger (diameter 25.4 mm). The peak height at 4 mm compression was called gel hardness (Carvajal-Millan *et al.,* 2005a).

2.9 Gel Swelling

After laccase addition, FAXN, FAXMB and FAXWB solutions were quickly transferred to a 5 ml tip-cut-off syringe (diameter 1.5 cm) and allowed to gelify for 2 h at 25°C. After gelation, the gels were removed from the syringes, placed in glass vials and weighted.

The gels were allowed to swell in 20 ml of 0.02% (w/v) sodium azide solution to prevent microbial contamination. During 20 h the samples were blotted and weighed. After weighing, a new aliquot of sodium azide solution was added to the gels. Gels were maintained at 25°C during the test.

The equilibrium swelling was reached when the weight of the samples changed by no more than 3% (0.06 g). The swelling ratio (q) was calculated as:

$$q = (Ws - Wd) / Wd$$

where Ws is the weight of swollen gels and Wd is the weight of polysaccharide in the gel.

2.10 Gel Structure

From swelling measurements, the molecular weight between two cross-links (Mc), the cross-linking density (ρc) and the mesh size (ξ) values of the FAXN,

FAXMB and FAXWB gels were obtained as reported by Carvajal-Millan *et al*. (2005b). Mc, ρc and ξ were calculated using the model of Flory and Rehner modified by Peppas and Merrill for gels where the cross-links are introduced in solution.

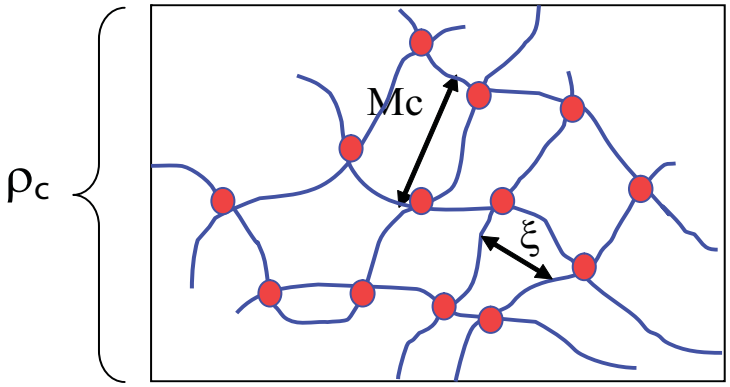

Figure 1. Schematic representation of gel structural parameters. Mc=Molecular weight between two cross-links, ρc= cross-linking density and ξ= mesh size.

3. Results and Discussion

In a previous report, FAXN and FAXMB have been extracted and characterized Niño-Medina (2009a). Yield of FAXN and FAXMB were 8 (w FAXN/v nejayote) and 29 % (w FAXMB/w maize bran), respectively.

In FAXN, pure arabinoxylans represented 81 % dry basis (db) of the recovered sample (Table 1). This value was estimated from the sum of xylose + arabinose. The ratio arabinose-to-xylose (A/X) was 0.65 indicating a moderately branched structure, similar to that reported by Singh *et al*. (2000) in maize bran arabinoxylans. Residues of glucose, galactose, mannose, proteins and ash were also detected in FAXN.

The ferulic acid content, [η] and Mw values were lower to those reported for ferulated arabinoxylans from other sources (Izydorczyk *et al.,* 1990; Dervilly-Pinel *et al.,* 2001; Vansteenkiste *et al*., 2004; Carvajal-Millan *et al.,* 2006).

Arabinose (%)	33.0 ± 0.70
Xylose (%)	39.0 ± 1.20
Glucose (%)	4.90 ± 0.15
Galactose (%)	3.90 ± 0.11
Mannose (%)	0.60 ± 0.04
Protein (%)	2.70 ± 0.20
Ash (%)	3.58 ± 0.14
Ferulic acid (%)	0.003 ± 0.05
A/X (mass ratio)	0.85 ± 0.20
[η] (mL/g)	208 ± 6.2

Table 1. Composition of ferulated arabinoxylans from nejayote (FAXN) (adapted from Niño-Medina (2009a)).

As presented in Table 2 , FAXMB presented an arabinoxylan content of 72% (w/w), a ferulic acid content of 0.003 (% w/w), an A/X ratio of 0.85, an intrinsic viscosity [η] of 208 mL/g and a molecular weight (Mw) of 190 kDa.

Arabinose (%)	32.0 ± 0.80
Xylose (%)	49.0 ± 1.90
Glucose (%)	5.10 ± 0.40
Galactose (%)	3.70 ± 0.20
Mannose (%)	0.60 ± 0.04
Protein (%)	4.50 ± 0.20
Ash (%)	5.10 ± 0.21
Ferulic acid (%)	0.002 ± 0.01
A/X (mass ratio)	0.65 ± 0.10
[η] (mL/g)	183 ± 5.4
Mw (kDa)	60 ± 6.0

Table 2. Composition and physico-chemical characteristics of ferulated arabinoxylans from maize bran (FAXMB) (adapted from Niño-Medina (2009a))

The higher ferulic acid content and [η] and Mw values in FAXMB in comparison with FAXN could be attributed to the differences in the extractions conditions used. FAXN are recovered after maize nixtamalization which consists of cooking maize grains in a lime solution, after soaking for 2 to 8 hours while FAXMB are alkali-extracted under mild conditions (NaOH 0.5 N solution at 25 °C in darkness for 8 h).

In this chapter is presented for the first time the extraction and characterization of FAXWB. FAXWB yield was 17% (w FAXWB/w wheat bran), which is higher than the value previously reported for FAXN (8% w/v) but lower than that found for FAXMB (29% w/w).

Composition of FAXWB is presented in Table 3. Arabinoxylan (AX) represented 76 % dry basis (db) of the recovered FAXWB. This value was estimated from

the sum of xylose + arabinose. This arabinoxylan content is in the range found for FAXN and FAXMB (86 and 72 %, respectively).

The ratio arabinose-to-xylose was high (A/X = 0.80) indicating a highly branched structure, similar to that reported by Schooneveld-Bergmans *et al.* (1999) in wheat bran arabinoxylans. A high A/X ratio was also found in FAXMB (0.85) in comparison to the moderate A/X value found in FAXN (0.65).

Arabinose (%)	33.00 ± 1.20
Xylose (%)	43.00 ± 1.30
Glucose (%)	3.10 ± 0.40
Galactose (%)	2.10 ± 0.30
Mannose (%)	0.30 ± 0.03
Protein (%)	2.70 ± 0.20
Ash (%)	2.30 ± 0.21
Ferulic acid (%)	0.0050 ± 0.05
A/X (mass ratio)	0.80 ± 0.40
[η] (mL/g)	198 ± 5.6
Mw (kDa)	60 ± 3.0

Table 3. Composition of ferulated arabinoxylans from wheat bran (FAXWB)

These differences in A/X ratio could explain the higher water solubility of FAXN as arabinose residues increase the molecule hydrophilic characteristic. As in FAXN and FAXMB, residues of glucose, galactose, mannose, proteins and ash were detected in FAXWB. The ferulic acid content (0.005%) was higher in FAXWB than in FAXN and FAXMB but lower than that obtained by Schooneveld-Bergmans *et al.* (1999) in wheat bran arabinoxylans recovered by using a different extraction method.

The intrinsic viscosity ([ŋ]) and a molecular weight (Mw) values in FAXWB were 168 mL/g and 60 kDa, respectively, which are in the range found in FAXN and FAXMB. Due their different nature, the ferulated arabinoxylans extracted from each low-value maize and wheat by-product showed different yield, composition and physico-chemical characteristics.

FAXN and FAXMB have been reported to form covalent gels in presence of a laccase. According to Niño-Medina (2009a), treatment of FAXN with laccase as oxidizing agent formed a gel after 4-6 hours at 25°C.

The formation of 4% (w/v) FAXN gels over time was rheologically investigated by small amplitude oscillatory shear. These authors found that the storage (G´) and loss (G'') modulus rose over the time to reach a plateau with a final G' value of 2 Pa, which is lower than those reported for arabinoxylan gels from other sources (20-40 Pa) at lower polysaccharide concentrations (1-2% w/v) (Carvajal-Millán *et al.,* 2005a). On the other hand, a final G' value of 20 Pa was found in 3.5% (w/v) FAXMB (Berlanga-Reyes *et al.,* 2009) after gelation by a laccase.

Concerning FAXWB, during laccase induced gelation G' modulus rose to reach a plateau (Fig. 1). The final G' value was 177 Pa in a 5% (w/v) FAXWB gel.

The mechanical spectrum of FAXWB gel is presented in Fig. 2. After 90 minutes gelation spectrum was typical of solid-like materials with a linear G' independent of frequency and G'' much smaller than G' and dependent on frequency (Doublier & Cuvelier, 1996). This behavior is similar to that previously reported for other arabinoxylan gels cross-linked by laccase or peroxidase/H_2O_2 system (Izydorczyk *et al.,* 1990; Dervilly-Pinel *et al.,* 2001; Vansteenkiste *et al.,* 2004; Carvajal-Millan *et al.,* 2006).

The hardness of FAXN, FAXMB and FAXWB gels are presented in Fig. 3. In agree with small deformation rheological results (G', G'') discussed above, large deformation measurements (gel hardness) showed that FAXWB can form gels more elastic than those from FAXN and FAXMB.

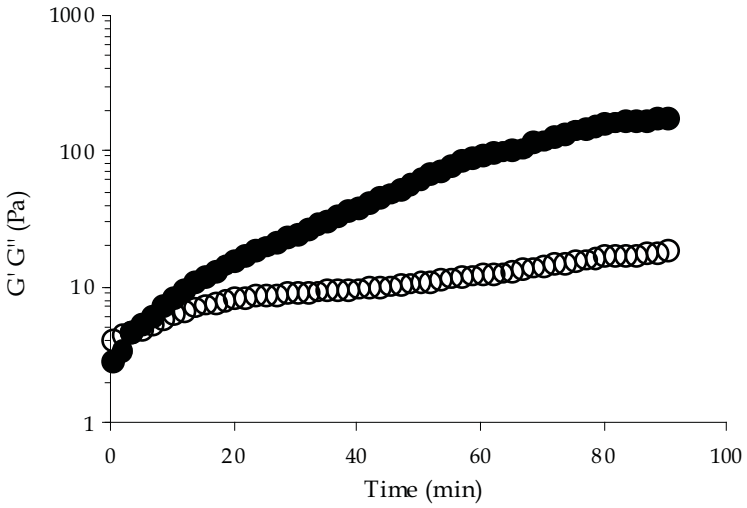

Fig 1. Laccase induced gelation of FAXWB solutions at 5 % (w/v) (G´ ●, G´´o). Rheological measurements made at 25°C, 0.25 Hz and 5% strain.

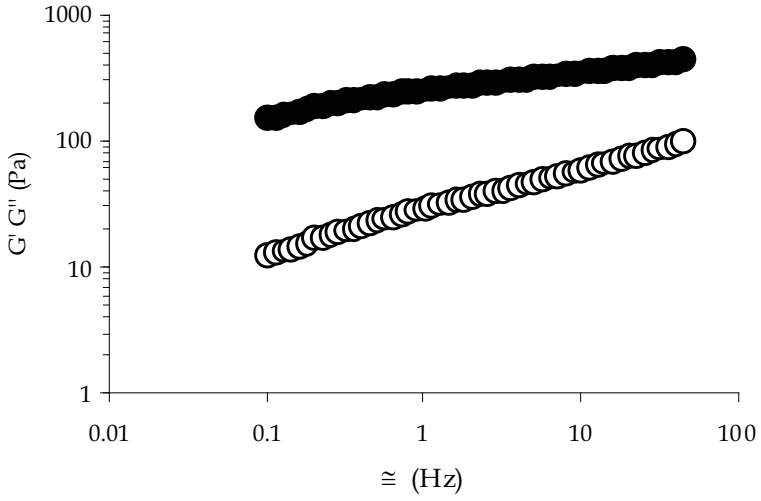

Fig 2. Mechanical spectra of FAXWB gels at 5.0 % (w/v) (G´ ●, G´´o). Rheological measurements made at 25°C and 5% strain.

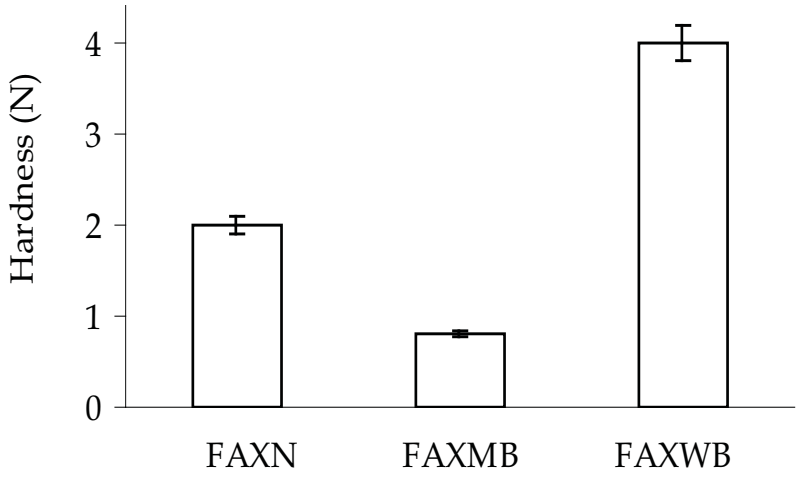

Fig 3. Hardness of FAXN, FAXMB and FAXWB gels at 4, 3.5 and 5.0 % (w/v), respectively. Rheological measurements made at 25°C.

The rheological differences between FAXN, FAXMB and FAXWB gels might have its origin in the structural and/or conformational characteristics of these macromolecules. Clearly, further studies on the distribution of arabinose and feruloyl groups along the polymer chain backbone of these different arabinoxylans are needed to establish relationships between the molecular structure, gelling ability and gels properties.

The equilibrium swelling of FAXN, FAXMB and FAXWB gels was reached between 15-20 h. The swelling ratio (q, g water/g polysaccharide) in FAXN, FAXMB and FAXWB gels were 40, 22 and 20, respectively (Table 4).

AX Source	Swelling ratio (q, g water/g AX)	M_c [a] x10^3 (g/mol)	ρ_c [b] x10^{-6} (mol/cm^3)	ε [c] (nm)
FAXN	40 + 1.5	95 + 0.1	9.0 + 0.01	183 + 4
FAXMB	22 + 1.9	20 + 0.1	75 + 0.01	48 + 1
FAXWB	20+ 1.7	29 + 0.1	59 + 0.01	58 + 1

[a] Molecular weight between two cross-links

[b] Cross-linking density

[c] Mesh size

Table 4. Structural characteristics of FAXN, FAXMB and FAXWB gels

The lower swelling ratio values obtained for FAXMB and FAXWB can be related to the more compact polymeric structure that limits the water absorption in comparison to the FAXN gels. The higher water uptake of gels made from FAXN can be explained in terms of a decrease in ferulic acid content and therefore the existence of longer un-cross-linked polysaccharide chains sections in the network. Uncross-linked polymer chains sections in the gel can expand easily conducting to higher amounts of water uptake.

The molecular weight between two cross-links (Mc), the cross-linking density (ρc) and the mesh size (ξ) values of the different gels are presented in Table 3. Higher Mc and ξ and lower ρc values have been reported in laccase induced water soluble arabinoxylan gels from wheat at similar AX concentrations (Carvajal-Millan *et al.,* 2005b).

The latter could be related to a high molecular weight in arabinoxylans from wheat endosperm (400-600 kDa) in comparison to alkali-extracted arabinoxylans from maize and wheat bran (60-240 kDa) used in the present

study. The involvement of physical interactions between polysaccharide chains could also be responsible of these differences. Different arabinoxylan gel structural characteristics were therefore obtained by modifying the polysaccharide source (FAXN, FAXMB, FAXWB).

The results discussed above indicate that by changing the arabinoxylan source gels with different rheological and structural properties can be obtained. To illustrate how the possible covalent cross-links content in the gel can affect network structure, we propose in Fig. 4 a model of the FAXN, FAXMB and FAXWB gels. As showed in Fig. 4 a decrease in the initial ferulic acid content could decrease the covalent bonds content. These differences in the network structure could induce changes in the functional properties of the gel.

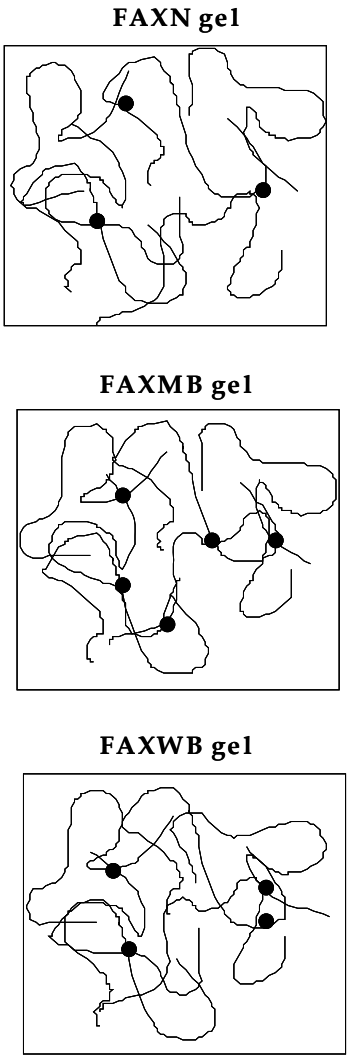

Fig 4. Schematic representation of FAXN, FAXMB and FAXWB gels.

4. Conclusion

Nejayote (a maize processing waste water), maize bran and wheat generated from flour industries can be potential sources of ferulated arabinoxylans as added-value hydrocolloids for the food industry. Due their different nature, the arabinoxylans extracted from each showed different physico-chemical and gelling properties. Recuperation of these hydrocolloids from low-value maize

and wheat by-products could represent a commercial advantage face to other polysaccharides commonly used in the food industry.

5. Future Considerations

New sources of polysaccharides continue to be investigated and different functional properties are being discovered. Concerning ferulated arabinoxylans from low-value low-value maize and wheat by-products, several questions remained to be elucidated, especially those concerning the relationships between the molecular structure, gelling ability and gels properties.

Additional studies will also be required on the application of these polysaccharides in food products. In this regard, technological and nutritional evaluation of these food products would be necessary. FAXN, FAXMB and FAXWB would have health benefits such as lowering of blood cholesterol and sugar as well as antioxidants properties but complementary studies are required.

6. Acknowledgements

Some of the results presented in this contribution are part of a research project supported by Fondo Institucional SEP-CONACYT, Mexico (grant 61287 to E. Carvajal-Millan). The authors are pleased to acknowledge Kevin Hicks (Eastern Regional Research Center, NAA, ARS, USDA), Valerie Micard (SupAgro Montpellier, France) and Mirko Bunzel (University of Minnesota, U.S.) for their participation in this research project. The authors are grateful to Alma C. Campa and Karla Martínez for their technical assistance.

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CAPITULO IV

Enzymatic Cross-linking of Alkali Extracted Arabinoxylans: Gel Rheological and Structural Characteristics

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Received: 23 June 2011; in revised form: 24 August 2011 / Accepted: 25 August 2011 / Published: 9 September 2011

Int. J. Mol. Sci. (2011), 12, 5853-5861

Abstract

Ferulated arabinoxylans were alkali-extracted from wheat bran at different incubation times (0.0, 0.5, 1.0, 1.5 and 2.0 h). Wheat bran ferulated arabinoxylans (WBAX) arabinose-to-xylose ratio, ferulic acid content, intrinsic viscosity and viscosimetric molecular weight values decreased as the incubation time of extraction increased. WBAX enzymatic cross-linking capability was affected by incubation time while an increase in WBAX concentration from 5 to 6% (w/v) favored gelation. The WBAX gels formed presented a macroporous structure with mesh size ranging from 40 to 119 nm and hardness values varying from 1.7 to 5 N.

Keywords: Arabinoxylans; ferulic acid; laccase; gelation.

1. Introduction

Wheat bran is a by-product of the commercial wheat milling process, which could be a potential source of added-value biomolecules such as hydrocolloids. Wheat bran contains fiber (approximately 58.4%, where 21.4% corresponds to arabinoxylans) [1]. The major part of the arabinoxylans portion in wheat bran is water-unextractable [2] and can be isolated by alkali extraction [3,4]. The extractability of these polysaccharides is based on their physical interactions and the covalent ester bonds between ferulic acid (FA) and other components such as lignin [5].

Arabinoxylans (AX) are built up of pentose sugars, mostly arabinose and xylose residues, and are therefore often referred to as pentosans [6]. Arabinoxylans consist of a backbone chain of β-(1□4)-D-xylopyranosyl units to which α-L-arabinofuranosyl substituents are attached through O–2 and/or O–3 [7]. Some of the arabinose residues are ester linked on (O)–5 to FA being called ferulated arabinoxylans [8].

Once extracted, arabinoxylans form highly viscous solutions with gelling capability by covalent cross–linking through dimerization of FA substituents under oxidative conditions (e.g., use of enzymatic free radical generating agents as laccase and peroxidase/H2O2 system) [9,10]. Diferulic acids (di–FA) and triferulic acid (tri–FA) [11,12] have been identified as covalently cross–linked structures in laccase–gelled AX. Both, covalent bridges (di–FA, tri–FA) and physical interactions between arabinoxylans chains, have been reported to be involved in the gelation process and the final gel properties [11,12]. The arabinoxylans properties such as solubility, viscosity and gelling capability are closely related to their chemical structure, conformation, and molecular interaction. Arabinoxylans gels present interesting properties like neutral taste and odor, high water absorption capability and stability to pH or electrolyte susceptibility [13].

In this study, ferulated arabinoxylans from wheat bran by-product have been alkali extracted and characterized and their gelling capability and the gel rheological and structural characteristics have been investigated.

2. Results and Discussion

2.1. WBAX Extraction and Characterization

In Figure 1, WBAX yields are shown as a function of extraction time. The yield of WBAX showed a statistically non–significant decrease as the time of extraction increased from 0.5 to 2 hours where yields values ranged from 3.8% to 3.0% (w WBAX/w wheat bran). By using calcium hydroxide 0.02 M (4 hours) and barium hydroxide 0.05 M (2 hours) at 20 °C a previous research [14] reported lower yield values (2% and 3%, respectively). The WBAX physico–chemical characteristics and enzymatic cross-linking capability were affected by the length of extraction time. Physico-chemical characteristics of the extracted WBAX are presented in Table 1. The ratio of arabinose-to-xylose (A/X) decreased from 0.83 to 0.76 as the time of extraction increased from 0.5 to 2.0 hours. The A/X ratios found in the present study indicate a highly branched structure, similar to that reported before [14–16] in wheat bran arabinoxylans. On the other hand, the FA content in WBAX was greatly affected by extraction time, registering values from 0.009 to 0.006 µg/mg WBAX. Higher FA contents (1–3 µg/mg) have been previously reported in wheat bran arabinoxylans extracted under mild conditions [15,16]. The intrinsic viscosity [ɳ] and viscosimetric molecular weight (Mv) of WBAX registered a decrease from 206 to 184 mL/g and from 74 to 66 kDa, respectively, as the extraction time increased from 1.0 to 2.0 hours. [ɳ] and Mv values obtained are in agreement with previous reports on ferulated arabinoxylans extracts of wheat bran [15,16]. [ɳ] and Mv values could not be determined in WBAX extracted after 0.5 hours as this sample presented very low water solubility. In general, the effect of NaOH

exposure on the yield and physico-chemical characteristics of WBAX can be attributed to the chemical hydrolysis of the different linkages in the arabinoxylan molecule (xylose-to-xylose, xylose-to-arabinose, arabinose-to-ferulic acid).

Figure 1. Yields of wheat bran ferulated arabinoxylans (WBAX) extracted under different incubation times in 0.5 M NaOH at 25 °C and 100 rpm in darkness. The presented results are averages of three replicates. Mean values with different letters are significantly different ($P < 0.05$).

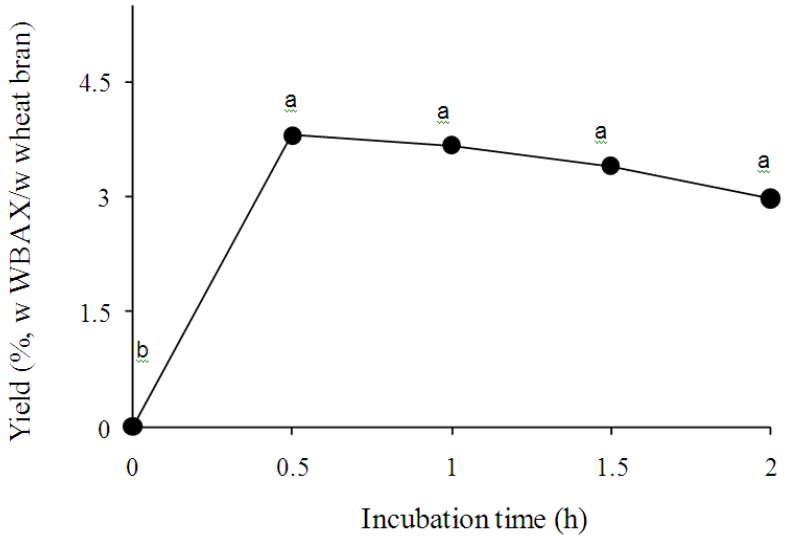

Table 1. Physico–chemical characteristics of wheat bran ferulated arabinoxylans (WBAX) extracted wth different incubation time.

Incubation time (h)	A/X ratio	FA (μg/mg WBAX)	[η] mL/g	Mv (kDa)
0.5	0.83 a	0.009 a	–	–
1.0	0.80 a	0.008 a	206 a	74 a
1.5	0.79 a	0.008 a	194 a	70 a
2.0	0.76 b	0.006 b	184 b	66 b

All values are means of three determinations. Mean values in the same column with different letters are significantly different ($P < 0.05$).

2.2. WBAX Enzymatic Cross–Linking and Gel Hardness

WBAX extracted with 1.0, 1.5 and 2.0 hours of NaOH exposure produced firm and brittle gels at 5 and 6% in WBAX (w/v) in the presence of laccase as a gelling agent. WBAX recovered after 0.5 hours of NaOH treatment did not form a homogenous solution; therefore gelation tests were not performed. The hardness of the WBAX gels obtained is presented in Figure 2. A significant increase in the gel hardness was registered when the polysaccharide concentration augmented from 5 to 6% (w/v) for WBAX extracted after 1.0, 1.5 or 2.0 hours NaOH exposure. Nevertheless, at the same polysaccharide concentration, increasing the WBAX extraction time decreased gel hardness by 57 and 60% for gels formed at 5 and 6% in WBAX (w/v), respectively. In general, WBAX gels hardness values (1–5 N) were higher than those previously reported for wheat flour and maize bran arabinoxylans (0.5–1 N) [12,17]. This decrease in gel hardness could be explained by the differences in the physico-chemical characteristics of the

WBAX recovered as discussed above (Table 1). Arabinoxylans FA content and A/X ratio are related to gel hardness. A higher FA content could conduce to a higher content of cross-linking structures in the gel. The solubility of arabinoxylans is closely related to the presence of arabinose. The amount of arabinose affects the potential of arabinoxylan chains to interact with each other. A low A/X ratio (<0.4) may lead to precipitation of polysaccharide chains, affecting the gelling capability [13]. In the present study, WBAX recovered after 2 hours incubation presented A/X ratio, FA content, [ŋ] and Mv values statistically significant lower than those of WBAX samples extracted after 1.0 and 1.5 hours of alkali treatment. In this context, the fact that increasing WBAX extraction time decreased gel hardness could be related to the reduction in the A/X ratio which decreases arabinoxylans solubility, to the lower FA content which could conduce to a low content of cross-linking structures and to the lower [ŋ] and Mv values as a decrease in chain length could reduce the potential of the polymer chain to form intermolecular aggregates [13].

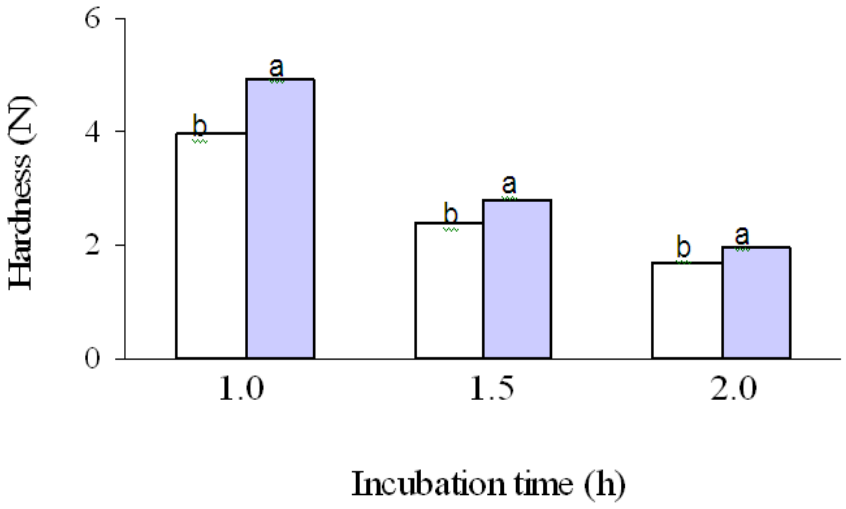

Figure 2. Hardness of WBAX gels at 5 (□) and 6 (■)% in WBAX extracted using different incubation times. Values are means of triplicate measurements. All the given values are means of three determinations. Mean values for the same incubation time with different letters are significantly different ($P < 0.05$).

2.3. WBAX Gels Structure

The equilibrium swelling of the different WBAX gels at 5 and 6% (w/v) was reached between 8 and 10 h. The swelling ratio (q, g water/g WBAX) in WBAX gels increased with the length of the incubation time used for WBAX extraction (Table 2). This increase in q could be related to a decrease in the gel hardness (Figure 2). For all WBAX samples, an increase in WBAX concentration in the gel from 5 to 6% (w/v) reduced q values. The lower q values could be related to a more compact polymeric structure that limits water absorption [18,19]. The structural characteristics of WBAX gels (molecular weight between two cross-links, Mc; cross-linking density, ρc and mesh size, ξ) are presented in Table 2.

As the extraction time of WBAX increased, higher Mc and ξ values of the gels formed were found while lower ρc were registered, which is consistent with the formation of weaker gels (Figure 2). When WBAX concentration in the gel was modified from 5 to 6% (w/v), Mc and ξ values decreased and ρc increased, confirming the formation of a stronger gel (Figure 2). Similar structural parameters have been reported for maize bran arabinoxylans gels [19] while in wheat flour arabinoxylans gels higher mesh sizes values (200–400 nm) were found [18]. The latter could be related to the differences in the structural characteristics of arabinoxylans from bran or endosperm cereal cells, such as molecular weight, ferulic acid content and A/X ratio.

Table 2. Structural characteristics of WBAX gels formed at different polysaccharide concentrations and with distinct incubation times during WBAX extraction.

	WBAX (% w/v)							
	5.0				6.0			
Incubation time (h)	q (g water/ g WBAX)	Mc × 10^3 (g/mol)	$\rho_c \times 10^{-6}$ (mol/cm^3)	ξ (nm)	q (g water/ g WBAX)	Mc × 10^3 (g/mol)	$\rho_c \times 10^{-6}$ (mol/cm^3)	ξ (nm)
1.0	8.6 b	29 c	59 a	57 c	6.6 b	16 b	102 a	40 b
1.5	13.4 a	40 b	41 b	72 b	11.6 a	19 a	84 b	44 a
2.0	11.2 a	80 a	21 c	119 a	10.3 a	18 a	95 b	41 b

All the given values are means of three determinations. Mean values in the same column with different letters are significantly different ($P < 0.05$).

3. Experimental Section

3.1. Materials

Ferulated arabinoxylans were isolated from wheat bran by–product kindly provided by a commercial wheat milling industry in Northern Mexico (Molinos La Fama). Laccase (benzenediol: oxygen oxidoreductase, E.C.1.10.3.2) from Trametes versicolor, sodium hydroxide, citric acid, sodium phosphate dibasic, hydrochloric acid and sulfuric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.2. Methods

3.2.1. WBAX Extraction

Wheat bran was milled to a 20-mesh particle size using a CyclotecTM 1093 Mill (FOSS, Sweden). Wheat bran (500 g) was suspended in ethanol (2,500 mL) over 12 h at 25 °C and 100 rpm to remove lipophilic components. The ethanol treated bran was then filtered and subjected to starch gelatinization and enzyme inactivation (boiling for 30 min in 3500 mL of water). After boiling, wheat bran was recovered by filtration and treated with 2,500 mL of 0.5 N NaOH solution at 25 °C in darkness for 0, 0.5, 1.0, 1.5 or 2.0 hours with shaking (100 rpm). Residual bran was eliminated by filtration and the filtrate was centrifuged (12,096 g, 20 °C, 15 min). Supernatant was acidified to pH 4 with 3N HCl and then centrifuged (12,096 g, 20 °C, 15 min). Supernatant was then recovered and precipitated in 65% (v/v) ethanol for 4 h at 4 °C. Precipitate was recovered and dried by solvent exchange (80% (v/v) ethanol, absolute ethanol and acetone) to render wheat bran ferulated arabinoxylans (WBAX) powder.

3.2.2. Laccase Activity

Laccase activity was measured at 25 °C from a laccase solution at 0.125 mg/mL in 0.1 M citrate-phosphate buffer pH 5.5 [10,12]. Syringaldazine 0.0216 mM in methanol was used as substrate. The enzymatic reaction was followed for 4 min at 530 nm.

3.2.3. Sugar Composition

Sugar composition was determined after WBAX hydrolysis with 2 N trifluoroacetic acid at 120 °C for 2 h. The reaction was stopped on ice, the extract was evaporated under air at 40 °C and rinsed twice with water (200 µL) and resuspended in water (500 µL). All samples were filtered through 0.45 µm (Whatman) and analyzed by high performance liquid chromatography (HPLC) using a Supelcogel Pb column (300 × 7.8 mm; Supelco, Inc., Bellefont, PA, USA) eluted with 5 mM H2SO4 (filtered 0.2 µm, Whatman) at 0.6 mL/min and 50 °C [17]. A Varian 9012 HPLC with Varian 9,040 refractive index detector (Varian, St. Helens, Australia) and a Star Chromatography Workstation system control version 5.50 were used. Series of sugar calibration standards were prepared in HPLC grade water at appropriate concentrations for creating a calibration curve for each sugar of interest (xylose, arabinose, galactose, mannose and glucose) in the range of 0.2–12.0 mg/mL. Inositol was used as internal standard.

3.2.4. Ferulic Acid

FA was quantified by reverse phase high–performance liquid chromatography (RP-HPLC) after a deesterification step, as described elsewhere [11]. An Alltima (Alltech, Deerfield, IL, USA) C18 5 µm column (250 x 4.6 mm) was used. Detection was by UV absorbance at 320 nm. Gradient elution was performed using acetonitrile and 0.05 M sodium acetate buffer, pH 4.0, at 1 mL/ min at 35 °C, in linear gradients from 15:85 to 35:65 in 30 min, from 35:65 to 60:40 in 0.5

min, from 60:40 to 15:85 in 4.5 min, and finally maintained at 15:85 for 5 min. A Waters 996 (Millipore Co., Milford, MA, USA) photodiode array detector was used to record the ferulic acid spectrum.

3.2.5. Intrinsic Viscosity and Viscosimetric Molecular Weight

Specific viscosity (ηsp) of WBAX solutions was measured by registering WBAX solutions flow time in an Ubbelohde capillary viscometer at 25 ± 0.1 °C, immersed in a temperature controlled water bath. WBAX solutions were filtered using 0.45 μm membrane filters before viscosity measurements. The intrinsic viscosity ([η]) was estimated from relative viscosity measurements (ηrel) of WBAX solutions by extrapolation of Kraemer and Mead and Fouss curves to "zero" concentration [20,21]. The viscosimetric molecular weight (Mv) was calculated from the Mark-Houwink relationship, Mv = ([η]/k)1/α.

3.2.6. WBAX Enzymatic Cross-Linking

WBAX solutions at 5 and 6% (w/v) were prepared in 0.1 M citrate phosphate buffer pH 5.5. WBAX solutions were mixed with 50 μL of laccase (1.675 nkat/mg WBAX). Gels were allowed to form for 6 h at 25 °C.

3.2.7. Rheology

The hardness of 5% and 6% (w/v) WBAX gels made in 6 mL glass flasks of 30 mm height and 25 mm internal diameter was analyzed with a TA.XT2 Texture Analyzer (Stable Micro Systems, Godalming, England). The gels were deformed by compression at a constant speed of 1.0 mm/s to a distance of 4 mm from the gel surface using a cylindrical plunger (diameter 15 mm). The maximum force obtained from the force vs. distance curve was recorded as a measure of gel hardness [17].

3.2.8. Gel Structure

After laccase addition, 2 mL WBAX solutions were quickly transferred to a 5 mL tip–cut–off syringe (diameter 1.5 cm) and allowed to gel for 6 h at 25 °C. After gelation, the gels were removed from the syringes, placed in glass vials and weighed. The gels were allowed to swell in 20 mL of 0.02% (w/v) sodium azide solution to prevent microbial contamination. During 10 h the samples were blotted and weighed. After weighing, a new aliquot of sodium azide solution was added to the gels. Gels were maintained at 25 °C during the test. The equilibrium swelling was reached when the weight of the samples changed by no more than 3%. The swelling ratio (q) was calculated as:

$$q = (Ws - Wd)/Wd$$

where Ws is the weight of swollen gels and Wd is the weight of WBAX in the gel.

From swelling measurements, the molecular weight between two cross-links (Mc), the cross-linking density (ρc) and the mesh size (ξ) values of the different WBAX gels were obtained as described before [18].

4. Conclusions

Wheat bran arabinoxylan gels with different rheological and structural characteristics can be obtained by modifying the polysaccharide structural features such as molecular weight, A/X ratio and ferulic acid content or by using different polysaccharide concentrations. The incubation time of extraction of wheat bran showed a direct effect on the structural features of arabinoxylans investigated in this study. These differences could be reflected in the functional properties of the arabinoxylans gels such as the water absorption capacity.

Further research is undergoing in order to explore the controlled release properties of these arabinoxylans gels.

Acknowledgments

This research was supported by Fondo Institucional SEP-CONACYT, Mexico (grant 134301 to E. Carvajal-Millan). The authors are pleased to acknowledge Valérie Micard, Cécile Barron and Aurélie Putois (SupAgro, INRA, Montpellier, France) and Alma C. Campa-Mada, Karla G. Martínez-Robinson, M. Carmen Granados Nevarez and Francisco Vazquez Lara (CIAD, A.C.) for collaboration in this project.

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CAPITULO V

Arabinoxylan Gels as Delivery Devices of Proteins: Defining Mass Ratio Compatibility by Confocal Laser Scanning Microscopy

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En preparación para ser enviado al journal Carbohydrate Polymers

Abstract

Arabinoxylan gels entrapping model proteins at different mass ratios were formed. The distribution of protein through the network was investigated by confocal laser scanning microscopy. In mixed gels, protein aggregates forming clusters were detected at protein/polysaccharide ratios higher than 0.25. These clusters were not homogenously distributed, suggesting that arabinoxylan and protein are located in two different phases. The apparent diffusion coefficient (Dm) of proteins during release from mixed gels was investigated for mass ratios of 0.06 and 0.12, respectively. For insulin, Dm increased significantly from $2.64x10^{-7}$ to $3.20x10^{-7}$ cm^2/s as the mass ratio augmented from 0.06 to 0.12. No significant difference was found for Dm values of ovalbumin and bovine serum

albumin released from the mixed gels. The results indicate that homogeneous protein/arabinoxylan gels can be formed at low mass ratios, allowing the estimation of Dm by using an analytical solution of the second Fick's law.

Keywords: Arabinoxylans; ferulic acid; proteins; phase separation; controlled release.

1. Introduction

Gels are polymeric three-dimensional networks, which swell on contact with water but do not dissolve (Peppas & Khare, 1993). The water absorption property of gels confers them interesting properties in applications like food additives, enzyme immobilization and controlled release devices (Peppas *et al*., 2000). Gels as controlled release matrices have been used in food, medicine, agronomy and cosmetic industries. Gels made from synthetic polymers, gelling native or tailored polysaccharides; recently, non-toxic and highly biocompatible, are receiving increasing attention (Rodriguez *et al*., 2003). Studies concerning the utilization of polysaccharide gels as controlled release matrices included chitosan, alginate, modified dextran and starch derivatives (Gonzalez-Rodriguez *et al*., 2002, Hennink *et al*., 2002, Mulhbacher *et al*., 2001). Gel-based delivery devices can be used for oral, rectal, ocular, epidermal and subcutaneous application. In oral administration, gels can deliver drugs to four major sites: mouth, stomach, small intestine, and colon. The gastrointestinal (GI) tract is the most common route for drug delivery, because of the ease of administration and its large surface area for systemic absorption. However, it is also the most complex route, so that the gel-based devices design is needed to deliver drugs in specific sites in the GI tract (Peppas *et al*., 2000).

Research on the controlled release of peptides and proteins is expanding as they are becoming an increasingly important class of therapeutic agents.

Nevertheless, oral delivery of peptides and proteins to GI tract is complicated because of protein inactivation by digestive enzymes in the GI tract and poor epithelial permeability of these drugs (Cummings *et al.,* 1992; Metcalf *et al.,* 1987). The colonic region, due to its lower proteolytic activity in comparison to that in the small intestine, has been considered as a possible absorption site for orally administrated peptides and proteins (Hennink *et al*., 2002). Gels made from polysaccharides like dextran, amidated pectine and arabinoxylans, which form gels resistant to enzymic digestion but degraded in the presence of colonic enzymes or microflora, are potential devices for colon-specific drug delivery (Brondsted *et al*., 1996; Vansteenkiste *et al*., 2004). Thus, oral administrated polysaccharide gels loaded with peptides or protein therapeutic agents can protect the drug while passing through the stomach and small intestine and allow its release in the colon during gel degradation.

Arabinoxylans (AX) are non-starch polysaccharides from the cell walls of cereal endosperm and pericarp constituted by a linear backbone of β-(1→4)-linked xylose units containing α-L-arabinofuranosyl substituents attached trough O-2 and/or O-3 (Izydorczyk & Biliaderis, 1995). Arabinoxylans have been classified as water extractable (WEAX) or water-unextractable (WUAX). AX may have some of the arabinose residues ester-linked on (O)-5 to ferulic acid (FA) (3-methoxy, 4 hydroxy cinnamic acid), being so called “ferulated”. Ferulated WEAX can gel by covalent cross-linking involving FA oxidation by some chemical or enzymatic (laccase and peroxidase/H_2O_2 system) free radical-generating agents (Geissman & Neukom, 1973; Hoseney & Faubion, 1981; Izydorczyk *et al*., 1990; Figueroa-Espinoza & Rouau, 1998). Diferulic acids (di-FA) (Figueroa-Espinoza *et al*., 1998; Schooneveld-Bergmans *et al*., 1999; Vansteenkiste *et al*., 2004) and tri-ferulic acid (tri-FA) (Carvajal-Millan *et al*., 2005a) have been identified as covalent cross-links structures in laccase gelled WEAX. Both, covalent bridges

(diFA, tri-FA) and physical interactions between AX chains have been reported to be involved in the WEAX gelation process and the final gel properties (Vansteenkiste *et al*., 2004; Carvajal-Millan *et al*., 2005a, b). WEAX gels present interesting properties like neutral taste and odour, high water absorption capacity (up to 100 g of water per gram of dry polymer) and absence of pH or electrolyte susceptibility (Izydorczyk & Biliaderis, 1995). The macroporous structure of AX gels with mesh sizes varying from 48 to 400 nm (Carvajal-Millan *et al*., 2005b; Berlanga-Reyes *et al*., 2009a; Martínez-López *et al*., 2011), and the dietary fiber nature of arabinoxylans (resistant to digestive enzymes but degraded by colonic microflora (Van Laere *et al*., 2000) confer them potential applications for colon-specific protein delivery. In this study, model proteins of different molecular weight were entrapped in arabinoxylans gels at different protein/polysaccharide mass ratio and the distribution of protein through the network was investigated by confocal laser scanning microscopy. Protein release capacity of mixed gels was then investigated and related to the gel structure.

2. Experimental

2.1. Materials

Arabinoxylans were obtained from wheat bran (WBAX) as previously described by Berlanga-Reyes *et al*. (2011). They presented an A/X ratio of 0.80, a ferulic acid content of 0.008 µg/mg arabinoxylans, a molecular weight of 74 kDa, and a $[\eta]$ of 206 mL/g. Laccase (benzenediol: oxygen oxidoreductase, E.C.1.10.3.2) from *Trametes versicolor*, and all chemical reagents were from Sigma Chemical Co. (St. Louis, MO, USA). Laccase activity was measured as reported elsewhere (Carvajal-Millan *et al*., 2005a).

2.2. Preparation of WBAX Gels

An arabinoxylan solution at 5% (w/v) in polysaccharide was prepared in citrate phosphate buffer 0.1 M pH 5.5. This solution was mixed with laccase (1.675 nkat/mg WBAX). Gels were allowed to form for 6 h at 25°C.

2.3. Phenolic Acids Content

Ferulic acid (FA), dimers of ferulic acid (di-FA) and trimer of ferulic acid (tri-FA) contents were determined in arabinoxylans gels by RP-HPLC after esterification step. FA and di-FA were quantified as described by Vansteenkiste *et al*. (2004). Response factors of di-FA determined by Saulnier *et al*. (1999) were used. Tri-FA (tri-FA 4-O-8', 5'-5''dehydrotriferulic acid) was quantified as described by Rouau *et al*. (2003). An Alltima C_{18} column (250 $\times$ 4.6 mm) (Alltech associates, Inc. Deerfield, IL) and a photodiode array detector Waters 996 (Millipore Co., Milford, MA) were used. Detection was followed by UV absorbance at 320 nm.

2.4. Rheological Tests

Rheological tests were performed by small amplitude oscillatory shear by using a strain controlled rheometer (ARES 2000, Rheometric Expansion System, Rheometric Scientific, Champ sur Marne, France) as reported before (Vansteenksite *et al*., 2004; Berlanga-Reyes *et al*., 2009a). WBAX gelation was studied for 6 h at 25°C. All measurements were carried out at 0.25 Hz frequency and 5% strain (in linear domain).

2.5. WBAX Gel Swelling and Structure

WBAX gels were allowed to swell as described elsewhere (Carvajal-Millan *et al*., 2005b). The equilibrium swelling was reached when the weight of the samples changed by no more than 3%. The swelling ratio (q) was calculated as:

$q= (W_s-W_d)/W_d$ (1)

where W_s is the weight of swollen gels and W_d is the weight of WBAX in the gel. From swelling measurements, the molecular weight between two cross-links (Mc), the cross-linking density (ρ_c) and the mesh size (ξ) values of the different WBAX gels were obtained as reported by Carvajal-Millan *et al*. (2005b). Mc, ρc and ξ was calculated using the model of Flory and Rehner (1943) modified by Peppas and Merrill (1976) for gels where the cross-links are introduced in solution (Peppas *et al*. (1985, 2000)).

2.6. Preparation of Protein/ WBAX Gels

WBAX and insulin (5 kDa), ovalbumin (43 kDa) or bovine serum albumin (67 kDa) solutions in 0.05 M citrate phosphate buffer pH=5.5 were mixed in order to prepare five protein/ WBAX mixtures at mass ratios of 0.06, 0.12, 0.25, 0.5 and 1.0) and control gels containing only WBAX. Laccase (1.675 nkat per mg WBAX) was added as cross-linking agent. Gels were allowed to form for 6 h at 25°C.

2.7. Confocal Laser Scanning Microscopy (CLSM)

CLSM was performed with an IRBE microscope integrated with a model TCS-SP confocal scanner head (Leica Microsystems, Mannheim, Germany). CLSM was used in the fluorescent mode, with rhodamine iso thyocyanate (RITC) at 0.02% (w/v) used as fluorescent probe. Samples were mounted in a universal stage, on slides or within microwell dishes (MatTek Inc., Ashland, Mass., U.S.A.), and then illuminated with the 543-nm line from an argon laser through an RSP500 dichroic filter. Reflection from sample surfaces was collected with a long working distance (1.15 mm) 20×lens (numerical aperture = 0.5) in a single, 8-bit channel and the optimal detector pinhole; begin and end limits of the vertical series were set visually in the Z-wide scan control. Gain and black levels

for the channel 2 photomultiplier were adjusted through digital control in the glow/over/under look-up-table and two 512 × 512 pixel frames were averaged in each optical section. Image stacks with the optimal number of sections for maximal z-resolution (for the lens) were transformed into topographical images for measurements in the Leica LCS Materials software package or converted to 3-D images.

2.8. Protein Release

Protein release from protein/WBAX gels was measured followed to Carvajal-Millan *et al*. (2005c). Quantification of released protein was performed by Bradford's assay (Bradford, 1976). Protein release was characterized by calculating an apparent diffusion coefficient (Dm). This Dm was estimated from the release kinetics curve, fitted by using an analytical solution of the second Fick's law, which gives the solute concentration variation as a function of time and distance (Crank, 1975).

$$\frac{\mathrm{Mt}}{\mathrm{Mo}} = \frac{4}{\mathrm{L}}\left(\frac{\mathrm{Dm}\,t}{\pi}\right)^{0.5}$$

Where Mt is the accumulated mass of protein released at time *t*, Mo is the mass of protein in the gel at time zero, L is the sample thickness (0.3 cm) and Dm is the coefficient. By plotting the relative solute mass released (Mt/Mo) at time *t*, versus the square root of time, a simplified determination of Dm can be made assuming that Dm is constant and that the sample is a plate with a thickness (L). In this study the apparent Dm was calculated from the linear part of the Mt/Mo (*t*) curves (Favre & Girard, 2001). The percentage of protein released at the end of the test was also calculated.

3. Results and Discussion

3.1. WBAX Gel Characterization

The formation of 5% (w/v) WBAX gels over time was rheologically investigated by small amplitude oscillatory shear. The storage (G´) and loss (G'') modulus registered final G' and G'' values of 177 and 20 Pa, respectively (Table 1), which are close to those recently reported in maize bran arabinoxylans gels at 4% (w/v) (Martínez-López *et al*., 2011). FA, di-FA and tri-FA contents of the WBAX were measured before and after 6 h of gelation (Table 1). FA was oxidized (75% of initial FA content) during the gelation process. After gelation the di-FA content in a WBAX did not increase, but rather decreased from 0.01 to 0.008 µg/mg polysaccharide, respectively. The tri-FA was present only in low amounts. Nevertheless, the tan δ (G''/G'=0.11) value at the end of gelation confirms the formation of a true gel after laccase exposure. These results could be related to the formation of ferulated cross-linking structures which cannot be released by mild alkaline hydrolysis and/or to physical interactions between arabinoxylan chains, as suggested before by several authors in different arabinoxylans gels (Vansteenksite *et al*. 2004; Carvajal-Millan *et al*., 2005b; Berlanga-Reyes *et al*., 2009; Martínez-López *et al*., 2011). In the present study, the di-FA in WBAX gels were 8-5' (81%), 5-5' (3%) and 8-O-4' (16%).

The equilibrium swelling of WBAX gels was reached between 15-20 h presenting a swelling ratio (q, g water/g WBAX) of 9. This high water uptake could be explained in terms of low covalent cross-links content (di-FA, tri-FA) and therefore, the existence of longer uncross-linked arabinoxylan chains sections in the network. Uncross-linked polymer chains sections in the gel can expand easily conducting to higher water uptake values (Meyvis *et al*., 2000). From swelling measurements, the molecular weight between two cross-links (Mc), the cross-linking density (ρc) and the mesh size (ξ) values of arabinoxylan

gels were calculated registering values of 29 x10^3 g/mol, 59 x10^{-6} mol/cm^3 and 57 nm, respectively (Table 1).

3.2. Protein Distribution in the Gels

The distribution of protein through the network was investigated by carrying out confocal laser scanning microscopy observations of mixed protein/WBAX gels. Bright zones corresponding to protein aggregates forming clusters were detected at protein/polysaccharide ratios higher than 0.25, the size of which increased with the content of entrapped protein. These clusters were not homogenously distributed, suggesting that polysaccharide and protein are located in two different phases as a result of thermodynamic incompatibility (Fig. 1). The same behaviour was registered for the three different model proteins used in the present study (insulin, ovalbumin, bovine serum albumin). It has been reported between a protein and neutral polysaccharide mixture, the protein is usually enhanced by self-association of the protein molecules resulting in a segregative phase separation (Grinberg & Tolstoguzov, 1997).

3.3. Protein Release

The release of proteins from gels was investigated at different protein/WBAX mass ratios of 0.06 and 0.12, respectively, which presented a homogenous distribution of protein through the network (Section 3.2), allowing the estimation of Dm by using an analytical solution of the second Fick's law. Fig. 2 shows the protein release profiles of these model proteins at those protein/WBAX mass ratios. At the same mass ratio, different release rates were obtained for the three proteins tested. As shown in Fig. 2, linear relationships between Mt/Mo and the square root of time were found for protein release from gels, allowing the calculation of Dm, which are given in Table 2. The diffusion coefficients of these proteins in water (Do) as reported in the literature (Nauman *et al*., 2007; Amsden, 1998; Cole *et al*., 1998) are given for comparison. For insulin, Dm

increased significantly from $2.64x10^{-7}$ to $3.20x10^{-7}$ cm^2/s as the mass ratio augmented from 0.06 to 0.12 while no significant difference was found for Dm values of ovalbumin and BSA. The Dm values were in the range reported for proteins in hydrated gels reported by Gehrke *et al*., (1998). The entrapment and release of insulin in maize bran arabinoxylans gels at 2.5 % (w/v) has been previously investigated (protein/arabinoxylan mass ratio of 0.04, G' value of 9 Pa, mesh size of 80 nm and di-FA and tri-FA content of 0.03 and 0.015 μg arabinoxylan, respectively) (Berlanga-Reyes *et al*., 2009a,b). That study reported an insulin Dm value of $0.99x10^{-7}$ cm^2/s, which is lower than that obtained in the present research ($2.64\text{-}3.20x10^{-7}$ cm^2/s). The latter could be due to the lower insulin/arabinoxylan mass ratio used in that study (0.04) in comparison to those considered in the present study (0.06 and 0.12). A higher amount of protein entrapped in the gel could induce a reduction in the connectivity of arabinoxylan network and/or an increase of protein gradient concentration between the gel and the recovery medium (Amsden, 1998). In another report, a method where different proteins were loaded into cured wheat WEAX gels by making protein solution to diffuse into the gel has been developed; however, the method wastes protein and requires long time. From protein/arabinoxylans mass ratios from 0.1 to 0.4 these authors found Dm values from $3.0x10^{-7}$ to $6.8x10^{-7}$ cm^2/s for ovalbumin and from $1.9\ x10^{-7}$ to $5.9\ x10^{-7}$ cm^2/s for BSA; which are higher than those found in the present research for the same proteins. These differences in Dm could be related to the higher mesh size values of WEAX gels used in that study (201-331 nm) in relation to that found in our study (58 nm). The differences in Dm values for the model proteins tested in the different protein/WBAX gels were also reflected in the percentage of protein released at the end of gels incubation. The percentages of protein released were from 3.2 to 7.5 (Table 2). In general, a low amount of the entrapped protein was released by diffusion. This result indicates that as delivery matrices, protein/ WBAX gels would liberate most of entrapped protein

only after gel degradation in specific sites such as the colon where arabinoxylans can be degradated by colonic bacteria.

4. Conclusions

Model proteins did not avoid WBAX enzymatic gelation but at protein/polysaccharide mass ratios higher than 0.25 these biomolecules are located in two different phases. For a low molecular weight protein such as insulin, release rate and quantity can be significantly modified by varying the amount of protein entrapped. Only a low amount of the protein entraped in WBAX gels is released by diffusion. These result indicates that WBAX gels could be suitable delivery matrices for protein releasein specific sites such as the colon after polysaccharide degradation.

Acknowledgments

Authors are very thankful to Valérie Micard and Cécile Barron (SupAgro, Montpellier, France) for phenolics analysis and to Stephanie Simon, Douglas S. Soroka, Guoping Bao and Winnie Yee (USDA, USA) for the CLSM micrographs. M.Sc. Berlanga-Reyes thanks CONACYT, Mexico for providing a doctoral scholarship.

Table 1. Characteristics of WBAX before and after 6 h gelation

	T = 0 h	t = 6 h
FA (μg/mg WBAX)	0.008 ± 0.001	0.002 ± 0.001
di-FA (μg/mg WBAX)	0.010 ± 0.001	0.008 ± 0.001
tri-FA (μg/mg WBAX)	0.0005 ± 0.001	0.0003 ± 0.001
G' (Pa)	4	177
G'' (Pa)	6	20
$Mc^a \times 10^3$ (g/mol)	-	29 ± 1
$\rho c^b \times 10^{-6}$ (mol/cm^3)	-	59 ± 2
ξ^c (nm)	-	57 ± 8

[a] Molecular weight between two cross-links; [b] Cross-linking density; [c] Mesh size; All values are means ± standard deviation from triplicates.

Table 2. Apparent diffusion coefficients of proteins of different molecular weight in WBAX gels at different protein/polysaccharide mass ratios.

			$Dm^b \times 10^{-7}$ (cm^2/s)		Protein released (%)	
Protein	Mw (kDa)	$Do^a \times 10^{-7}$ (cm^2/s)	Protein/WBAX mass ratio 0.06	0.12	Protein/WBAX mass ratio 0.06	0.12
Insulin	5	15.9	2.64 ± 0.07	3.20 ± 0.06	6.2 ± 0.4	7.5 ± 0.5
Ovalbumin	43	8.40	1.47 ± 0.01	1.49 ± 0.04	5.1 ± 0.5	4.4 ± 0.3
BSA	67	6.80	1.20 ± 0.01	1.25 ± 0.03	3.5 ± 0.2	3.2 ± 0.3

[a] Diffusion coefficient of proteins in water from the literature (Nauman et al., 2007; Amsden, 1998; Cole et al., 1998)
[b] Apparent diffusion coefficient of proteins in the WBAX gels
All values are average from duplicates

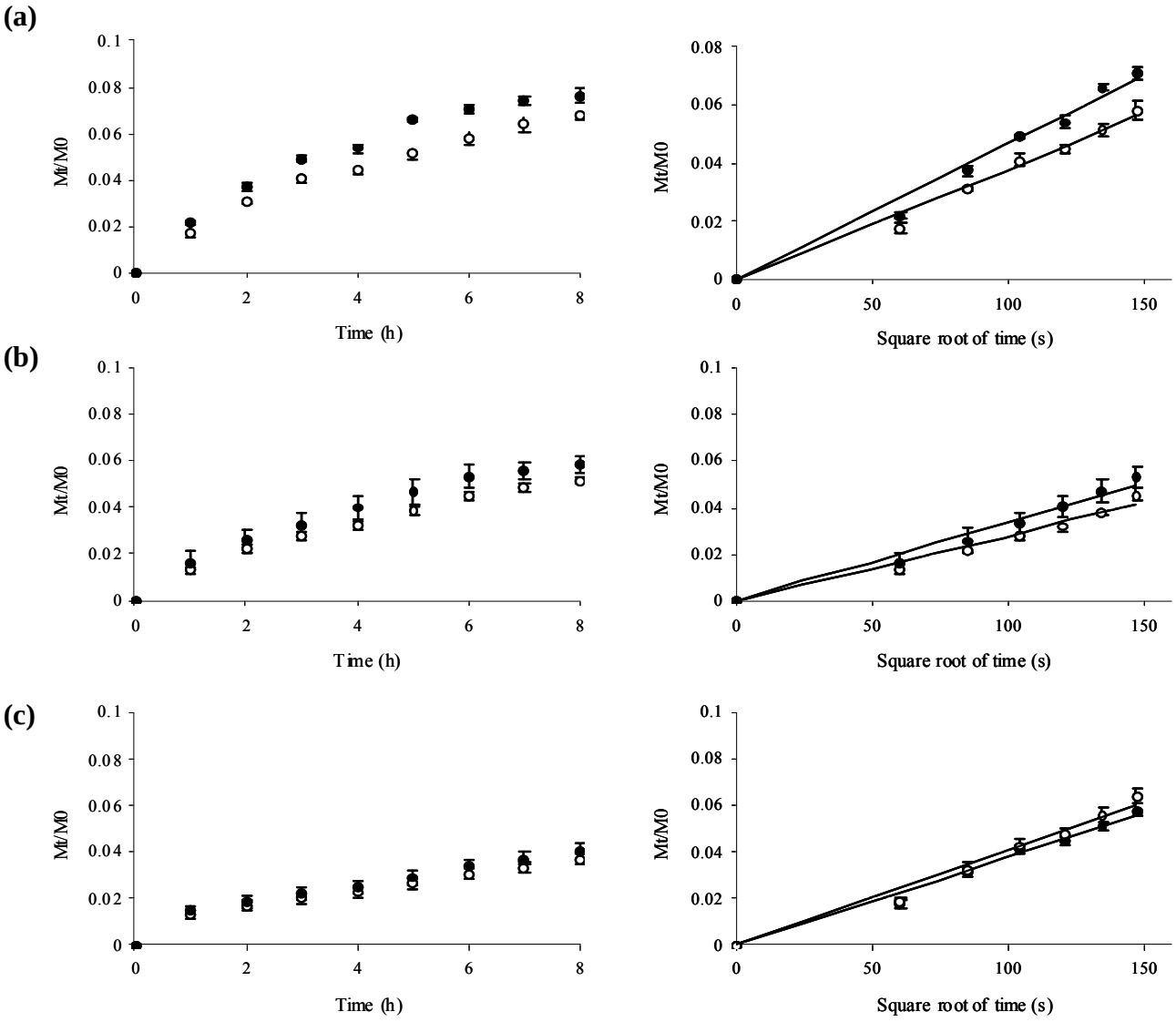

Figure 1. Cumulative release (Mt/Mo) of insulin (a), ovalbumin (b) and bovine serum albumin (c) from WBAX gels as a function of time and as a function of square root time. Protein release was followed at 25°C and 90 rpm during 8 h. Gels at protein/WBAX mass ratios of 0.06 (○) and 0.12 (●).

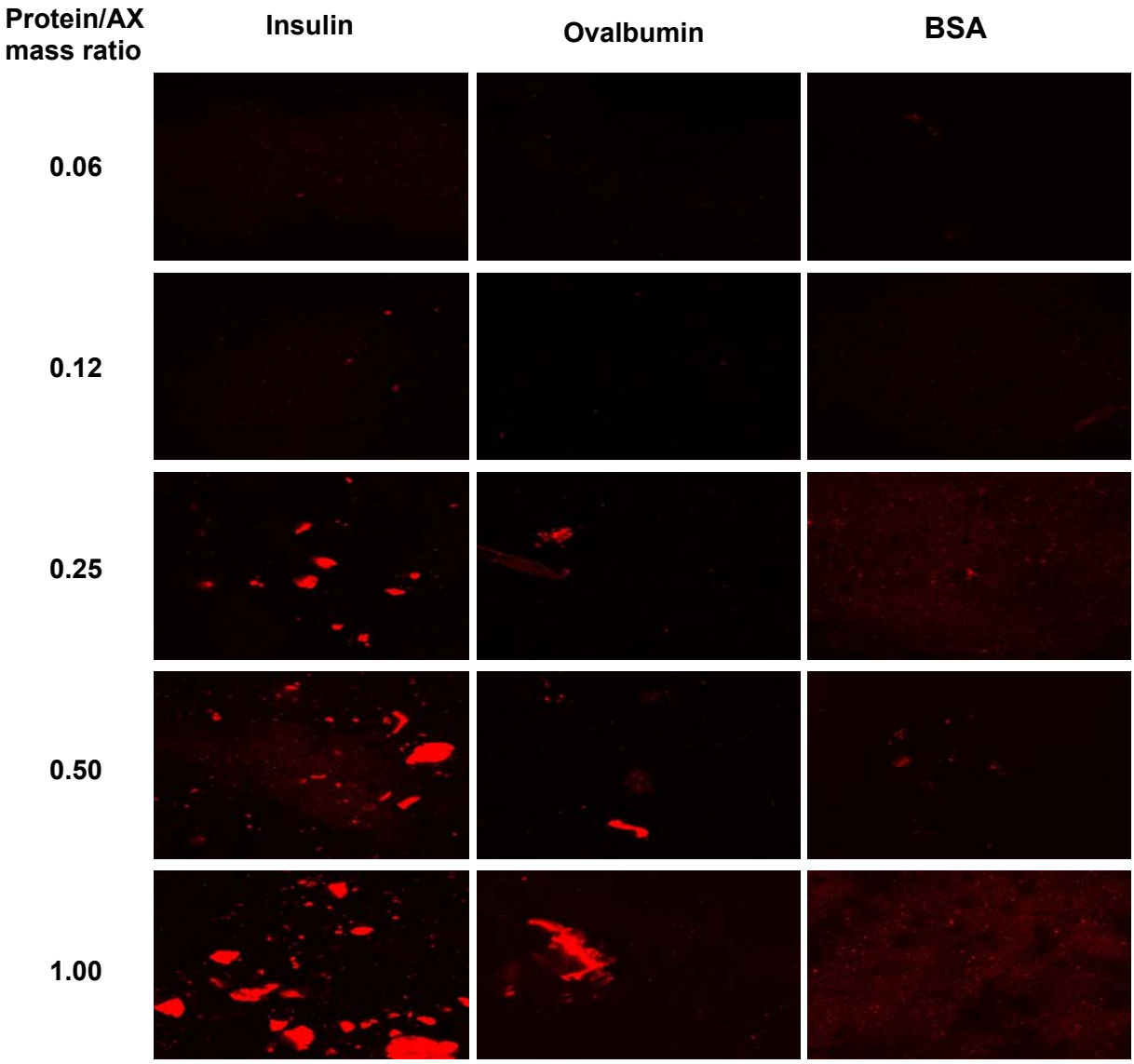

Figure 2. CLSM observations of WBAX gels at different protein/polysaccharide mass ratios 0.06 to 1 at 25 °C. Proteins stained by rhodamine iso thiocyanate (RITC) at 0.02% (w/v). Protein phase is shown as a bright zone.

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