UNIVERSIDAD NACIONAL
AUTÓNOMA DE MÉXICO

FACULTAD DE QUÍMICA

PROGRAMA DE MAESTRÍA Y DOCTORADO
EN CIENCIAS BIOQUÍMICAS

ESTUDIO DE LA RELACIÓN ESTRUCTURA-FUNCIÓN DE UNA α-ZEÍNA CON PROPIEDADES EMULSIFICANTES

T E S I S

QUE PARA OBTENER EL GRADO DE:

DOCTORA EN CIENCIAS (BIOQUÍMICAS)

P R E S E N T A :

VANESSA DEL CARMEN
CABRA LEDESMA

Tutora: DRA. AMELIA FARRÉS GONZÁLEZ-SARAVIA

MÉXICO, D. F. Septiembre 2006.

ESTUDIO DE LA RELACIÓN ESTRUCTURA-FUNCIÓN DE UNA α-ZEÍNA CON PROPIEDADES EMULSIFICANTES

RECONOCIMIENTOS

Esta tesis doctoral se realizó bajo la dirección de la Dra. Amelia Farrés González-Saravia en el laboratorio 312, en el Departamento de Alimentos y Biotecnología del Conjunto "E" de la Facultad de Química y en los Laboratorios del Departamento de Bioquímica del Instituto de Química de la Universidad Nacional Autónoma de México.

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Se reconoce la colaboración del Dr. Guillermo Mendoza, del Departamento de Bioquímica de la Facultad de Medicina, en cuyo laboratorio se llevó a cabo la secuenciación de la proteína.

Se reconoce la asesoría técnica de la M. en C. Idalia Flores Argüello en los experimentos de evaluación de las propiedades emulsificantes.
Se reconoce la asesoría técnica de la M. en C. Sandra Pérez Munguía en los experimentos de caracterización de la proteína.

El proyecto fue apoyado parcialmente por CONACYT (35948-B) y PAEP-UNAM (203304). Durante los estudios de doctorado goce de una beca otorgada por CONACYT con no. 159981 y DGEP-UNAM para la realización de la presente tesis.

El Jurado de Examen Doctoral estuvo constituido por:

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A MIS PAPÁS, gracias por ser la luz que guía mi camino e ilumina mi vida. Su inagotable cariño ha sido la fuerza que me ha permitido llegar hasta este momento. Gracias por reir y llorar conmigo, por compartir mis alegrías, sueños, y triunfos pero también mis tristezas y desvelos, por estar siempre conmigo sin necesidad de pedírselos, por conocerme quizá más de lo que me conozco yo misma. Gracias por tanto amor. Dios me bendijo con unos padres maravillosos. Los amo.

A MIS HERMANOS:
JOSÉ, porque con hechos más que con palabras me haces saber a cada momento que me apoyas, que cuento contigo. Gracias por tantas horas de espera, por traerme y llevarme cada vez que lo necesité, por cuidarme, pero sobretodo, por quererme. Te quiero mucho.
ALEJANDRO, porque tus palabras me arrancan una sonrisa en los momentos que más lo necesito. Gracias por hacerme sentir siempre unida a ti, eres un ser mágico y muy especial al que quiero con todo mi corazón.

A MIS TIOS:
Inés, por tu cariño inmenso e incondicional, te quiero y admiro muchísimo.
Yula, Augusto(†), Hermilo y Amelia, por sus palabras de aliento y muestras constantes de cariño.
Haroldo, por ser un hermano más para mis padres y un super-tío para mis hermanos y para mí, te queremos mucho.

A LA DRA. AME, gracias por todos estos años, por haber creído y confiado en mí desde el principio de esta fantástica experiencia, por ser una gran tutora, pero sobretodo, una gran amiga de la cual aprendí y aprendo cada día. La admiro y quiero mucho. Siempre será un gran ejemplo a seguir.

AL DR. ROBERTO, por haber sido parte fundamental de todo esto. Gracias por su apoyo incondicional. Disfruté y aprendí muchísimo del trabajar con usted. Gracias por ser un excelente guía, pero especialmente, por ser un gran ser humano.

AL DR. RAFAEL, por estar desde el inicio en esta aventura, fue un gran placer escuchar sus sugerencias, críticas y observaciones. Gracias por todo su apoyo.

A LOS MIEMBROS DEL JURADO, por que cada observación enriqueció el presente trabajo, pero lo más importante, aportó a mi formación académica. Muchas gracias.

AL DR. BARBARÍN, por el oportuno consejo y las divertidas charlas de las que siempre aprendí algo y disfruté mucho.

A MIS AMIGOS, Anel, Carmen, Paty, Alicia, Caro, Lilia, Ana María, Mariana, Adriana, Denisse, Oliver, Tere, Chucho, Miguel, Francisco, Héctor, Lalo, Gaby, Maricruz y Rodrigo. Dicen que la amistad es como cada una de las caras de un diamante, que difracta la luz de diferente forma, pero sigue siendo una luz. Gracias a cada uno de ustedes por su cara de diamante.

A LETY Y ADE, gracias por tantos y tantos problemas resueltos, papeleos y trámites que "urgían para ayer", mil gracias por todo!.

A LAS DOCOTRAS AMANDA Y MARICARMEN Y A TODOS MIS COMPAÑEROS DEL LABORATORIO 312, por todas las experiencias, horas de trabajo, conocimientos y especialmente festejos compartidos a lo largo de todos estos años, fueron una parte muy importante de mi formación académica y humana.

A LOS DRES. EDGAR VÁZQUEZ, ENRIQUE GARCÍA, ADELA RODRÍGUEZ Y ABEL MORENO DEL DEPARTAMENTO DE BIOQUÍMICADEL INSTITUTO DE QUÍMICA, por estar siempre dispuestos a ayudarme y resolver mis dudas, por compartir con migo su tiempo, conocimientos y espacio (y una que otra pizza, pastelito o antojito).

A LA UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

A la que llevo orgullosamente tatuada en el corazón.

CONTENIDO

III. ABREVIATURAS

ACN: Acetonitrilo
ANS: Ácido 1-anilinonaftalen-8-sulfónico (1-anilinonaphthalene-8-sulfonic acid)
ASB: Albúmina sérica bovina
BME: β-mercaptoetanol
CHAPS: 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate
DC, CD: Dicroismo circular (Circular dichroism)
DH: Grado de hidrólisis (Degree of hydrolysis)
DIG: Digoxigenina
DLS: Dispersión dinámica de la luz (Dinamic Light Scattering)
DTNB: Ácido 5'5'-ditiobis(2-nitrobenzóico) (5'5'-dithiobis(2-nitrobenzoic acid))
DTT: Ditiotreitol
EDTA: Ácido etilendiaminotetraacético (Ethilendiaminotethracetic acid)
EE, ES: Estabilidad de emulsión (Emulsion stability)
FI: Intensidad de fluorescencia (Fuorescence intensity)
GD, DD: Grados de desamidación (Deamidation degree)
HIC: Cromatografía de interacción hidrofóbica (Hydrophobic interaction chromatography)
HMW: Alto peso molecular (High Molecular Weight)
IEX: Cromatografía de intercambio iónico (Ion exchange chromatography)
IFE: Isoelectroenfoque (Isoelectric focusing)
IPA: 1-isopropanol
LMW: Bajo peso molecular (Low molecular weight)
MALDI-TOF- MS: Matrix-assisted laser desorption ionization time-of-flight mass Spectrometry
O/W: Aceite en agua (Oil in water)
PG: Protein glutaminasa (Protein glutaminase)
PGase: Peptidoglutaminasa (Peptidoglutaminase)
PI: Punto isoeléctrico
PVDF: Polifluoruro de vinilideno (PolyVinylidine DiFluoride)
RP-HPLC: Cromatografía de fase reversa (Reverse phase chromatography)
SDS: Dodecil sulfato de sodio (Sodium dodecyl sulphate)
SDS-PAGE: Electroforésis en gel de poliacrilamida con dodecil sulfato de sodio (Sodium dodecyl sulfate – polyacrylamide gel electrophoresis)
SP: Sulfopropil
S_o: Índice de hidrofobicidad superficial (Surface hydrophobicity index)
TFA: Ácido trifluoroacético (Trifluoroacetic acid)
W/O: Agua en aceite (Water in oil)

IV. INTRODUCCIÓN

La relación estructura-función en proteínas

Las proteínas desempeñan papeles cruciales en casi todos los procesos biológicos, llevan a cabo una gran variedad de funciones como lo son la catálisis enzimática, el transporte y almacenamiento de pequeñas moléculas (ej.: la hemoglobina), el soporte mecánico (ej.: colágeno), la protección inmune, la generación y transmisión de impulsos nerviosos, el control del crecimiento y diferenciación celular, entre otras. Las funciones de las proteínas están determinadas por sus estructuras tridimensionales. Dada la importancia que las proteínas tienen para la vida, es esencial la comprensión de la relación entre su estructura y su función. A pesar de los avances en el campo de estudio de las proteínas, estamos lejos de entender completamente esta compleja relación. En los últimos años, la obtención de estructuras proteicas se ha acelerado de forma tal que, hasta el momento se han determinado cerca de 38,198 estructuras de proteínas y otras macromoléculas, lo que aporta información acerca de la relación entre la estructura y la función (tan sólo hace 6 años se contaba con solo 13,629 estructuras) (Protein Data Bank, página accesada el 15 de agosto de 2006: http://www.rcsb.org/pdb/contentGrowthChart.do?content=total&seqid=100).

Una forma de estudiar la relación estructura-función de las proteínas es la modificación de las cadenas laterales de los aminoácidos (Richardson, 1985), la que puede traer como consecuencia diversos cambios en la estructura proteica como lo es el desplegamiento parcial o total de la cadena polipeptídica o la alteración de la superficie hidrofóbica de la proteína la cual puede resultar en importantes cambios en la función de la misma.

Las interacciones hidrofóbicas son extremadamente importantes en el plegamiento de las proteínas así como en sus asociaciones (Janin y Chothia, 1978).

El hombre les ha encontrado otra utilidad a las proteínas, que es la de ser ingredientes al incluirlas en la formulación de alimentos procesados debido a sus propiedades funcionales, término que define, en el campo de la industria alimentaria, a todas aquellas propiedades fisicoquímicas de las proteínas que les permiten contribuir con características deseables en los productos alimenticios (Fligner y Mangino, 1991). Es así que las proteínas se utilizan para mejorar el sabor, retener mayor cantidad de agua, aumentar o disminuir la viscosidad, gelificar, contribuir en la formación de masas porque pueden retener agua, participar en la formación de geles, modificar la textura

(viscosidad), promover la formación de extrudidos y obtener fibras más resistentes así como estabilizar espumas y emulsiones en los alimentos.

El presente trabajo está enfocado en conocer y establecer la relación que existe entre las características estructurales de la α-zeína Z19 y sus propiedades emulsificantes. La generación de una emulsión involucra el mezclado de una fase oleosa y una acuosa. Cuando las regiones no polares de una proteína se exponen a la fase acuosa tienden espontáneamente a asociarse de manera tal que los contactos con el agua se minimizan. En las emulsiones aceite en agua la proteína rodea a los glóbulos grasos, los estabiliza y mantiene en suspensión. Ejemplos de emulsiones alimenticias de este tipo son la mayonesa, los aderezos para ensaladas y la leche. Para la industria alimentaria las propiedades emulsificantes de las proteínas son de gran importancia por lo que el estudio de sus estructuras y la relación que tienen con la funcionalidad que presentan es determinante para lograr contar con mejores proteínas emulsificantes.

Aspectos importantes en el estudio de la relación estructura-función emulsificante de las proteínas son su hidrofobicidad, flexibilidad y estabilidad térmica. Existe una correlación positiva entre el índice de actividad emulsificante y la hidrofobicidad (Kato y Nakai, 1980). Ésta relación, junto con la propiedad de flexibilidad facilita la interacción con la superficie del aceite. En términos generales la desnaturalización puede promover mejoras en las propiedades emulsificantes lo que es atribuido al incremento de la movilidad conformacional de la proteínas (Poon, Clarke y Shultz., 2001; Sze y Sathe, 2001).

Origen del presente trabajo

En nuestro grupo de trabajo, el estudio de las proteínas del gluten de maíz se inició con la evaluación de sus propiedades funcionales y el análisis de los procesos de desamidación e hidrólisis enzimática, como alternativas para mejorarlas. Se determinó que estas proteínas en general presentan limitadas propiedades funcionales, sin embargo, se encontró, que la capacidad para emulsificar mejoraba si se sometía el gluten de maíz a un proceso de desamidación química (Flores, 1997, **Figura 1)**, este hecho fue verificado también por otros investigadores (Wu, 2001).

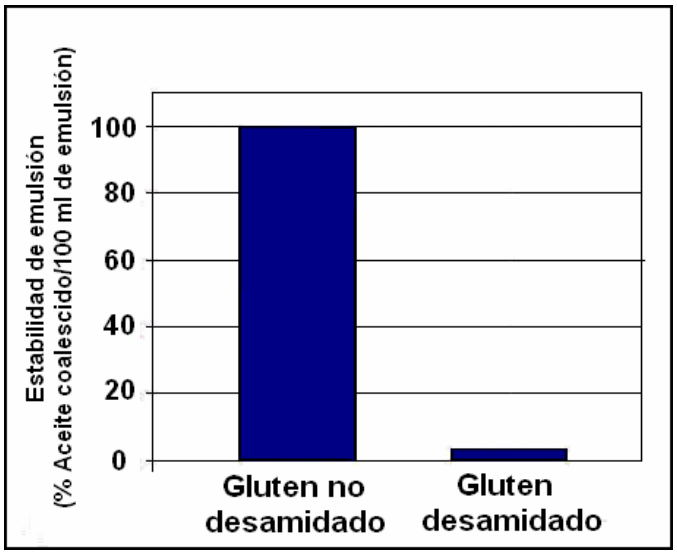

Figura 1. Efecto de la desamidación química del gluten de maíz con HCl (Flores, 1997)

Las proteínas mayoritarias del gluten de maíz son las zeínas (68%), el 27% corresponde a las glutelinas y el 1.2% a las globulinas. Para saber cuáles de todas estas proteínas participaban en la emulsificación del aceite, se llevó a cabo la investigación correspondiente (Cabra, 2002). Lo primero que se realizó fue la extracción cuantitativa de todas las proteínas presentes en la emulsión elaborada con gluten de maíz. Posteriormente, las proteínas se sometieron a un fraccionamiento con base en su solubilidad (albúminas, solubles en agua; globulinas, solubles en soluciones salinas; prolaminas, solubles en soluciones alcohólicas y glutelinas, solubles en soluciones alcalinas en presencia de agentes reductores) (Osborne y Mendel, 1914). Se resolvieron los patrones electroforéticos de las fracciones obtenidas y los pesos moleculares de las proteínas presentes, con los cuales se identificaron a cada una de ellas (Dickey y Dallmer, 1998) encontrando que las proteínas más abundantes en la emulsión, y por lo tanto, las principales responsables de las propiedades emulsificantes del gluten de maíz, son las zeínas (**Figura 2**).

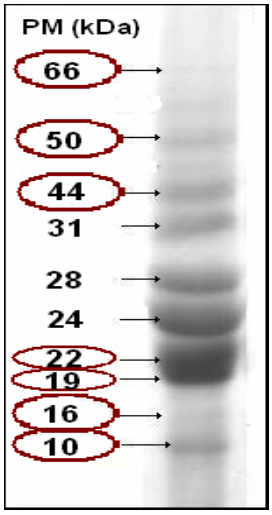

Figura 2. Perfil electroforético desnaturalizante (SDS-PAGE, 20% T) de las proteínas extraídas de la emulsión elaborada con proteínas del gluten de maíz. Los pesos moleculares (kDa) señalados con círculos corresponden a zeínas. La banda de 31 kDa corresponde a una glutelina y las restantes de 28 y 24 kDa a globulinas. Las muestras fueron tratadas con β-mercaptoetanol y urea 5 M y sometidas a ebullición durante 10 minutos. El gel se tiñó con azul de Coomassie (Cabra, 2002).

Las zeínas se clasifican en α, β, γ y δ con base en su solubilidad y peso molecular (Esen, 1986; Esen, 1987 Wilson, 1991). El más abundante de estos 4 grupos, es el de las α-zeínas que comprende entre el 75 al 85% del total de las zeínas y está compuesto por los grupos Z19 y Z22 con pesos moleculares aparentes de 23.8 y 26.7 kDa respectivamente (Esen, 1987; revisado en Hamaker y col., 1995).

A partir del análisis densitométrico del gel de la **Figura 2**, se determinó que las proteínas más abundantes en la emulsión eran las α-zeínas. Para confirmar lo anterior, se realizó el fraccionamiento de las proteínas extraídas con base en su solubilidad diferencial en etanol. Las α-zeínas son solubles en etanol al 95%, mientras que el resto de las zeínas se solubilizan en soluciones acuosas de etanol (40:70%, v:v). Tras haber obtenido dos fracciones, una de la mezcla de ambas α-zeínas y otra con las β, γ y δ-zeínas, se procedió a evaluar y comparar sus propiedades emulsificantes. Los resultados demostraron que las α-zeínas emulsifican mejor que el resto de las zeínas (**Figura 3**). Con base en estos resultados se decidió investigar algunas de las características estructurales de las α-zeínas para determinar si estaban directamente relacionadas con su capacidad para estabilizar interfases aceite/agua.

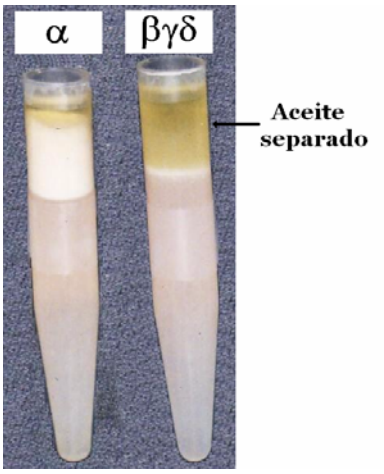

Figura 3. Resultados de estabilidad de emulsiones elaboradas con la mezcla de α-zeínas y la de las β, γ y δ zeínas. Menor cantidad de aceite separado indica mejores propiedades emulsificantes. (Método de Dagorn y col., 1987).

Otras razones importantes por las cuales se consideraron a las α-zeínas como objeto de estudio de esta investigación fueron las siguientes:

- Son abundantes, ya que constituyen aproximadamente el 60% del total de las proteínas presentes en el maíz (Watson, 1987), del 71 al 85% del total de las zeínas (Hamaker et al., 1995), y el 68% del contenido proteico del gluten del maíz (Wu, 2001).
- Mejoran sus propiedades emulsificantes cuando los residuos de aminoáciodos poseen carga negativa (Cabra, 2002).
- Poseen alto contenido de hélice α, característica importante en las proteínas emulsificantes (Argos y col., 1982).
- Presentan perfiles de hidropaticidad periódicos.
- Alto contenido de residuos de aminoácidos hidrofóbicos (superiores al 70%), índices alifáticos promedio de entre el 116-125 y de hidrofobicidad de 0.300-0.435.
- Poseen pocos residuos de cisteínas, lo que les da mayor flexibilidad.
- Tienen un alto contenido de glutamina, lo que les permite ser objeto de desamidación como forma de modificación.

La desventaja que presentan las α-zeínas en general, es que, debido a su gran hidrofobicidad, son insolubles en agua a pH 7 en la cual forman agregados que son difíciles de manejar e incorporar en los alimentos.

Para conocer los aspectos estructurales y funcionales en términos de emulsificación de estas proteínas se requirió hacer estudios con técnicas que implicaban trabajar con proteínas puras. Contar con este material fue la etapa inicial del presente trabajo a partir de la adaptación de diversos protocolos de purificación de proteínas vegetales.

V. MARCO TEÓRICO

Las proteínas en los alimentos

Las preferencias por determinados productos alimenticios por parte de los consumidores están predominantemente basadas en las propiedades organolépticas como lo son el color, el sabor y la textura. Las proteínas constituyen uno de los principales grupos de ingredientes que contribuyen a las propiedades sensoriales y nutrimentales de los alimentos.

Como ya se mencionó con anterioridad, en el área de la ciencia de estudio de los alimentos, se suele definir a las propiedades funcionales de las proteínas como aquellas propiedades fisicoquímicas que determinan su comportamiento durante el procesamiento, almacenamiento, preparación y consumo de los alimentos y que afectan su calidad y aceptación (Matil, 1971; Kinsella, 1981). La funcionalidad de las proteínas permite contribuir a que los alimentos exhiban características deseables. Las proteínas que presentan una gran variedad de propiedades funcionales importantes pueden encontrarse de manera natural en los alimentos (por ejemplo, la ovoalbúmina en los huevos, las proteínas del suero y la caseína de la leche, la miosina en la carne) o bien, ser adicionadas durante el procesamiento o manufactura de los mismos (Dickinson y McClements, 1992). Las propiedades fisicoquímicas y estructurales que influyen en el comportamiento funcional de las proteínas en los alimentos incluyen su forma, tamaño, composición y secuencia de aminoácidos, carga neta, distribución de carga, hidrofobicidad, hidrofilicidad, estructura (secundaria, terciaria y cuaternaria), flexibilidad molecular y rigidez en respuesta a factores del ambiente externo (tales como pH, temperatura y fuerza iónica), e interacción con otros de los constituyentes del alimento (grasas, lípidos y carbohidratos) (Damodaran y Paraf, 1997). La gran reactividad de las proteínas les permite reaccionar con azúcares reductores, grasas y sus compuestos de oxidación, polifenoles y muchos otros componentes de los alimentos. Las cantidades y propiedades físicas de la mayoría de los componentes de los alimentos así como los tratamientos mecánicos, térmicos, químicos y enzimáticos, provocan diversas modificaciones en las proteínas. Debido a ello, las propiedades funcionales de las proteínas se ven afectadas por estos factores, que son tanto intrínsecos como ambientales.

Cabe señalar que no siempre es correcto suponer que las proteínas deben estar dotadas de una elevada solubilidad inicial como prerrequisito para que manifiesten propiedades

funcionales, como por ejemplo, la emulsificación y el espumado. La absorción y retención de agua por un ingrediente proteico puede mejorarse, a veces, mediante una desnaturalización e insolubilización previas. Igualmente, la capacidad de formar geles puede no perderse durante la desnaturalización e insolubilización parciales. (Fennema, 1993; Velev y col., 1993; Mimouni et al., 1994; Linarés et al., 2000). Todo esto coincide con el hecho de que la formación de emulsiones, geles y espumas implica diversos grados de desplegamiento, agregación o insolubilización de las proteínas (Fennema, 1993).

Propiedades de superficie de las proteínas

Las propiedades de superficie de las proteínas son el resultado de la composición y distribución de los residuos de aminoácidos, de la flexibilidad molecular y, en menor grado, de la forma y tamaño de la molécula proteica (Morr y Ha, 1993).

Emulsificación y espumado: Ambas propiedades requieren de la capacidad de las proteínas para actuar como surfactantes y estabilizar emulsiones y espumas gracias a su capacidad inherente de adsorberse en las interfases, lo cual provoca una reducción considerable de la tensión interfacial y la formación de una película cohesiva (**Figura 4**)

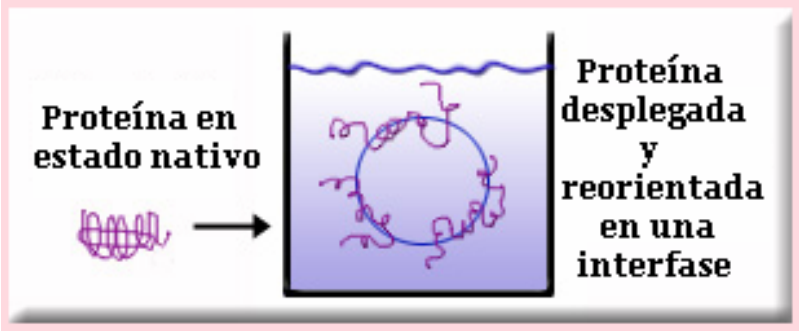

Figura 4. El acomodo de una proteína en una interfase líquido/líquido o líquido/gas provoca la pérdida parcial o total de la estructura secundaria, terciaria y cuaternaria.

Dependiendo del contenido, tanto de los residuos de aminoácidos hidrofóbicos como del de los hidrofílicos y de la distribución y acomodo de los mismos, las proteínas presentaran un determinado grado de anfifilicidad. El grado de anfifilicidad es una de las causas por las que el grado de adsorción y la capacidad de reducir la tensión interfacial y formar películas cohesivas en la interfase difiere ampliamente en las proteínas (Mangino, 1984). Esto se debe principalmente a diferencias de conformación

así como de cambios en las propiedades fisicoquímicas de la superficie de contacto entre las fases dispersa y continua en las emulsiones y espumas. Debido a que el tema del presente trabajo se refiere a emulsiones estabilizadas por proteínas, es importante considerar que las emulsiones son mezclas de al menos dos líquidos inmiscibles. Al líquido que está dispersado como gotas se le llama fase discontinua, dispersa o interna. El líquido circundante es llamado fase continua o externa. El tipo de emulsión que se forme dependerá de la proporción en que se encuentren dichas fases (**Figura 5**).

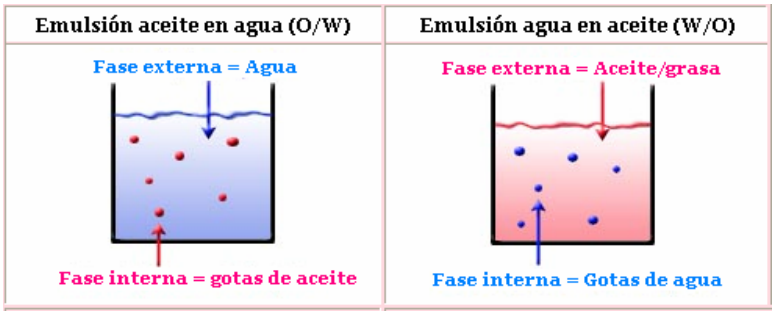

Figura 5. Tipos de emulsiones generadas a partir de la mezcla de fases líquidas polares y no polares

Específicamente los factores que afectan la adsorción y la formación de películas de las proteínas en las interfases son la estabilidad conformacional, la adaptabilidad a las fronteras de las fases y la distribución simétrica o asimétrica de los grupos hidrofóbicos e hidrofílicos en la superficie de la proteína (Hettiarachy y Ziegler, 1994) (**Figura 6**).

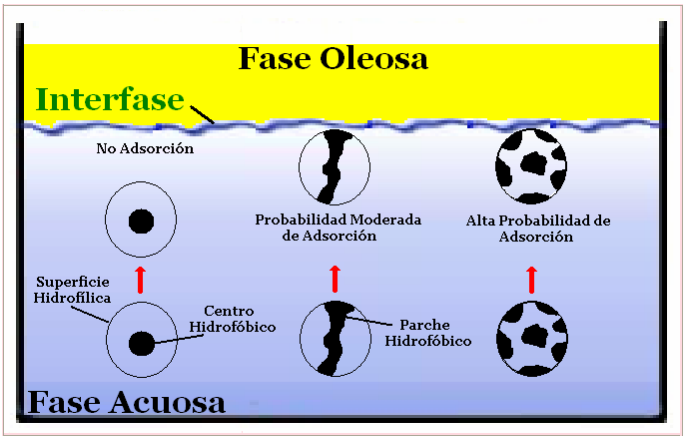

Figura 6. Dependencia de la probabilidad de adsorción de una proteína del número de regiones hidrofóbicas de su superficie (Hettiarachchy y Ziegler, 1994).

Se piensa que la adsorción de proteínas en interfases aceite-agua y aire-agua es un proceso controlado por difusión. Se asume que cuando una interfase está recién creada, las moléculas proteicas que están bajo la superficie se adsorben instantáneamente en la interfase (MacRitchie y Saraga, 1984). Cuando la superficie se satura se detiene la migración de moléculas proteicas hacia ella, lo que crea un gradiente entre la sub-superficie y la superficie.

El proceso de emulsificación, la estabilización de emulsiones por proteínas, así como los aspectos fisicoquímicos involucrados en los mismos se consideran de manera más profunda como documento **Anexo**, que consiste en el artículo de revisión titulado "*Emulsifying properties of proteins*" que se realizó como actividad académica. En él se presenta de forma organizada la información bibliográfica relacionada con el tema sobre el cual se llevó a cabo el presente trabajo de investigación. Dicho artículo fue aceptado para su publicación en el "El boletín de la Sociedad Química de México" en agosto de 2006.

Proteínas vegetales como emulsificantes

El mercado de las proteínas emulsificantes para alimentos hasta hace poco estaba dominado por las proteínas de origen animal como las caseínas y las proteínas del suero de leche, la ovoalbúmina y las miosinas de la carne, sin embargo, problemas de alergenicidad y enfermedades asociadas, aunados con elevados precios han promovido la búsqueda de nuevas fuentes proteicas más seguras y baratas. Las proteínas vegetales constituyen una buena opción ya que son abundantes, pueden presentar buenas propiedades funcionales, son de bajo costo, y para una gran cantidad de ellas, su consumo no representa riesgos para la salud. Estas son algunas de las razones por las que en los últimos años el interés en ellas ha aumentado notablemente, lo que ha llevado a que se haya realizado un intenso esfuerzo en la investigación que promueve su utilización. Es así que hoy en día se comercializan proteínas provenientes de la soya, el trigo, el amaranto, el cacahuate, el ajonjolí, la cebada, el chícharo y el girasol entre otras. Sin embargo, algunas de estas proteínas presentan desventajas como lo son la baja solubilidad en sistemas acuosos, baja funcionalidad y baja digestibilidad si no son modificadas química y/o enzimáticamente, por lo que es necesario continuar con la investigación para su mayor aprovechamiento.

Las proteínas del maíz, otra opción de proteínas emulsificantes vegetales

En contraste con las proteínas emulsificantes extraídas de semillas y leguminosas como lo son las de cacahuate, girasol, semilla de algodón, soya y ajonjolí, las proteínas de cereales son menos aprovechadas, con excepción de las del gluten de trigo las cuales son utilizadas en sistemas alimenticios debido a sus singulares propiedades de textura. El maíz es una de estas fuentes de proteínas poco explotadas. La mayoría de las proteínas del maíz se concentran en el gluten. El gluten (**Figura 7**) es un subproducto de la molienda húmeda del maíz para la obtención de aceite y almidón, con un contenido proteico de entre el 50 y el 70% en base seca. La mayor parte del mismo se destina a alimentación animal.

Figura 7. Imagen del gluten de maíz

Debido al proceso de extracción que sufre el grano de maíz, la mayor parte de las fracciones de albúminas, globulinas y glutelinas se remueven, lo que da como resultado un gluten enriquecido en zeínas (prolaminas) (Watson, 1987). Las zeínas se localizan en los cuerpos proteicos del endospermo del grano (**Figura 8**) y son proteínas de reserva. Son pobres en Lys, Trp y Met y ricas en Gln y Pro.

A los valores de pH y fuerza iónica de los sistemas acuosos alimentarios, el gluten de maíz presenta baja funcionalidad (Kinsella, 1976), sin embargo diversos trabajos (Cabra, 2002; Wu, 2001; Flores, 1997; Mannheim y Cheryan, 1992; Casella y Whitaker, 1990) han mostrado mejoras sustanciales al hidrolizar, desamidar, disminuir el tamaño de partícula y cambiar el pH y la fuerza iónica en el sistema, lo que convierte al gluten de maíz en potencial fuente de proteínas emulsificantes de uso en la industria alimentaria, pero para ello se requiere invertir en proyectos de investigación y desarrollo.

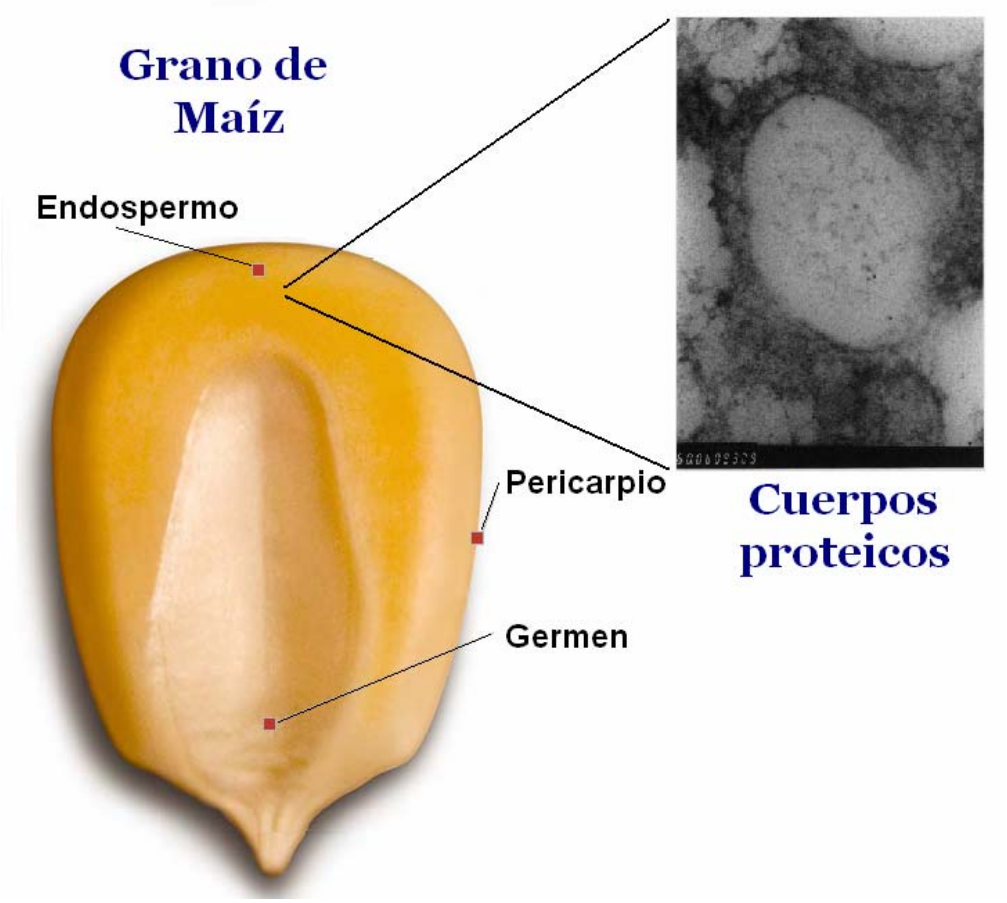

Figura 8. Esquema del grano de maíz y localización de los cuerpos proteicos en los que se encuentran las zeínas.

VI. PLANTEAMIENTO DEL PROBLEMA

Hasta el momento, los estudios de la relación estructura-función emulsificante para proteínas son escasos e insuficientes para entender adecuadamente los factores que determinan su funcionalidad. Los estudios se centran en proteínas de origen animal, de estructura globular, por lo que se requiere comprender el fenómeno en proteínas de origen vegetal con estructuras terciarias más diversas y poco conocidas.

VII. HIPÓTESIS

Si la relación carga/hidrofobicidad es determinante de las propiedades emulsificantes de las α-zeínas, entonces la modificación de esta relación mediante la desamidación provocará cambios en dichas propiedades.

VIII. OBJETIVOS

GENERAL

Determinar que cambios estructurales provocados por la desamidación permiten mejorar las propiedades emulsificantes de las α-zeínas.

PARTICULARES

1.- Purificar y caracterizar bioquímica, estructural y funcionalmente en términos de sus propiedades emulsificantes a las α-zeínas involucradas en la estabilización de emulsiones.

2.- Determinar las condiciones de desamidación que mejoran las propiedades emulsificantes.

3.- Comparar las características estructurales y propiedades emulsificantes de la α-zeína desamidada con las de la nativa en términos de composición de estructura secundaria, estabilidad térmica, hidrofobicidad, y poder emulsificante.

X. ESTRATEGIA EXPERIMENTAL

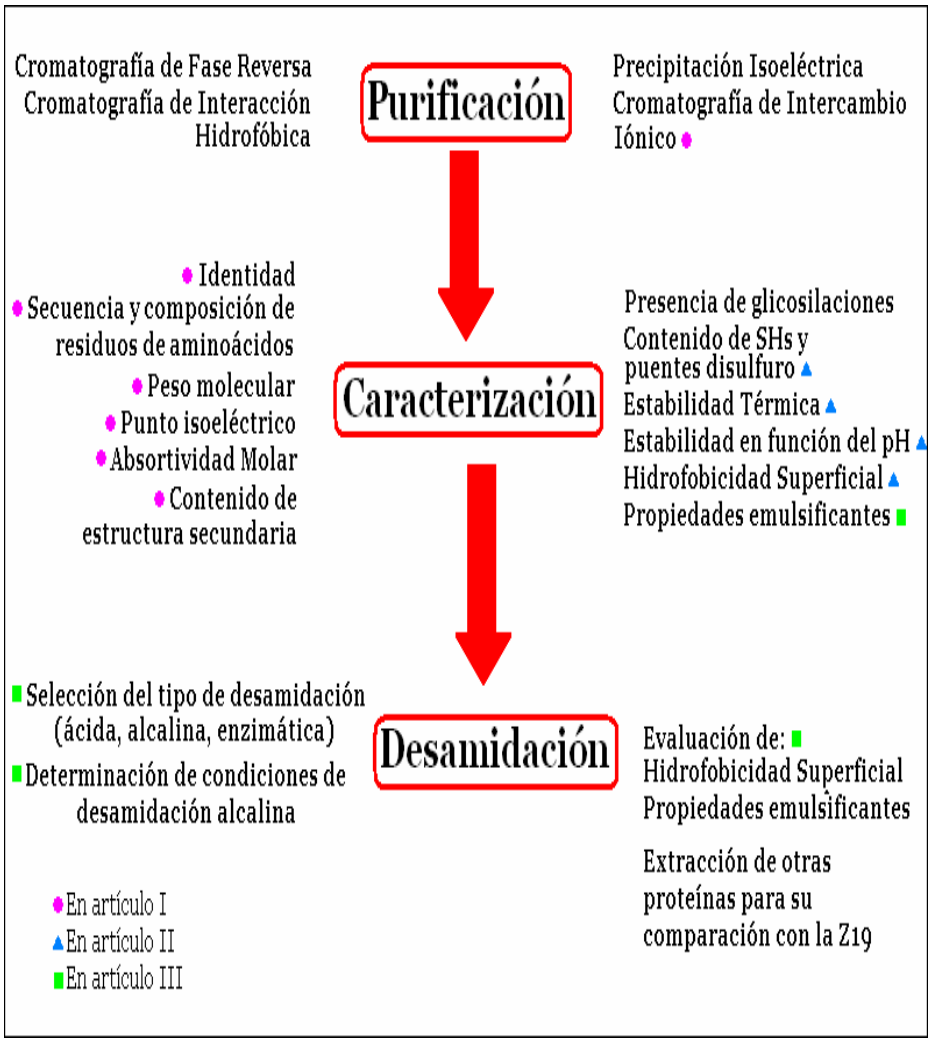

Figura 9. Estrategia experimental general

X. RESULTADOS

1) PURIFICACIÓN

Los estudios ideales sobre aspectos estructurales y funcionales de las proteínas implican trabajar con proteínas puras. Por tanto, en la primera etapa de este trabajo se implementó un protocolo de purificación de la Z19 que permitiera obtenerla con un alto grado de pureza y en cantidades suficientes para la realización de los estudios subsecuentes.

a)Cromatografía de fase reversa:

El mecanismo de separación en la cromatografía de fase reversa depende de la interacción y unión hidrofóbica entre la proteína en la fase móvil y el ligando hidrofóbico inmovilizado, es decir, la fase estacionaria. Es por ello que una característica importante en la separación es el grado de hidrofobicidad que presentan las proteínas, del cual dependerá la fuerza con la cual interaccione con el ligando. Con base en el hecho de que las α-zeínas están compuestas por un alto porcentaje de residuos de aminoácidos hidrofóbicos (mas del 70%), con índices alifáticos y promedios de hidrofobicidad superficial altos (en promedio, aproximadamente 120 y 0.35 respectivamente), así como en el hecho de que sus perfiles de hidrofobicidad sugieren la presencia de zonas idóneas para la interacción con el ligando hidrofóbico, esta técnica indicaba ser una buena opción para la separación de las proteínas. Se encontraron reportes de separación exitosa de las mismas mediante la cromatografía de fase reversa (Wilson, 1991; Paulis y Bietz, 1986). Con base en esta información, se decidió utilizar una columna C18 (longitud= 15 cm y diámetro=0.1 mm, poros de 5 μ) en un cromatógrafo de líquidos (Perkin Elmer, Canada) con detector de arreglo de diodos. Se utilizó una fase móvil de agua-acetonitrilo con TFA como modificador de pH. El corrimiento se monitoreó a 275 nm. Para su inyección, la muestra se disolvió en 70% etanol, 0.5% acetato de sodio, 5% BME y se utilizó un flujo de 0.4 ml/min.

Se obtuvo un primer perfil cromatográfico que presentaba picos definidos, los cuales se corrieron en un SDS-PAGE al 15% de acrilamida en condiciones reductoras para saber qué proteínas se encontraban en cada uno de ellos. El gel mostró que las proteínas no se separaron: la fracción colectada correspondía a la mezcla de las dos α-zeínas. Posteriormente se probaron condiciones isocráticas, con las cuales se obtuvo un solo pico, por lo que se hicieron corrimientos posteriores con gradientes de ACN a diferentes valores de pH. Finalmente se obtuvo un perfil cromatográfico con 6 picos resueltos cuando se utilizó un gradiente de ACN de 50 a 80% y después de 80 a 50%. Cada pico se colectó y se analizó en un SDS-PAGE (**Figura 10**). Nuevamente, en cada fracción se encontraban las

dos proteínas en cantidades semejantes. El que se hayan obtenido 6 picos sugiere que las 2 proteínas se asociaron en diferentes arreglos de tamaños distintos. Los resultados mostraron que, a pesar de que las propiedades de hidrofobicidad que presentan las α-zeínas son ligeramente diferentes, esta diferencia no es suficiente para separarlas e incluso es una posible causa de la formación de agregados que impiden su separación por cromatografía de fase reversa.

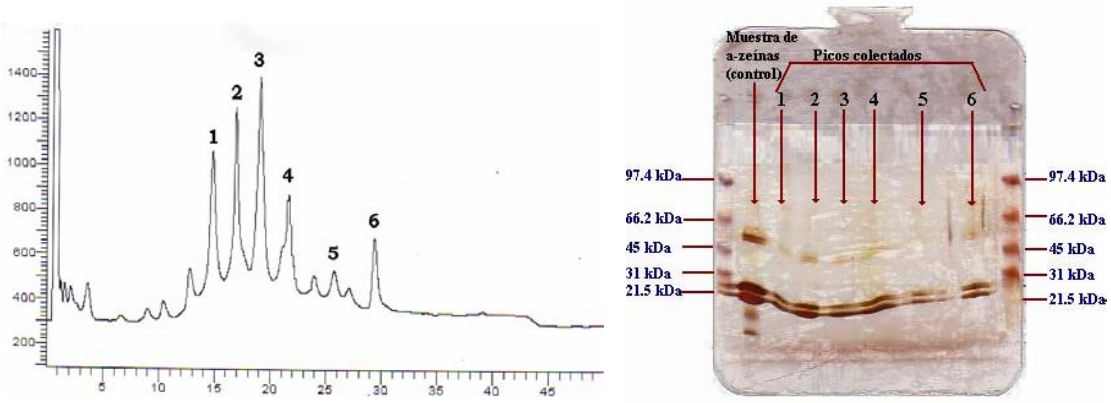

Figura 10. Izquierda: Perfil cromatográfico utilizando un gradiente de ACN 80-50%. Derecha: Perfil electroforético de cada pico en SDS-PAGE al 20% de acrilamida.

b)Cromatografía de Interacción Hidrofóbica:

Como se indicó anteriormente, el alto grado de hidrofobicidad de la α-zeínas sugirió la utilización de esta técnica cromatográfica, que se basa en la interacción de los "parches hidrofóbicos" de las proteínas con el grupo hidrofóbico unido a la matriz. Es muy similar a la fase reversa, pero la diferencia radica en el grado y tipo de sustitución de la matriz (contenido de carbono). En la fase reversa el grado de sustitución con ligandos hidrofóbicos es mucho mayor que el que presenta las matrices de interacción hidrofóbica, razón por la cual normalmente la unión de las proteínas a los adsorbentes de fase reversa es mucho más fuerte y se requieren solventes no polares para su elusión. En resumen, la cromatografía de interacción hidrofóbica significó otra opción para explotar las propiedades de hidrofobicidad de estas proteínas, a pesar de que no se encontraron reportes de su utilización para la separación de las mismas. Debido a que no se conocía con certeza el grado de hidrofobicidad superficial que presentan las α-zeínas, se decidió probar tres matrices de distinto grado de hidrofobicidad para encontrar la más idónea para la separación. Las matrices probadas, en orden de menor a mayor grado de hidrofobicidad fueron la butil, octil y fenil-sefarosa.. Se utilizó un FPLC (Pharmacia) Modelo 501 Plus con una sola bomba, por lo que todas las eluciones se hicieron isocráticas. Se utilizó como fase móvil, fosfato de sodio 20 mM con BME 1 M, pH 7. La muestra de α-zeínas se disolvió en

fosfato de sodio 20 mM con BME 1 M y sulfato de amonio, pH 7. La respuesta se monitoreó a 275 nm. Para las tres matrices se probaron distintos flujos (0.1, 0.5 y 1 ml/min) y diferentes concentraciones de sulfato de amonio (0.01, 0.1, 1 y 10 M).

En los casos de la butil y octil-sefarosa, bajo todas las condiciones se obtuvo un solo pico en el cual se encontraban las dos proteínas. Para el caso de la fenil-sefarosa, no fue posible obtener un perfil cromatográfico debido a que las proteínas se adhirieron tan fuertemente a la matriz que fue necesaria la utilización de SDS al 10% y urea 5 M para desorberlas. Con base en lo anterior, bajo las condiciones empleadas, la cromatografía de interacción hidrofóbica no fue adecuada para la separación de las α-zeínas, posiblemente debido a que se forman agregados con grandes superficies hidrofóbicas que interaccionan fuertemente con las distintas matrices.

d) Precipitación isoeléctrica

Los puntos isoeléctricos de las α-zeínas no habían sido determinados con exactitud debido a la cercanía entre estos y a la poca solubilidad de dichas proteínas en agua. Existen reportes (Brink y col., 1989; Esen, 1988), que muestran el resultado de isoelectroenfoques en los cuales se ven claramente separadas las zeínas de 19 y 22 kDa lo que permitió considerar la separación por punto isoeléctrico como paso adicional de purificación que ayudara a obtener fracciones más puras. Inicialmente la muestra de α-zeínas se solubilizó en agua con IPA (isopropanol) al 20% y BME 0.01 M a pH 7.5. Debido a la baja solubilidad, la concentración de proteína soluble fue muy baja (25 μg/ml). A dicha muestra se le adicionó una mezcla de anfóteros al 3%, pH de 5-7 y se inyectaron 50 ml en un Rotofor (Bio-Rad). Se corrieron a 12 v durante 6 h y tras esto, se colectaron las fracciones a cada una de las cuales se les midió el pH. Posteriormente se analizó su composición en un SDS-PAGE al 20% de acrilamida. La fracción en la que se obtuvo una sola banda de 19 kDa fue la de pH 6.4, sin embargo, se observaron agregados de las dos proteínas en todo el intervalo de pH evaluado. Debido a que la cantidad de proteína soluble fue muy baja, se incrementó a un 70% la concentración de IPA con lo que la concentración de proteína soluble aumentó a 100 μg/ml. No se observó precipitación durante la corrida, sin embargo, se volvieron a formar agregados en todo el rango de pH e incluso donde se había separado la proteína de 19 kDa en las condiciones anteriores. Debido a que la agregación continuó, se probó disolviendo la muestra en glicerol, el cual, de acuerdo a Bondos y Bicknell (2003), previene la agregación y aumenta la solubilidad de las proteínas. Además, se adicionó 1.5% de digitonina, que es un detergente no iónico que no afecta de manera importante a las proteínas y si mejora su solubilidad (manual del Rotofor). Por último, se adicionó urea 5 M,

que mejora la solubilidad y que puede eliminarse posteriormente mediante diálisis. La adición de tantos aditivos aumentó la viscosidad, lo que posiblemente evitó que las proteínas se enfocaran adecuadamente, razón por la cual se eliminó la digitonina y la muestra se solubilizó únicamente en urea 5 M y BME 0.01 M, con un 1.5% de anfolitos de intervalo de 5-7. La muestra se corrió a 12 v/ 7 h y las fracciones se colectaron, se midió el pH a cada fracción y se corrieron en un SDS-PAGE al 20% de acrilamida con urea 5 M y se tiñó con azul de Coomassie. En las fracciones con pH 5.85-6.75 se obtuvo una sola banda de19 kDa, por lo que dichas fracciones se recircularon a 12 v / 2 h para mejorar la separación. Nuevamente se colectaron las fracciones, se les midió pH y se corrieron en geles al 20% de acrilamida. En las fracciones de pH entre 2.7 y 3.8 se obtuvieron nuevamente las dos bandas, pero con una proporción visiblemente más alta de la de 19 kDa.

d) Cromatografía de intercambio catiónico:

De acuerdo con los resultados experimentales anteriores, las α-zeínas a pHs menores a 4-5 poseen una carga neta total positiva porque sus puntos isoeléctricos van de 4.5 a 7 (Casella y Whitaker; 1990), por lo que se planteó la estrategia de separarlas utilizando esta propiedad.

De hecho, Esen (1982) realizó una semi-purificación de la α-zeína de 19 kDa utilizando una columna de intercambio catiónico de SP-sephadex de 2.5 x 62 cm. En este trabajo se empleó una columna de SP-sefarosa de 2.6 x 11 cm, que era la más parecido a la manejada en dicho artículo. Se utilizó un buffer de inicio con 45% de IPA, ácido láctico 0.02 M y BME 0.01M pH 3.5 y uno de elusión con NaCl 1 M. La muestra se disolvió en el buffer de inicio y se inyectó en un cromatógrafo de líquidos (Beckman) System Gold con detector de arreglo de diodos. El corrimiento se monitoreó a 275 nm. El perfil de elusión obtenido presentó 8 picos. Los puntos muestreados se corrieron en geles de SDS-PAGE en los cuales se observó que a bajas concentraciones de NaCl se obtenía una sola banda. Sin embargo, la cantidad obtenida de las mismas era muy baja debido a la baja solubilidad de la proteína a estas concentración de IPA y aunado a esto, se observó que la utilización del ácido láctico a estas concentraciones además de formar una gran cantidad de cristales que aumentaban rápidamente la presión en el equipo y causar una evidente corrosión en el mismo, puede provocar la plastificación de las zeínas (Fu y col., 1999) razones por la cuales se buscaron distintas opciones para sustituir dichos reactivos. Lawton (2001) reportó que se pueden hacer soluciones de zeínas al 20% (p/v) utilizando soluciones acuosas de acetona al 70%, lo que representó una mayor cantidad de proteína solubilizada en la muestra. En lo

concerniente al regulador de pH, el manual de la columna indicaba que el buffer de citratos 0.02 M es el recomendado para utilizar a pH 3.5 con esta matriz. Con base en ello, la fase móvil quedó compuesta por amortiguador de citratos 0.02M con 70% de acetona pH 3.5. La muestra se solubilizó en dicha fase y se le adicionó 0.01M de BME con una concentración final de 25 mg/ml. El buffer de elusión contenía además 0.7 M de NaCl. El perfil cromatográfico presentó 4 picos de los cuales los 2 primeros eluían a bajas concentraciones de NaCl. Al correrlos en los geles SDS-PAGE se obervaron bandas puras de 22 kDa, además de bandas alrededor de los 66 kDa correspondientes a los agregados. En este caso, el gradiente de NaCl se hizo de 0 a 5 mM. La purificación se optimizó de forma tal, que se obtuvo un pico de la proteína pura. Este protocolo final es el reportado en el artículo I (página 29):

Cabra, V., Arreguín, R., Gálvez, A., Quirasco, M., Vázquez-Duhalt, R. and Farrés, A. (2005) Characterization of a 19 kDa alpha-zein of high purity. *J Agric Food Chem.* 53,725-9.

En dicho artículo se presentaron también los primeros resultados de la caracterización de esta proteína.

2) CARACTERIZACIÓN

Ya contábamos con proteína pura y en cantidad suficiente para iniciar la caracterización, sin embargo, no sabíamos con certeza de cual se trataba porque el peso molecular que se determinó por SDS-PAGE era de 22 kDa.

Tras la identificación por secuenciación se confirmó que se trataba de la Z19. En este punto es importante aclarar algo que ha causado controversia en la identificación y nomenclatura de las α-zeínas. Cuando se iniciaron los estudios de estas proteínas, se identificaron sólo por geles de electroforesis, los pesos aparentes en ellos variaban de los 19 a los 25 kDa, (Osborne, 1924; McKinnery, 1958; Pomes, 1971) dependiendo de las condiciones en las que se haya corrido el gel y tratado las muestras de proteína. La mayoría de los reportes coincidía en dos bandas de aproximadamente 19 y 22 kDa, por lo que se les asignaron los nombres de α-zeínas Z19 y Z22. El uso de técnicas de análisis más modernas y exactas como lo es la espectrometría de masas y el cálculo a partir de secuencias de cDNA del peso molecular han concluido que los pesos moleculares de estos dos grupos de proteínas van de los 23 a los 25 kDa. Por sus características de hidrofobicidad y agregación, presentan un corrimiento errático en geles de electroforesis, es así que su migración varía dependiendo de factores como lo son la adición de desnaturalizantes como la urea y el cloruro de guanidinio, el tiempo de desnaturalización en ebullición, la adición de agentes reductores como el BME y el DTT, la pre-solubilización en soluciones etanólicas e incluso la forma en que fue concentrada la muestra, pues procedimientos como la liofilización dificultan la posterior solubilización. Los resultados del cálculo del peso molecular confirmaron que para la α-zeína Z19 purificada es de 24 kDa.

El punto isoeléctrico determinado experimentalmente para la Z19 purificada fue de 6.8, valor que coincidía con los valores reportados para las α-zeínas, que van de 6-7 (Casella y Whitaker, 1990) y que explicaba su elevada insolubilidad a valores de pH menores a 7. Para determinar la absortividad molar se utilizó un método experimental y dos teóricos a partir de la secuencia. De acuerdo con Pace y col., (1995), el método de Hedelhoch sobreestima el resultado para proteínas que poseen pocos o ningún triptofano, es por esto que el valor obtenido por este método es superior a los otros 2.

Para la determinación del contenido de estructura secundaria se utilizó un espectropolarímetro con el cual se obtuvo el espectro de dicroismo circular que se presenta en el artículo y a partir de cuyos datos se hizo el cálculo teórico de su composición en

hélice α y lámina β. Esto confirmó el alto porcentaje de estructura en hélice α que tiene la Z19.

DETERMINACIÓN DE GLICOSILACIONES

La presencia de cadenas de polisacáridos unidos a las proteínas es un factor importante en la estructura de las mismas. Entre sus funciones biológicas está la de permitir el correcto plegamiento y la de conferir estabilidad durante su secreción (modificación postraduccional). Adicionalmente, estos carbohidratos cumplen otras funciones sutiles como la de protección contra enzimas proteolíticas y la de moduladores en las actividades biológicas de las proteínas (Reiko et al., 2005). Estos carbohidratos influyen en las características electrostáticas de la superficie de la proteína lo que es importante al considerar las interacciones proteína-proteína que se llevan a cabo.

Con la finalidad de saber si la Z19 purificada presentaba glicosilaciones en su estructura y con ello explicar mejor la diferencia en pesos moleculares encontrada por espectrometría de masas y análisis de la secuencia, se realizó la determinación de éstas utilizando el "DIG Glycan Differentiation kit" (Roche, Cat. No. 1210238) que utiliza la unión específica de las lectinas a los carbohidratos para su identificación. Las lectinas aplicadas están conjugadas con el esteroide digoxigenina que permite la detección inmunológica de las lectinas unidas. La diferenciación entre estructuras de carbohidratos se da cuando las lectinas reconocen selectivamente los azúcares terminales. Adicionalmente a las lectinas individuales, el kit contiene glicoproteínas control para demostrar la especificidad de las lectinas. La metodología fue la siguiente:

1) Elaboración de gel desnaturalizante de poliacrilamida al 20% de 1 mm de espesor

La proteína purificada se dializó contra agua desionizada. Posteriormente se liofilizó, se resuspendió en volúmenes iguales de agua desionizada y buffer de tratamiento de muestra (0.125 M Tris-Cl, 4% SDS, 20% Glicerol, 10% BME, urea 5 M y azul de bromofenol 0.01%, pH 6.8) y se sometió a calentamiento a 100 °C por 15 min. En el gel se corrieron también las siguientes glicoproteínas que sirvieron como controles positivos: Carboxipeptidasa, Transferrina, Fetuina, y Asialofetuina. Los marcadores de peso molecular utilizados (Bio-Rad) fueron los siguientes: Fosforilasa b, 97400; Albúmina sérica bovina, 66200; Ovoalbúmina, 45000; Anhidrasa carbónica, 31000; Inhibidor de tripsina de soya, 21500; y lisozima, 14400. El SDS-PAGE se llevó a cabo de acuerdo a Laemmli (1970).

2)Transferencia de las proteínas a membrana

Tras el término de la corrida del gel, este se enjuagó con agua desionizada por 10 minutos y posteriormente se incubó durante 15 minutos en el buffer de transferencia (25 mM Tris, 190 mM glicina, 10% metanol) tras lo cual se transfirió a una membrana PVDF (Nalgene) prehumedecida en buffer de transferencia por 30 min. La transferencia se corrió a 52 mA durante 1 h (Pharmacia Biotech Multiphor II).

3) Detección de glicosilaciones

La membrana se tiñó con el colorante rojo de Ponceau para la visualización y marcado con lápiz de las bandas de las proteínas transferidas. Posteriormente se incubó durante 30 minutos a 4 ºC en la solución bloqueadora. Tras esto se lavó 2 veces durante 10 minutos con solución TBS (0.05 M Tris, 0.15 M NaCl pH 7.5) y después 1 vez por 10 minutos con buffer 1 (0.05 M Tris, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1mM $CaCl_2$, pH 7.5). A continuación se incubó durante 1 h con las siguientes lectinas: GNA (*Galanthus nivalis aggltinin*), SNA (*Sambucus nigra agglutinin*), MAA (*Maackia amurensis agglutinin*), PNA (*Peanut agglutinin*) y DSA (*Datura stramonium agglutinin*).

Tras la incubación con las lectinas, el gel se lavó con solución de TBS y se incubó 1h más con antidigoxigenina-AP y se lavó nuevamente con solución de TBS. La membrana se reveló con una solución de cloruro de tetrazolio azul (4-Nitro).

Los resultados se muestran en la **Figura 11**:

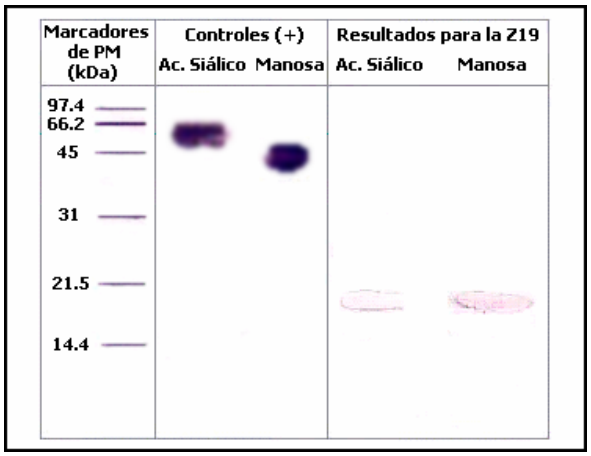

Figura 11. Resultados de la determinación de glicosilaciones en las α-zeínas

Los resultados indicaron la unión de ácido siálico y manosa a la Z19.

La glicosilación de las prolaminas de maíz ya había sido descrita por Pismenestkaia y Rybak (1992), quienes determinaron que las lectinas concanavalina A (Con A), Lens culinaris lectina (LCL), lectinas de *Arachis hypogaea* (PNA), de *Triticum vulgaris* (WGA), de *Dolichos biflorus* (DBA), de *Glycin max (*SBA), de *Lotus tetragonolobus* (LTA), de *Laburnum anagiroides* (LAL), de *Ricinus communis* (RCA), y de *Phaseolus vulgaris* (PHA) interactúan con las zeínas lo que indicó la unión de manosa, galactosa, fucosa y aminoazúcares.

En su camino hacia su destino final, las zeínas transitan a través del aparato de Golgi y de vacuolas de almacenamiento proteico donde también se adicionan glicanos, estas últimas posteriormente se convierten en los cuerpos proteicos. No está claro si todas o sólo algunas de las zeínas están glicosiladas, pero se sabe que varias proteínas de almacenamiento de cereales son glicosiladas tras ser depositadas en los cuerpos proteicos para generar conformaciones más adecuadas para el empaquetamiento o bien, para mediar procesos de agregación y asociación con membranas (Müntz, 1998).

La presencia de manosa no resulta sorprendente ya que es un tipo de glicosilación común en plantas, pero no lo es para el ácido siálico, ya que se sabe que este tipo de modificación pos-traduccional es propio de mamíferos y no de plantas. Existen algunos reportes de la presencia de ácido siálico en plantas (Shah et al., 2003; Séveno et al., 2004), sin embargo, no se ha reportado para el caso del maíz.

La comprobación de presencia de glicosilaciones en la estructura de la Z19 sugiere que la diferencia de 475 kDa entre la determinación del peso molecular por espectrometría de masas MALDI-TOF y el análisis de la secuencia de residuos de aminoácidos se puede deber a la presencia de 2 a 3 unidades de manosa (475/180.16=2.63 unidades).

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2005, 53, 725−729 725

Characterization of a 19 kDa α-Zein of High Purity

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A highly pure α-zein was extracted from corn flour using ethanol (95%). Subsequently, ion-exchange chromatography was performed, using SP-Sepharose that yielded a highly homogeneous protein. This protein migrated as a single band in 20% SDS−PAGE and in pH gradient gels, showing an isoelectric point of 6.8. Mass spectrometry (MALDI-TOF-MS) showed a single peak with a molecular mass of 24 535 Da. It was identified as Z19, when comparing the sequence obtained in an automatic Edman sequencer with the Swissprot database using BLAST. The molar extinction coefficient, determined by dry weight in 70% methanol, was 12 415.49 M^{-1} cm^{-1} at 280 nm. Light scattering showed its presence in a monodispersed state of 44−66 kDa aggregates in methanol (70%). Circular dichroism spectra allowed the estimation of an α-helix content that was lower than the one found for a mixture of two α-zeins but with a higher content of β sheets.

KEYWORDS: Zein; protein characterization; Z19; MALDI-TOF-MS; circular dichroism

INTRODUCTION

The structural analysis of hydrophobic proteins poses a challenge to traditional techniques because of their limited solubility in aqueous solvents (*1*). Such is the case of corn prolamins, also known as zeins, which are storage proteins that are the most abundant group in corn endosperm and are poor in lysine and tryptophan (*2*, *3*). They are characterized by their high hydrophobicity and are classified as α, β, γ, or δ depending on their solubility, molecular weight, immunological response, and structure (*4*−*6*). Among these four groups, α-zeins are the most abundant and comprise 75−85% of total zeins; Z19 and Z22 proteins are included in this group and have apparent molecular weights of 23.8 and 26.7 kDa, respectively (*7*).

There are several reports on the separation of these proteins (*9*− *16*), but their close similarity has made separation and purification in a native state a difficult task, with low yields and poor quality of performance in further characterization studies. Therefore, the development of a suitable method to achieve a proper characterization remains an interesting challenge (*16*).

Zeins are more hydrophobic than prolamins from other cereals and tend to form aggregates and to precipitate under standard separation conditions used successfully to purify prolamins from wheat, rye, and rice (*1*, *11*). Thus, most of the physicochemical and structural studies performed with these proteins have used the mixture of α-zeins (*12*−*16*). Furthermore, results produced with these mixtures have so far generated controversies about conformation (*13*, *14*), which Forato, et al. (*17*) attribute to the use of mixtures and not to individual α-zeins in the published studies. This is why the individual purification and characterization of each of the α-zeins has become an important issue that will allow the study of behavior as defined mixtures or as individual proteins. Also, a high degree of sample homogeneity is required to perform structural studies.

The purpose of this work was to achieve such a preparation and to show, with different analysis criteria, the high purity of the obtained protein: single bands in sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and isoelectric focusing (IFE), a single peak in matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and light scattering. This study differs from those cited above since this comprises the structural characterization of an individual, highly pure Z19 and thus allows comparison of its behavior when it is a member of the mixture with Z22.

MATERIALS AND METHODS

Biological material. Mixtec waxy yellow dent corn seeds (*Zea mays* L.) kindly donated by Arancia Corn Products, S. A. de C. V. (Tlalnepantla, Mexico), were ground in a disk mill (Weber BROS. & Wiltz, Metal Works, Inc., U.S.A.).

α-Zein Extraction from Grain. The extraction was performed according to Dickey et al. (*18*) with a slight modification. Lipids from flour were extracted with hexane, and carotenes and xanthophylls were extracted with a chloroform−methanol mixture (2:1). Defatted, decolored flour was mixed with 95% ethanol (5:1 solvent−flour (v/w)) using orbital agitation for 12 h at 25 °C (New Brunswick Scientific Model R76, Edison, NJ). It was then centrifuged at 12 000 × *g* for 30 min at 4 °C and the supernatant was recovered.

Ion-Exchange Chromatography. A cationic exchange column was used (SP-Sepharose 2.6 cm × 11 cm Amersham Biotech, Uppsala, Sweden). The mobile phase was a citrate buffer, 0.02 M with 70%

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10.1021/jf048508a CCC: $30.25
Published on Web 01/12/2005

methanol, pH 3.5. The sample was solubilized in this phase to reach a final concentration of 0.5 mg mL^{-1}. It was then filtered with a 0.22 μM membrane (Millipore, Ireland) and injected into a high-pressure liquid chromatograph (AKTA prime, Amersham Pharmacia Biotech, Uppsala, Sweden). The elution buffer had 0.7 M NaCl, and a gradient from 0 to 1.2 mM NaCl was performed. A 0.5 mL min^{-1} flux was used, and elution was monitored at 280 nm through a UV detector. The collected peak was concentrated by ultrafiltration using YM10 membranes (Amicon, Millipore, U.S.A.).

SDS−PAGE. All protein preparations were dialyzed against deionized water and lyophilized. They were resuspended in equal volumes of deionized water and buffer (0.125 M Tris-Cl, 4% SDS, 20% Glycerol, 10% BME, 5 M Urea and bromphenol blue 0.01%, pH 6.8) and then submitted to heating at 100 °C for 10 min. SDS−PAGE was performed according to Laemmli (20). Gels were silver-stained (Amersham Biosciences, Uppsala, Sweden) for band visualization.

Amino Acid Sequencing and Identification. After SDS−PAGE, the protein was electroblotted to a polyvinylidenedifluoride membrane (Immobilon, Bio-Rad), by the Towbin method (21), for further sequencing. The *N*-terminal sequence was determined by automated Edman degradation (22) on a gas-phase protein sequencer (LF 3000, Beckman Instruments, Irvine, CA) equipped with an online high-performance liquid chromatography system (Beckman System Gold, Beckman Instruments, Irvine, CA). HPLC equipment included a diode array detector with settings at 268 and 293 nm for signal and reference, respectively. The HPLC column used was a Beckman Spherogel Micro PTH (2 mm × 150 mm) column; standard Beckman sequencing reagents were used for analysis.

Molar Absorption Coefficient Determination. This coefficient was determined for the purified Z19 protein by the dry weight method (23), which was slightly modified as follows: Protein solution in 70% methanol was dialyzed at 4 °C against 70% methanol for 48 h, through a 10 000 MW-cut Spectrapore membrane. Dilutions from 5 to 30 mg mL^{-1} of protein solution were performed, and absorbance at 280 nm was assayed in cells with a light path of 1 cm; 70% methanol was used as a blank. Two milliliters of each dilution were taken and dried at 95 °C for 48 h. The samples were weighed, and the molar protein concentrations were calculated. The molar extinction coefficient was the slope of the absorbance graph against protein concentration. The assay was performed in quadruplicate, and the standard deviation was no higher than 10% of the medium value.

Mass Spectrometry. For MS measurements, the purified zein was dissolved in 200 μL of a methanol−water mixture (70:30). Nanoelectrospray tandem mass spectra were obtained on an Esquire ion-trap mass spectrometer (Bruker-Flurzen Analytical, Gmbh, Germany) in the positive-ion mode, as described by Jensen et al. (24).

Isoelectric Point Calculation. A prepared IPE acrylamide gel (Amersham Pharmacia, Uppsala, Sweden) was used, with a 3−9 pH gradient. The lyophilized sample was solubilized in 5 M Urea; and 1 μg was used per lane. Bands were visualized using a silver-staining kit (Amersham Biosciences, Uppsala, Sweden).

Circular Dichroism (CD) Spectroscopy. The CD spectra were recorded on a Jasco J-715 spectropolarimeter, baseline and solvent corrected. Spectra were run at 25 °C. Concentrations used were in the 0.08−0.25 mg mL^{-1} range for the purified Z19 and 0.003−0.05 mg mL^{-1} for the α-zein mixture to avoid the aggregation effect. Three scanning acquisitions were accumulated and averaged to yield the final spectrum in both cases. The results are expressed as mean residue ellipticity, $[\theta]_{MR}$, using a value of 109.867 g mol^{-1} for the molecular weight of a mean residue. To estimate the secondary structure content, the CD spectrum was analyzed with the SELCON3 deconvoluting program (25). The spectrum was also analyzed with the CLUSTER software (26).

Dynamic Light Scattering. Light scattering measurements were performed in a multi-angle light scattering instrument (Zetasizer nanoseries Malvern Instruments, U.K.). To make the analysis, the α-zein mixture and the purified Z19 were dissolved in 100 μL of a methanol−water mixture (70:30) and filtered by 0.22 μm membranes (Millipore, Ireland). The measurements were made at 25 °C. Three measurements were accumulated and averaged. The results were analyzed with the Zetasizer nanoseries software (2003).

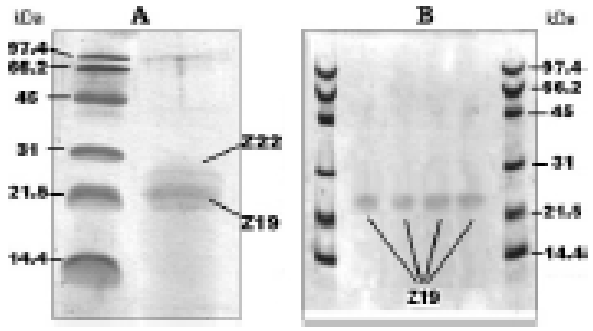

Figure 1. SDS−PAGE in acrylamide 20% of (A) the α-zein mixture after extraction with 95% ethanol and (B) the purified Z19 with 70% methanol and citric acid 0.02 N, pH 3.5.

RESULTS AND DISCUSSION

Purification and Identification of α-Zein. The mixture of α-zeins is observed as two typical bands of approximately 24 and 26 kDa, corresponding to Z19 and Z22 proteins, respectively, in SDS−PAGE (**Figure 1A**). The use of 95% aqueous ethanol allows the extraction of just the two α-zeins, avoiding solubilization of β, γ, and δ zeins, which yields an adequate work solution with an α-zein mixture concentration of 1.7 mg mL^{-1}. The final purification extraction yield was 2.63 ± 0.3 mg purified protein/100 g corn flour, which are remarkably good and enough for the next characterization experiments.

Various methods of protein purification were assayed according to several reports (results not shown). Wilson (6) and Paulis and Bietz (9) concluded that they had excellent zein separation using reversed-phase high-pressure liquid chromatography (RP-HPLC), but, according to our results, slight variations in equipment or accessories are enough to avoid having results such as the ones reported. Chromatographic profiles were different from those expected. Two peaks were obtained and were further analyzed by SDS−PAGE. Results showed the two α-zeins in similar amounts, which suggested the formation of different aggregates in both fractions.

Hydrophobic interaction chromatography (HIC) has not been reported as a tool for purifying this kind of proteins, but due to their highly hydrophobic properties, it seemed an interesting option. However, the evaluation of several matrixes and working conditions did not allow the separation of the mixed zeins. Instead, protein aggregation became an even greater phenomenon, possibly due to their hydrophobic surfaces that interact strongly with the support matrix.

Subsequently, ion exchange chromatography (IEX) was assayed. Solvent selection proved to be critical for this kind of chromatography because of its denaturing effects on the purified zeins. Aqueous buffers with nonpolar solvents led to just small amounts of pure, but denatured, Z19 being obtained. It must be said that Krueger and Bietz (27) reported that 22 kDa polypeptides present in cereals in high concentrations show a tendency to aggregate, mainly due to their hydrophobic nature. Thus, the protein yield they reported was not enough to allow complete characterization. It was demonstrated that a composition for the best solvent for zein is where the C/O (carbon oxygen) is around 0.7−1.3 (28), which is necessary to keep zeins soluble, but does not guarantee that they will not denature. According to Lawton (29), 70% aqueous acetone allows the handling of 20% (w/v) zein solutions, but our results showed that this solvent produces a highly denatured protein.

Besides, Forato et al. (30) showed that alcohol solubilization does not affect conformation for the zein mixture, so they were

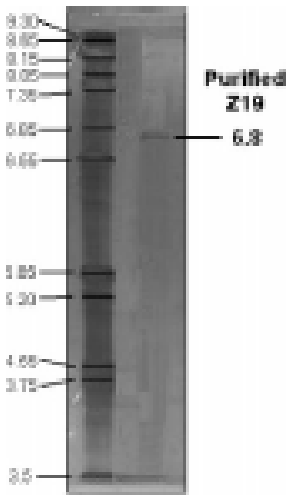

Figure 2. IFE of the purified Z19 protein. The pH gradient was 3–9, and the arrow shows the purified Z19 at pH 6.8.

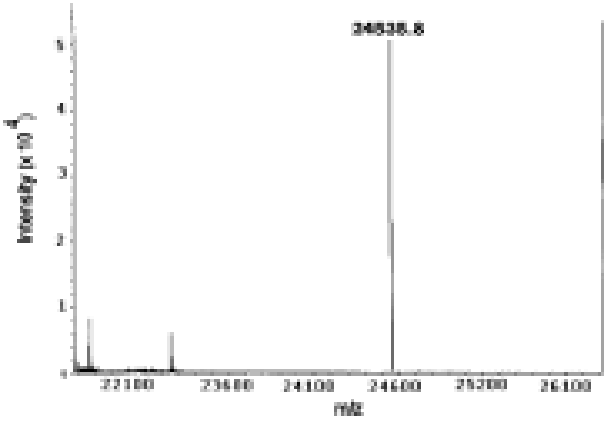

Figure 3. Purified Z19 profile by MALDI-TOF-MS.

able to validate secondary structure measurements in these solvents. Our results show that alcohols such as methanol, ethanol and 2-propanol, in concentrations ranging from 70 to 95%, make it possible to obtain native zeins in high concentrations in a nonaggregated state. Thus, zeins require careful solvent handling, as do materials and equipment, to avoid undesirable phenomena like aggregation and denaturation.

The purified protein obtained after IEX was eluted as a small fraction at 1.2 mM NaCl. **Figure 1B** shows a single band (19 kDa) corresponding to the purified protein.

IEF (**Figure 2**) made it possible to confirm that α-zein was the only protein present in the purified fraction and led to the determination that it has an isoelectric point of 6.8, just like the one reported for a zein mixture by Casella and Whitaker (*31*) and by Wilson (*32*).

A spectrum obtained for the purified protein using MALDI-TOF-MS is shown in **Figure 3**. A single peak appears at 24 535.8, a mass that can be assigned to one of the 19 kDa α-zeins (*15, 18*). No other peaks corresponding to molecular ions, double-charged peaks, or dimers could be seen, as in other studies, where two peaks of molecular ions have been reported for 19 kDa α-zeins (*15, 18*). The molecular weight for the present protein was determined using different methods. The molecular weights determined by SDS−PAGE and the analysis of the amino acid sequence (Predict Protein software) were 24 and 24.06 kDa, respectively, very close to that obtained

Table 1. Amino Acid Composition of the α-zein of 19 kDa Obtained from the Reported Sequence[a]

aa	Pred[a]	%	aa	Pred[a]	%
A	29	13.24	L	43	19.63
R	3	1.37	K	0	0.00
N	10	4.57	M	0	0.00
D	1	0.46	F	11	5.02
C	3	1.37	P	21	9.59
Q	43	19.63	S	18	8.22
E	1	0.46	T	7	3.20
G	2	0.91	W	0	0.00
H	1	0.46	Y	8	3.65
I	12	5.48	V	6	2.74

[a] Pred: amino acid residues per single polypeptide chain

experimentally using MALDI-TOF-MS, which was 24.535 kDa. The three methodologies used made it possible to obtain an average mass of 24.2 kDa for this Z19 protein. Comparison between the mass obtained by MALDI-TOF-MS and the one predicted for the corresponding amino acid sequence differs by 1.94%, which is remarkably good. Other authors (15, 18) reported molecular weights of 24.1 and 24.515 kDa from MALDI-TOF-MS for a Z19 and 24.706 kDa (18) from the amino acid sequence analysis. SDS−PAGE allows the classification of α-zeins into two groups (Z19 and Z22) according to the two bands obtained with this methodology, but techniques such as IFE (*29*), RP-HPLC (*1, 4−6*), or MALDI TOF-MS (*15, 18*) have shown that α-zeins consist of a mixture of at least 15 elements. Unlike other reports, in this study, just one of them was purified.

Sequencing and Identification of the Purified Zein. The sequence of the purified Z19 protein revealed that the first 20 amino acid residues were identified in a unique way, thus showing that the sample belonged to a homogeneous protein preparation. The *N*-terminus was TIFPQCSQAPIASLLPFYLP, which matches published sequences for zeins found in different data banks. The highest identity (100%) was obtained with the precursor of 19 kDa α-zein (100% identity) (Swissprot: accession number CAA26294). The analysis of the amino acid composition presented in **Table 1** shows that more than 50% of the amino acid residues are hydrophobic, with the highest levels corresponding to alanine, leucine, and proline, as with other α-zeins (*8, 34*). This structure yields high aliphatic indexes and high surface hydrophobicity (approximately 120 and 0.35, respectively) as calculated from the sequence analysis stored in the NCBI database using ProtScale software. The low amounts of polar, charged amino acids explain their high insolubility in water and the tendency to aggregate. The amount of aromatic residues is low, and there are no tryptophan residues.

The lack of tryptophan residues, together with the low tyrosine content, explains the low absorbance value at 280 nm and explains why the molar extinction coefficient obtained by the dry weight method (12 415.49 M^{-1} cm^{-1} ± 1350, r^2 = 0.9893) is higher than that obtained for proteins with a similar molecular weight but with a higher content of these residues (*35*). It is also higher than that predicted by Predict Protein software (10 240 M^{-1} cm^{-1}) or that performed according to the Hedelhoch method (*36*) (12 295 M^{-1} cm^{-1} in water at 280 nm). Another reason for obtaining such a high molecular coefficient would be the use of 70% methanol as solvent, which allows exposure of tyrosine residues and reduces insoluble aggregate formation, in contrast to the phenomena that occur when this protein is dissolved in water; furthermore, ϵ_{max} for tyrosine and phenylalanine increase when they are in alcoholic environments (*35*).

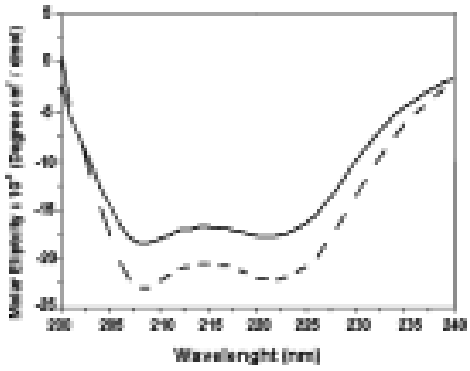

Figure 4. Far-UV CD Spectrum (solid line) for the pure Z19 protein an (dashed line) for the α- zein mixture in 70% (v/v) aqueous methanol at 25 °C

Table 2. Secondary Structure Content of a Zein Mixture and Purified Z19 as Determined by CD

	α-zein mixture (%)	purified Z19 (%)
α-helix	56.7	40.0
β-sheets	7.1	18.5
coils	8.2	15.4
not determined	28.0	26.1

Analysis of Secondary Structure by CD. CD spectra were determined both for the α-zein mixture and for the purified Z19. Aqueous methanol (70%) was used as solvent, and the 200−240 nm range was used (**Figure 4**). α-Helixes, β-sheets, and random coils were determined and are shown in **Table 2**.Corresponding spectra for both samples show two negative maxima around 207−208 nm and 222−224 nm that indicate high helical structure content. The highest percentage of α-helix was obtained for the two-zein mixture, with the same value as that reported in previous studies (*12*−*14*), while the β-sheet content was higher for the purified Z19. These results do not agree with those reported by Forato et al. (*17*) for a purified 19 kDa zein from a corn variety that does not produce Z22. They report an α-helix content of 46%, 22% of β-sheets, 23% of random coils and 13% of other structures. These differences might be due to the degree of homogeneity of the samples because the expression of Z22 in the modified corn used by Forato et al. (*17*) might be reduced but not totally blocked, while the expression of other zeins that are usually found in similar amounts could have been altered, resulting in a non-homogeneous mixture (*37*, *38*). The results obtained from the samples analyzed in this work correspond to the average of both the individual signals and those due to interactions among the mixture components. The prediction of a tertiary structure performed with the CLUSTER software indicated that the majority of the α-helixes in the Z19 folds into compact structures in both forms: purified and when it is found in the α-zeins mixture.

Light Scattering. Dynamic light dispersion is a useful diagnostic tool to obtain information about the aggregated state in proteins. The light-dispersion analysis made it possible to demonstrate that, under the conditions in which Z19 was obtained, it is found to be monodisperse in 2−3 monomer aggregates (**Figure 5A**). These forms seem to be very stable because they remain as such even under denaturing SDS−PAGE conditions (data not shown) with molecular weights between 44 and 66 kDa. This behavior is opposed to the one shown by the two zein mixtures (**Figure 5B**), which are found in larger sizes and molecular-weight aggregates, a fact that must explain

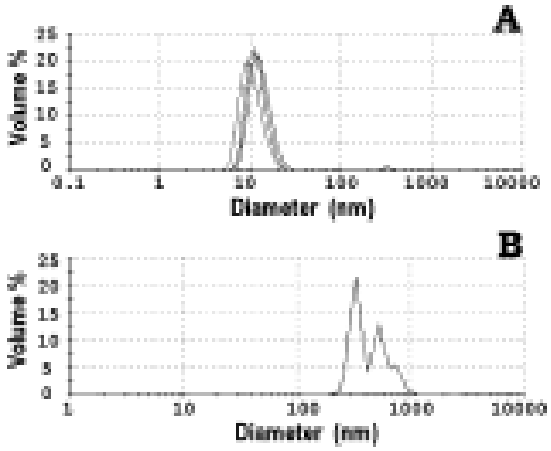

Figure 5. DLS profile, size distribution by volume for the (A) purified Z19 and the (B) α-zein mixture in 70% (v/v) aqueous methanol at 25 °C.

the differences in secondary structure content found by other authors.

The purification method reported here allows the acquisition of a highly pure, homogeneous preparation of a Z19 which provides the material required to understand its behavior, conformation, and structure, even though further studies are necessary. It is desirable to have the same quality of preparation for Z22 to allow a full understanding of the above features, both for the individual proteins and for the mixture, to be able to build a valid model of their structure and organization.

ACKNOWLEDGMENT

This work was partially funded by CONACyT project no. 35948-B. The authors thank Arancia Corn Products, S.A. de C.V. for the materials provided, Dr. Guillermo Mendoza (Medicine Faculty, UNAM) performed the sequencing of the peptides and Idalia Flores provided technical assistance. They appreciate the help of Jenniffer Lewis in translation. V.C. was a recipient of a CONACyT scholarship 159981 and of funds from PAEP 203304.

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Received for review September 3, 2004. Revised manuscript received November 5, 2004. Accepted November 9, 2004.

JF048530S

Las pruebas de estabilidad térmica son importantes debido a que durante la emulsificación se produce un aumento de temperatura debido a la fricción. Es de esperar que este incremento provoque cambios en la composición de estructura secundaria, que a su vez modificarán las propiedades emulsificantes de la misma.
Los resultados se presentan en el artículo II (página 36):

Cabra, V., Arreguín, Vázquez-Duhalt, R. and Farrés, A. (2006) Effect of temperature and pH on the secondary structure and processes of oligomerization of 19 kDa alpha-zein. *Biochim. Biophys. Acta - Proteins & Proteomics,* **1764**(6): 1110-1118.

Las pruebas realizadas mostraron que esta proteína presenta un plegamiento inestable, es muy sensible a altas temperaturas y presenta tendencia a la oligomerización y agregación. Este comportamiento durante el calentamiento nos sugiere que durante la emulsificación efectivamente se da el desplegamiento de la proteína, lo que teóricamente facilita la interacción en la interfase agua/aceite.
Debido a que se detectó la presencia de oligómeros y agregados de gran tamaño durante el calentamiento de la proteína, se cuantificaron los grupos sulfhidrilo de la proteína y se encontró que la mayoría de ellos se encuentran formando puentes disulfuro, interacciones covalentes determinantes de la función emulsificante de una proteína, ya que influirán en la flexibilidad de la misma. Estudios previos han demostrado que cuando se calientan a las prolaminas del maíz, su forma una gran cantidad de puentes disulfuro que llevan a la formación de grandes oligómeros. Con esta información, se sugiere que durante la emulsificación, la α-zeína Z19 se desnaturaliza y asocia formando agregados que influirán en la estabilización del glóbulo graso en la emulsión.

Las pruebas de estabilidad en función del pH revelaron una elevada dependencia del contenido de estructura secundaria del pH, la estructura en hélice α se favorece a valores de pH superiores al punto isoeléctrico, mientras que por debajo de el la estructura proteica está más desorganizada.

Para poder entender y explicar la función como emulsificante de la Z19, se requiere considerar a las interacciones que determinan las propiedades emulsificantes de las proteínas que son: las interacciones hidrofóbicas, las interacciones covalentes (puentes disulfuro) y las interacciones electrostáticas. En conjunto, todas las anteriores determinarán

la estabilidad que presente una proteína en función de la temperatura y el pH, al igual que la flexibilidad que desarrolle.

Debido a que la formación de agregados proteicos está favorecida cuando se tiene un alto contenido de residuos de aminoácidos hidrofóbicos con una determinada distribución tanto a lo largo de la cadena polipeptídica como regional en la estructura tridimensional de la proteína, la evaluación del grado de hidrofobicidad fue un paso importante para tratar de describir mejor el comportamiento de esta proteína, por lo que se procedió a realizar un análisis tanto de composición como de índices de hidrofobicidad a partir de la secuencia de la α-zeína Z19. Los resultados obtenidos así como su comparación con los resultados de otras proteínas similares, indicaron que la Z19 es una proteína altamente hidrofóbica, lo que sugiere que los fenómenos de agregación se deben a la formación de los puentes disulfuro y a la verificación de interacciones hidrofóbicas.

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Effect of temperature and pH on the secondary structure and processes of oligomerization of 19 kDa alpha-zein

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Received 9 February 2006; received in revised form 29 March 2006; accepted 3 April 2006
Available online 7 April 2006

Abstract

Highly hydrophobic protein Z19 zein shows a tendency towards oligomerization. The role of temperature and pH on the oligomerization process was studied monitoring the secondary structure content and the appearance of aggregates by Circular Dichroism Spectroscopy (CD) and Dinamic Light Scattering (DLS). Z19 zein suffers irreversible thermal denaturalization, as demonstrated by far-UV CD measurements. DLS data indicate that this denaturalization is accompanied by oligomerization processes which are strongly dependent on temperature. The aggregates that appear when the sample is heated maintain a certain amount of their native structure. Oligomers, showing high stability to temperature changes and other denaturing conditions with molecular weights of 45, 66 kDa and higher, were detected by SDS-PAGE. The secondary structure strongly depends on pH. Thus, at pH above pI (6.8), all the protein structure is in alpha helix. The formation of disulfide bonds plays an important role in the aggregation process, since most of the sulfhydryls in the protein (97.52%) form disulfide bonds and only 2.47% of them are free and superficially exposed. The sensitivity towards thermal denaturalization is also affected by pH rises.

Keywords: Alpha-zein; Protein aggregation; Circular Dichroism; Dinamic light scattering; Disulfide bond

1. Introduction

The factors that affect the secondary and tertiary structures of highly hydrophobic proteins, such as prolamins, are far less understood than those that affect water-soluble proteins [1,2]. Zeins, corn (*Zea mays* L.) prolamins, are synthesized during the endosperm development and stored in protein bodies [3]. Zeins are classified according to their solubility, molecular weight and immunological response into α, β, γ, and δ [4–6]. α-Zeins are the most abundant (75–85% of the total) and are divided in two types: Z19 and Z22 with approximate weights of 22 and 25 kDa respectively [7]. Several analytical efforts have been made to elucidate the molecular structure of zeins [8]. Physicochemical and structural characterization studies of the α-zeins mixture [9–15] suggest that these proteins have a high content of alpha-helix (40–60%). Nevertheless, there are no three-dimensional structures of α-zeins available. Proposed models from these studies suggest that the zeins show a compacted form within protein globules, with elongated molecular structures of prolates consisting of ellipsoids and a series of repeated and packed alpha-helixes [8]. In solution, zeins become an extended structure. It is well known that these proteins are organized in oligomers which are resistant to high temperatures and treatment with reducing reagents, and that they tend to form aggregates of high molecular weight (HMW) [16]. The aggregation process is complex, and it has not been completely elucidated, because it depends on a great number of physical and chemical parameters

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CD, circular dichroism; BME, beta mercaptoethanol; HMW, High Molecular Weight; DLS, Dinamic Light Scattering; EDTA, Ethilendiaminotetrabenzoic acid; DTNB, 5'5'-dithiobis(2-nitrobenzoic acid)

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doi:10.1016/j.bbapap.2006.04.002

(temperature, pressure, concentration, ionic force, pH, addition of denaturants, etc.) [17,18]. In general, protein aggregation seems to be associated mainly with conformational and structural changes that expose some of the non-polar residues [19]. In order to have a deeper understanding of the oligomerization process of highly hydrophobic proteins, spectroscopic analyses of a pure preparation of α-zein of 19 kDa and computational predictions of hydrophobicity were carried out in this work. The effect of temperature and pH, as well as the role of disulfide bonds formation during the aggregation process, were studied and discussed.

2. Materials and methods

2.1. Biological material

Waxy yellow dent from corn hybrid grains (*Zea mays* L.) were grounded in a disk mill until a particle size of 40 μm was obtained (Weber BIROS and White, Metal Works Inc., US). Corn was a generous donation of Arancia Corn Products, S. A. de C. V. (Hidalgotitlán, México).

2.2. α-Zein extraction and purification

The extraction was performed according to Dickey et al. [20] with a slight modification. Lipids from maize flour were extracted with hexane. Carotenes and xanthophylls were removed using a chloroform–methanol mixture (2:1). Defatted, decolorized flour was mixed with 95% ethanol (5:1 solvent-flour (v/w)) through agitation for 12 h at 25 °C. This mixture was then centrifuged at 12000×*g* for 30 min at 4 °C and the supernatant was recovered. The protein was purified using the previously described protocol [21]. The protein preparation was dialyzed against 70% ethanol and concentrated by ultrafiltration using YM10 membranes (Amicon, Millipore, US).

SDS-PAGE. Electrophoresis was carried out on PhastGel 20% (w/v) polyacrylamide gels (Amersham Biosciences, Uppsala, Sweden). Three eppendorf vials, containing 10 μL of protein extract in 70% ethanol, were dried under N_2 flux and dissolved in an electrophoretic sample buffer (0.125 M Tris–Cl, 4% SDS, 20% glycerol and bromophenol blue 0.01%, pH 6.8). Urea (5 M), 10% SDS and 10% BME were added to one of the vials to observe the monomer band. SDS (10%) and BME (10%) were added to the second vial, and the sample from the third vial was dissolved using only the sample buffer before mentioned. The three samples were submitted to heating at 100 °C for 15 min. Then, SDS-PAGE was performed according to Laemmli [22]. The gel was stained with silver (Amersham Biosciences, Uppsala, Sweden) for band visualization. The molecular weight standards (Bio-Rad, Hercules CA, USA) were: Phosphorylase b (97.4 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (31 kDa), Soybean trypsin inhibitor (21.5 kDa) and Lysozyme (14.4 kDa).

2.3. Analysis of sulfhydryl and disulfide contents

The free sulfhydryl group (SH) and total sulfhydryl group (including disulfide bonds) contents of purified Z19 were determined using the Ellman–Waimo reagent of *5,5′*-dithiobis(2-nitrobenzoic acid) and DTNB [23] and according Chang et al. method [24] with the modifications made by Hou-Chang [25] and Riener et al. [26]. Total free sulfhydryl content was determined adding 9 M urea in 0.1% EDTA solution (freshly prepared) to the protein solution (1.5 mg/mL), and the solution was heated in a boiling water bath for 15 min to unfold the protein structure. DTNB solution was then added for color reaction, and the absorbance was read at 412 nm. The surface free sulfhydryl (SH) content was determined using the same procedure without adding the denaturant (9 M urea in 0.1% EDTA solution). The SH value, after substracting the surface SH from the total free SH value, was defined as the internal free SH. The total sulfhydryl, including the S–S bond (total SH), was determined by using the same method (with urea) but after reduction of the proteins with sodium borohydride ($NaBH_4$). The disulfide group (SS) content was obtained as follows: (total SH − total free SH)/2. The calculations were made using a molecular mass of 24,535 g/mol.

2.4. Circular Dichroism (CD) Spectroscopy

The CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a Peltier thermostatic cell holder, using 1 mm path length quartz cell in the far-UV, base line and solvent corrected. The samples, containing a final concentration of 0.5–0.8 mg/mL of purified Z19, were filtered by 0.22 μm membranes (Millipore, Ireland). Three scanning acquisitions were gathered and averaged to obtain the final spectra in all cases. The results are expressed as a mean of the residue ellipticity [θ]mrw, using a value of 109.867 g/mol for the molecular weight of the mean residue. Secondary-structure estimation from the far-UV CD spectra was calculated using CDNN [27].

2.5. Thermal denaturation and renaturation

Thermal unfolding of the Z19 protein was studied by recording the CD signal at 221 nm as a function of temperature every 0.2 °C from 25 up to 90 °C. In addition, CD spectra were monitored between 200 and 260 nm at 10 °C intervals. Measurements were performed at thermal equilibrium using different heating rates (0.5, 1 and 1.5 °C/min).

2.6. pH experiments

The effect of pH on the secondary structure content, during the denaturalization and aggregation processes, was studied by CD and DLS spectroscopies. Spectra for thermal denaturalization and renaturalization were obtained at different pH values, from 3 to 9. All the buffers were made in ethanol 70% and 0.01 ionic strength adjusted. The protein solutions were buffer stabilized for 72 h at 4 °C.

2.7. Dynamic Light Scattering

Light scattering measurements were performed in a multi-angle light scattering instrument (Zetasizer Nano series Malvern Instruments, Ireland, U. K.). The samples were filtered in 0.22 μm membranes (Millipore, Ireland) to a final concentration of purified Z19 between 0.5 and 0.8 mg/mL. For the temperature dependence structure experiments, DLS measurements were carried out at the same temperature as the incubation temperature. For the pH dependence structure experiments, the measurements were carried out at 25 °C. Nine replicate measurements for each condition were collected and averaged. The results were analyzed with the Zetasizer Nano series Software (2004).

2.8. Computational prediction of the hydrophobicity

The amino acid sequence of the Z19 protein was analyzed for the hydropathical plot residues using the ProtScale program by means of the Kyte and Doolittle method [28], with a window size of 19, which is recommended for very hydrophobic proteins. The aliphatic index and the Grand hydropathicity average (GRAVY) were calculated using the ProtParam program.

3. Results and discussion

3.1. SDS-page of the Z19 protein

The purity of the protein was verified by SDS-PAGE, which, in addition, led to the detection of very stable 45 kDa

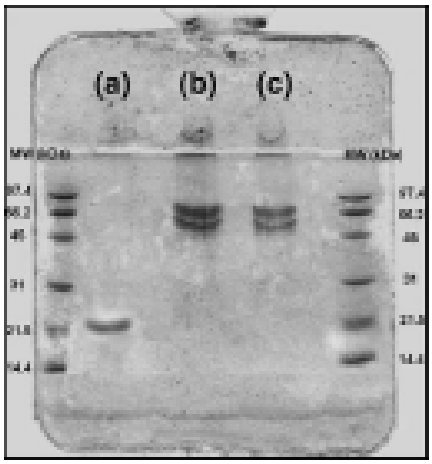

Fig. 1. SDS-PAGE of purified Z19 protein and its aggregates. (a) Z19 protein in monomeric form as a single band. Denaturalization treatment included the use of 10% SDS, 10% BME and 5 M Urea as described under Materials and methods. (b) 45 and 66 kDa dimer and trimer of the Z19 protein respectively. In this case, urea was no added. (c) Z19 protein boiling treatment without BME and urea.

and 66 kDa oligomers (Fig. 1). In order to visualize the monomer (Fig. 1, line a), the sample should be treated under drastic denaturing conditions (5 M Urea and 10% of additional SDS and 15 min in boiling water). Other denaturing conditions, such as those normally used in the SDS-PAGE, and even boiling for a long period of time using increased concentrations of detergent, chaotropic and reducing agents (Fig. 2, lines a and b), were not enough to produce the monomeric form. The high stability of Z19 protein oligomers is confirmed because these conditions were insufficient to disintegrate the 45 kDa, 66 kDa and higher molecular weight (HMW) oligomers. Nunes et al. [16] reported that incubating α-zeins at 100 °C for 20 min promotes the formation of these oligomers which are not affected by the treatment with

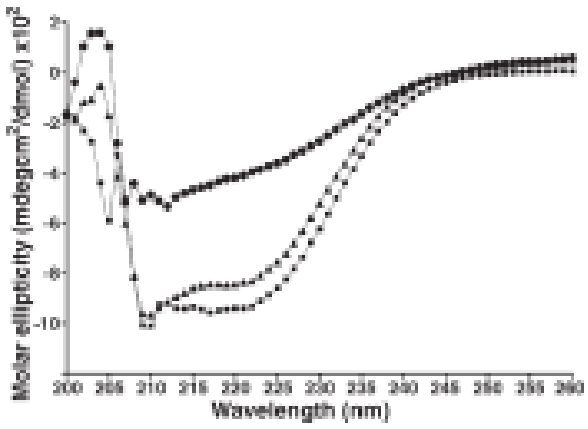

Fig. 2. Thermal denaturalization of the Z19 protein in ethanol 70%. Far UV-CD spectra measured at: (■) 25 °C the starting temperature, (●) 90 °C the highest temperature reached during the thermal unfolding and (▲) 25 °C after the heating-cooling process. Protein concentration was 0.6 mg/ml. CD spectra were measured at 10 °C interval during the heating cycle(25–90 °C). The heating rate was 1 °C/min.

Table 1
Sulfhydryl and disulfide contents (% per mole of protein) of the Z19 protein[a] based in the total SH[b] content including S–S

Surface free SH	2.50 (±0.05)
Internal free SH	0.0
Total free SH	2.47 (±0.05)
S–S content[c]	97.51 (±4.5)

[a] Data are expressed as means±standard deviation and are the mean of three replicates. All contents were expressed as cysteine equivalents except S–S, which is expressed in cystine equivalent. Data were calculated on the basis of a Z19 molecular mass of 24535 Da.

[b] Total SH is 13.98 (±0.05) moles per mole protein and contains all free and disulfide cysteine.

[c] S–S content was obtained from the difference of total SH and free SH contents in mole per moles of protein and divided by 2.

reducing agents. In agreement with the findings of other authors [29,30], our results show that a significant proportion of the Z19 protein can be found not only as monomers, but also dimers, trimers and oligomers, which are very stable. The electrophoretic patterns of cooked and uncooked prolamin samples of maize using increasing concentrations of the

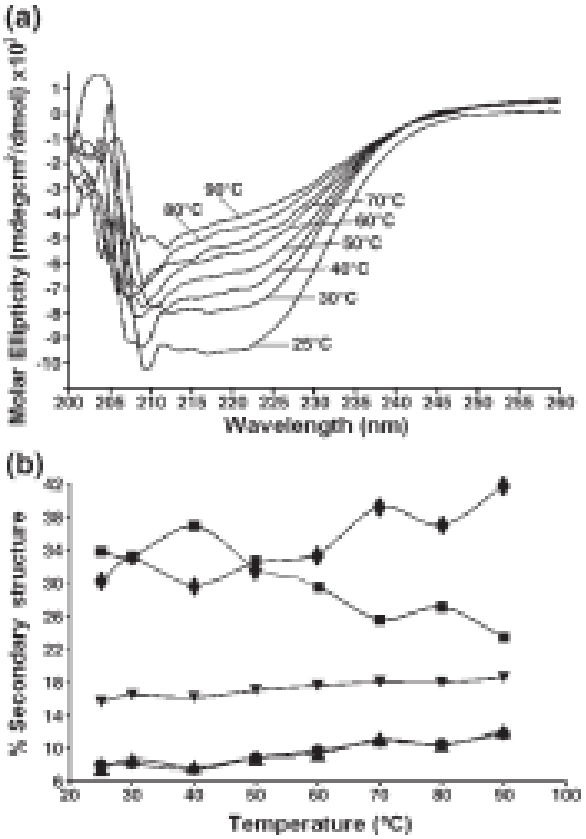

Fig. 3. (a) Far-UV CD spectra of Z19 protein (in ethanol 70%) at several different temperatures, from 25 to 90 °C, with 10 °C steps. (b) Changes of Z19 protein secondary structure, calculated using CDNN program [27] as a function of temperature (■) Helix, (●) Antiparallel, (▲) Parallel, (▼) Beta turn and (◆) Random coil.

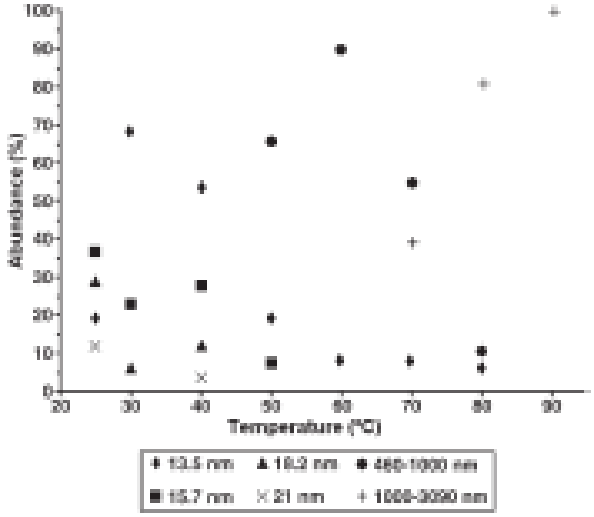

Fig. 4. Particle size distribution by volume for the Z19 protein in ethanol 70% in function of temperature measured by DLS. The results were analyzed by means of Zetasizer Nano series Software V1.4. The values are expressed as mean of three experiments. The populations with abundance minor than 5% were not plotted.

reducing-agent showed monomer bands intensity increased accompanied by a decrement in oligomer bands [29].

3.2. Determination of sulfhydryl groups and disulfide bond content

The proportion of disulfide bonds and free SH groups is a factor of flexibility, stability and tendency to oligomerization in a protein. Internal and superficial free SH groups were quantified (Table 1). Only 2.47% of the total SH are free and all of them are surface located, while most of the SH groups form disulfide bonds. No free SH was found inside the protein. In a previous work, three cysteins were described within the Z19 protein sequence [21]. Two of them are close neighbours in the sequence and they are separated by approximately 100 amino acid residues from the third. A considerable number of possible arrangements of monomers could be expected, resulting in the formation of 45 kDa and 66 kDa oligomers as well as higher molecular weight (HMW) oligomers. Disulphide-bonded protein oligomers were found in maize prolamins [29], suggesting their importance in the α-zeins structural organization. For kafirins (sorghum prolamins), it was suggested that the 44, 66 and HMW oligomers stability against heating and denaturizing conditions might be due to the presence of disulphide bonds that are inaccessible to the reducing agent [31]. Reduction-resistant oligomers may have conformations that do not allow easy access for the reducing agent to reach disulphide bonds [29].

3.3. Effect of temperature on the secondary structure and aggregation of the Z19 protein

Fig. 2 shows the far-UV CD spectra (200–260 nm) of Z19 protein in ethanol 70% obtained at 25 °C, after thermal unfolding at 90 °C, and after cooling at 25 °C. At 25 °C, the spectra show two strong ellipticity values around 210 and 222 nm (characteristic of α-helix structures) with average molar ellipticity values similar to those reported in previous studies for the Z19 protein [9,10]. The band near 222 nm in CD spectra of α-helical structures is due to the strong hydrogen-bonded environment and is largely independent of the length of the helix [32]. CD ellipticities at 222 nm have been widely used as an estimate of the α-helical content in proteins [33]. The changes on the helical content of Z19 zein after thermal treatment are shown in Fig. 2. A sharp decrease in ellipticity at 222 nm was observed for Z19 protein, when it was incubated at 90 °C indicating a complete loss of the protein secondary structure. Thermal denaturalization of the Z19 protein appears as an irreversible process, because the differences of the native and heated-cooled protein spectra. To corroborate the irreversibility of the thermal denaturalization the unfolding and refolding was monitored by the far-UV CD signal at 221 nm and plots with different slopes were obtained (data not shown). When

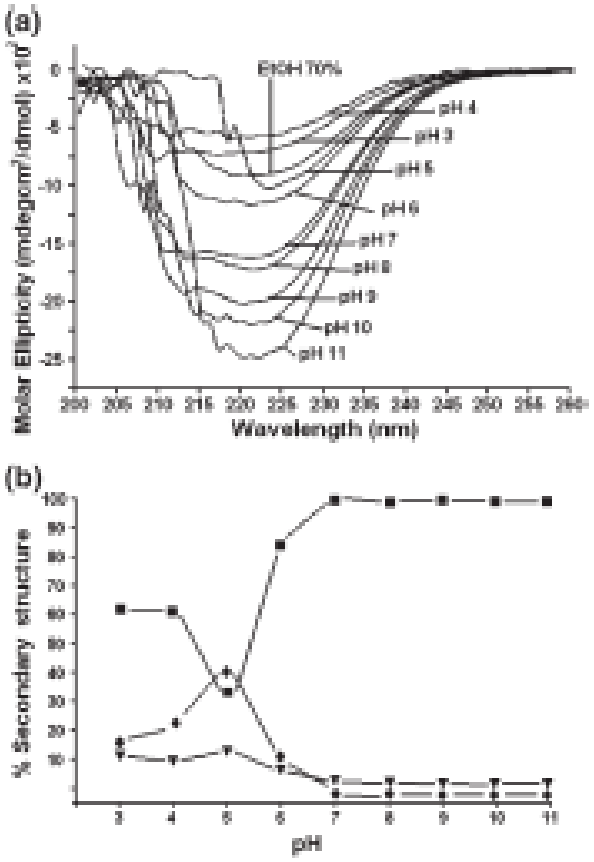

Fig. 5. (a) Far UV-CD Spectrum for the Z19 protein at 25 °C as function of pH. Protein concentration was 0.5–0.6 mg/mL. All buffers were made with ethanol 70% and 0.01 ionic strength adjusted. (b) Changes of Z19 protein secondary structure, calculated using the CDNN program [27] as a function of pH: (■) Helix, (▼) Beta sum and (●) Random coil. The Parallel and Antiparallel sheet contents were minor than 10% at all pHs.

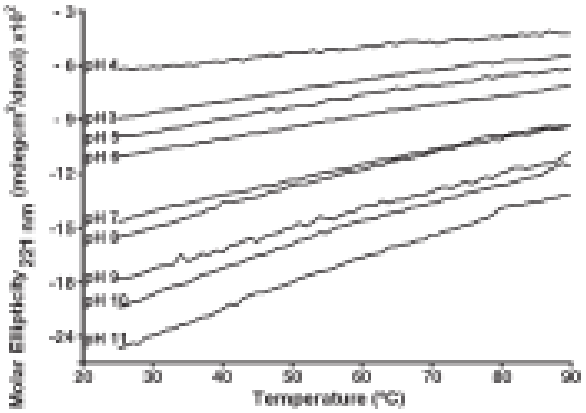

Fig. 6. Molar ellipticity of Z19 protein (in ethanol 70% buffer) was recorded at 221 nm as a function of pH and temperature (1 °C/min) from 25 to 90 °C during denaturalization.

the thermal unfolding is irreversible, the analysis of thermostabilities in terms of equilibrium thermodynamics is not applicable. During the heating-cooling cycle CD spectra as a function of temperature were collected, one every 10 °C from 25° to 90 °C and their far-UV CD data spectra were analyzed using the CDNN program [27] as shown in Fig. 3. In spite of the temperature increment, native structure was not completely lost, and the α-helix content decreased while β-pleated sheets, turns and random coils increased. From these results, it seems that the proteins remain soluble as the simultaneous formation of protein oligomers and order arrangements occur. This fact was illustrated in previous studies with kafirins (the sorghum prolamines) at 40 °C, in which the formation of aggregates is concurrent with an increment of β-intermolecular structures [34]. Other studies [35,36] reported that proteins with an α-helical content, higher than 40%, tend to form β-structures during the aggregation process, which showed the spectroscopic features of coiled coils [37]. Disulphide bonded polymers of the zein have also been reported to form when maize samples are heated. The electrophoretic analysis has shown that during the heating of maize a disulphide-mediated polimerization of α-zein [29,38] takes place. It seems that heating induces the formation of more disulphide crosslinked protein oligomers and polymers, resulting in a change in the protein secondary structure. Thus, we suggest that this polimerization avoids Z19 protein renaturalization.

Duodu et al. [39] showed that changes in the secondary structure occur when maize samples were heated wet. The protein tends to a more intermolecular β-sheet structure perhaps at the expense of some α-helical conformation. Such changes have been reported as characteristic of heat or solvent denatured and aggregated protein. It has also been observed in zein [40]. The change in the secondary structure of the protein (the increment on the β-sheet character) is due to heat denaturalization and aggregation.

The effect of pH on the thermal denaturalization of the protein structure was determined. Heating-cooling cycles were performed in a pH range between 3 and 11 and the irreversible character of thermal denaturalization was verified (data not shown). The particle size of a protein solution in 70% ethanol was analyzed during the thermal treatment (Fig. 4). The results obtained in DLS experiments at 25 °C showed a monomodal Gaussian distribution with 4 main populations with diameters between 13.5 and 21 nm (Fig. 4). These results are in agreement with those found by Bugs et al. [8], in which a molecular model of α-zein has been proposed with the following features: prolate ellipsoid with semiaxes of 13.8 nm and 3.4 nm, and with an axial radio of 4:1. The population with a diameter about 10–15 nm has been assigned as the monomeric form. Our results suggest that the population consists of monomers as well as dimers and trimers, with slightly bigger diameters than those expected due to the relaxation of the global protein structure. We suggest that this might be due to an increment in the random structure and a decrement in α helical structures. The molecule association phenomenon is more evident when the particle population distribution is observed as temperature is increased (Fig. 4). Smaller particles, representing the monomer, decreased as temperature was raised, while dimer and trimer populations increased.

3.4. Effect of pH on the secondary structure and oligomerization of the Z19 protein

Far-UV CD spectra for the Z19 protein were recorded in a range of pH from 3 to 11 (Fig. 5a). At a pH lower than 7, a shift to blue was observed, while at pH above the isoelectric point (6.8) the spectra shifted to red with no significant variations. The CDNN analysis of the far-UV DC data (Fig. 5b) indicates that when the pH is more alkaline than the isoelectric point, the protein structure is predominantly helical, suggesting that the α-helix structure is favoured when the protein possesses less positive electrostatic charges. Molar ellipticity at 221 nm vs. temperature was plotted (Fig 6) and the slopes were calculated (Table 2). The data suggest that denaturalization speed is a function of pH. Thermal denaturalization speed rises as pH became more alkaline.

Table 2
Slopes obtained from plotting Molar ellipticity vs. Temperature measured at 221 nm. during thermal denaturation

	pH 4	pH 3	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10	pH 11
Slope	14.44	27.4	29.90	30.04	41.40	47.85	52.35	59.77	66.90
S.D.	0.52	0.25	0.29	0.15	1.26	0.98	1.56	2.83	2.98
R^2	0.946	0.996	0.995	0.998	0.998	0.996	0.997	0.996	0.998

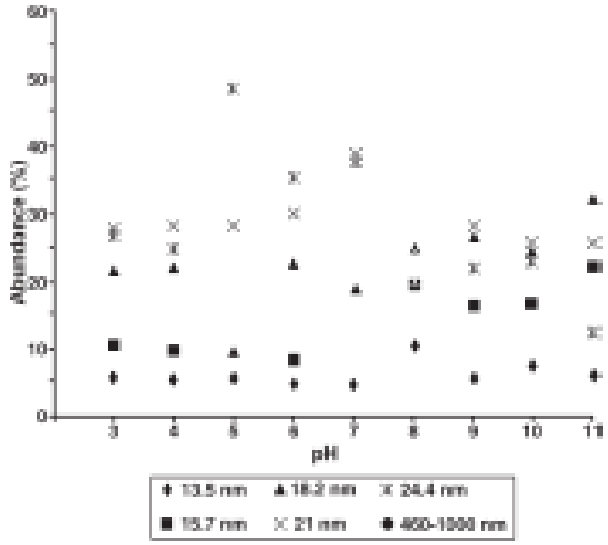

Fig. 7. Particle size distribution in function of pH measured by DLS at 25 °C. The results were analyzed by means of Zetasizer Nano series Software V1.4. The values are expressed as mean of three experiments. The populations with abundance minor than 5% were not plotted.

One possible explanation for this speed increment is the hydrolysis of glutamine transforming into glutamate at high pH values, which introduces negative charges to the protein structure and promotes electrostatic interactions and structural instability. The statistical analysis of protein particle size obtained by DLS (Fig. 7) shows that pH is important in the size distribution of the protein. Some of the bigger aggregates were observed in higher rates and when the protein was positively charged. On the other hand, the monomer is predominantly favoured in the presence of negative charges.

3.5. 19 kDa α-zein hydrophobicity

The "charge/hydrophobicity" theory proposes that the protein aggregation is a result of the balance between the charge and hydrophobicity distribution pattern along the sequence [41]. The zeins are known to be hydrophobic proteins [42,43]. The Goldman–Engelman–Steitz (GES) scale [44] considers the following: I, V, L, F, C, M, A, G, T, S, W, Y and P as hydrophobic amino acids on the basis of their free energy of transference from water to oil by calculating the surface area of each amino acid side chain in an α-helix. Using the GES scale for the analysis of the Z19 protein amino acid composition, the data presented in Table 3 shows that 76.25% of the amino acid residues are hydrophobic, with the highest levels corresponding to alanine, leucine, proline, and serine. The glutamine content in the water insoluble proteins (prolamines and glutenins) is equal or higher that the content in albumins and globulins. The abundance of glutamines could lead to the formation of intermolecular hydrogen bonds that would contribute to the oligomerization process and lead to the formation of more stable structures. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine and leucine). The Grand average of hydropathicity index (GRAVY) indicates the solubility in water of the proteins: hydrophobic proteins present positive values while hydrophilic proteins have negative GRAVY indexes [28]. As shown in Table 4, the Z19 protein amino acid sequence

Table 3
Mol% amino acid composition of 19 kDa α-zein compared with other seed proteins

Amino acid	Mol% amino acid											
	19 kDa rice globulin[a]	Oat globulin[a]	19 kDa sunflower albumin[a]	Maize albumin[a]	Barley albumin[a]	Wheat gliadin[a]	α-kafirin[b]	22 kDa α-zein[b]	Z19[c] protein	Maize glutenin[b]	Sorghum glutenin[b]	Wheat glutenin[a]
A	5.50	6.30	2.90	14.90	10.30	4.30	11.87	10.59	13.24	4.81	6.22	2.80
R	12.80	5.90	6.80	5.00	3.00	1.30	0.52	1.55	1.37	3.76	2.70	2.80
N	0.00	6.70	5.80	3.00	3.50	2.30	0.00	0.00	4.57	0.00	0.00	0.70
D	3.00	3.00	2.90	7.60	4.70	1.30	5.91	5.54	0.46	0.00	0.00	0.00
C	4.90	1.20	7.80	0.70	0.50	3.00	0.36	0.36	1.37	6.54	7.05	2.80
Q	12.80	14.00	7.80	3.60	5.30	29.80	23.80	22.58	19.63	17.89	14.18	33.80
E	9.80	5.90	9.70	5.60	5.70	0.70	0.00	0.00	0.46	0.00	0.00	1.40
G	8.50	7.30	4.90	7.30	6.50	2.60	0.67	0.67	0.91	3.52	4.95	3.20
H	0.60	2.20	2.90	1.30	2.50	2.30	2.30	1.38	0.46	8.94	9.03	1.10
I	1.20	5.30	2.90	3.60	4.80	6.00	4.66	5.07	5.48	1.89	2.55	4.90
L	5.50	7.10	8.70	5.50	11.20	7.00	17.48	18.71	19.63	11.81	11.20	8.50
K	0.00	3.00	3.90	6.30	5.50	1.00	0.87	0.43	0.00	0.00	0.57	0.40
M	4.90	0.80	15.50	1.70	1.80	2.30	1.77	2.22	0.00	1.07	1.74	1.00
F	2.40	5.50	0.00	3.00	5.80	5.30	4.40	4.91	5.02	1.19	1.92	4.20
P	4.90	4.80	5.80	5.90	3.80	16.60	7.50	7.52	9.59	21.14	19.65	13.70
S	11.00	7.30	4.90	5.60	9.50	4.30	4.98	5.93	8.22	3.79	4.89	7.40
T	1.80	3.40	1.00	8.90	5.30	4.60	2.82	3.54	3.20	3.86	4.16	2.80
W	0.60	0.80	1.00	2.30	0.80	0.30	0.61	0.00	0.00	0.00	0.00	0.70
Y	5.50	3.20	2.90	1.30	1.50	1.00	4.29	3.77	3.65	2.61	2.81	1.40
V	4.30	6.30	1.90	6.90	8.00	4.00	5.20	5.22	2.74	7.17	6.36	5.60

[a] Calculations from sequences obtained from the NCBI data bank.
[b] From Duodu et al. [39].
[c] From Cabra et al. [21].

Table 4
Comparison of free energy of hydration for amino acids, aliphatic and grand average of hydropathicity (GRAVY) indexes of 19 kDa α-zein and in other seed proteins

Amino acid	Free energy of hydration (kcal/mol)												
	Per residue [45]	19 kDa rice globulin[a]	Oat globulin[a]	19 kDa sunflower albumin[a]	Maize albumin[a]	Barley albumin[a]	Wheat γ-gliadin[a]	α-kafirin[b]	22 kDa α-zein[b]	Z19[c] protein	Maize glutenin[b]	Sorghum glutenin[b]	Wheat glutenin[a]
A	−0.66	−3.63	−4.16	−1.91	−9.83	−6.80	−2.84	−7.83	−6.99	−8.74	−3.18	−4.11	−1.85
R	−6.85	−87.68	−40.42	−46.58	−34.25	−20.55	−8.90	−3.56	−10.62	−9.38	−25.76	−18.50	−19.18
N	−3.15	0.00	−21.11	−18.27	−9.45	−11.02	−7.24	0.00	0.00	−14.39	0.00	0.00	−2.20
D	−3.11	−9.33	−9.33	−9.02	−23.64	−14.62	−4.04	−18.38	−17.23	−1.43	0.00	0.00	0.00
C	−0.27	−1.32	−0.32	−2.11	−0.19	−0.13	−0.81	−0.10	−0.10	−0.37	−1.77	−1.90	−0.76
Q	−3.15	−40.32	−44.10	−24.57	−11.34	−16.69	−93.87	−74.97	−71.13	−61.83	−56.35	−44.67	−106.47
E	−3.11	−30.48	−18.35	−30.17	−17.42	−17.73	−2.18	0.00	0.00	−1.43	0.00	0.00	−4.35
G	−0.23	−1.955	−1.68	−1.13	−1.68	−1.49	−0.60	−0.15	−0.15	−0.21	−0.81	−1.14	−0.74
H	−2.18	−1.31	−4.8	−6.32	−2.83	−5.45	−5.01	−5.01	−3.01	−1.00	−19.50	−19.69	−2.40
I	0.07	0.08	0.37	0.20	0.25	0.34	0.42	0.33	0.35	0.38	0.13	0.18	0.34
L	0.07	0.38	0.50	0.61	0.38	0.78	0.49	1.22	1.31	1.37	0.83	0.78	0.59
K	−3.77	0.00	−11.31	−14.70	−23.75	−20.73	−3.77	−3.28	−1.62	0.00	0.00	−2.15	−1.51
M	−0.10	−0.49	−0.08	−1.55	−0.17	−0.18	−0.23	−0.18	−0.22	0.00	−0.11	−0.17	−0.18
F	−0.28	−0.67	−1.54	0.00	−0.84	−1.62	−1.48	−1.23	−1.37	−1.40	−0.33	−0.54	−1.18
P	0.23	1.13	1.10	1.33	1.36	0.87	3.82	1.73	1.73	2.20	4.86	4.52	3.15
S	−2.36	−25.96	−17.23	−11.56	−13.22	−22.42	−10.15	−11.75	−13.99	−19.39	−8.94	−11.54	−17.46
T	−1.69	−3.04	−5.75	−1.69	−15.04	−8.96	−7.77	−4.77	−5.98	−5.41	−6.52	−7.03	−4.73
W	−0.88	−0.53	−0.70	−0.88	−2.02	−0.70	−0.26	−0.54	0.00	0.00	0.00	0.00	−0.62
Y	−2.82	−15.51	−9.02	−8.18	−3.67	−4.23	−2.82	−12.10	−10.63	−10.29	−7.36	−7.92	−3.95
V	0.04	0.17	0.25	0.08	0.28	0.32	0.16	0.21	0.21	0.11	0.29	0.25	0.22
Total hydration free energy		−220.458	−187.664	−176.418	−167.066	−151.028	−147,102	−140.36	−139.78	−131.228	−124.52	−113.63	−163.258
Aliphatic index[d]		44.02	72.63	53.98	70.99	96.09	66.19	–	–	119.13	–	–	71.34
Hydropathicity index[d]		−0.957	−0646	−0.586	−0.406	0.040	0.681	–	–	−0.273	–	–	−0.715

[a] Calculations from sequences obtained from the NCBI data bank according to Shewry et al. [45].
[b] From Duodu et al. [39].
[c] From Cabra et al. [21].
[d] Data calculated from the protein sequences by the ProtScale tool.

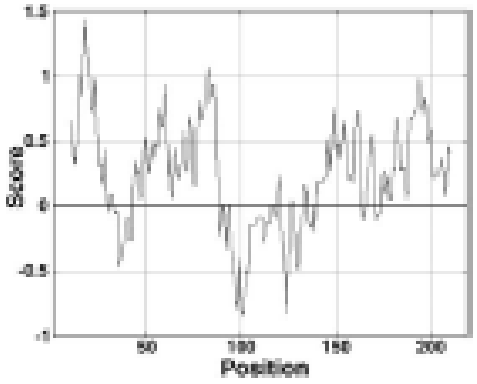

Fig. 8. Hydrophobicity plot for the Z19 protein and calculated by the Kyte and Doolittle method [28].

(analysis using ProtScale software) yields a high aliphatic index and a positive Grand average of hydropathicity (119.3 and 0.273, respectively) which suggests the highly hydrophobic character of this protein. In addition, there are low amounts of polar charged amino acids which contribute to the high insolubility in water and the tendency to aggregate shown by the Z19 protein. In general, this amino acid composition is shared with the other water insoluble proteins.

The relative hydrophobicity of the Z19 protein may be determined by calculating its free energy of hydration. The higher and more negative free energy of hydration, the less hydrophobic the protein [39,45]. Table 4 shows the free energies of hydration calculated for Z19 protein and proteins which are soluble in solvents such as water or saline, alcoholic, and alkaline solvents with reducing agent solutions (namely albumins, globulins, prolamins, and glutenins respectively).The calculations were made based on their amino acid sequences. The hydration free energy value obtained for the Z19 protein is less negative than those calculated values for albumins and globulins which are soluble in water and saline solutions, respectively. This indicates that the Z19 protein, along with other prolamins, is more hydrophobic than albumins and globulins. The reducing agent used in wheat glutenin extractions promotes differences in their structure that yield a more hydrophilic character even if their hydration free energies allow them to absorb water and form the viscoelastic gluten [39].

The use of hydropathical indexes is a useful tool to identify some important characteristics of proteins [46]. Hydrophobic and hydrophilic properties of the amino acid residues determine the protein structure and folding. The hydropathical indexes are assigned to each amino acid based on its relative hydrophobicity (positive values) or relative hydrophilicity (negative values). In the method by Kyte and Doolittle [28], the hydropathical indexes are assigned according to the water–vapor transfer free energies and the interior–exterior distribution of amino acids. In Fig. 8, the Z19 protein hydropathic plotting is shown, almost all the points in the graphic are located in the hydrophobic zone of the plot. It has three wide hydrophobic regions that include most of the structure separated by three small hydrophilic zones.

In conclusion, the Z19 protein showed a tendency towards oligomerization. In addition, these processes are responsible of the irreversible thermal denaturalization. We propose the disulphide-crosslinking and the hydrophobic character of the Z19 protein as the causes of the oligomerization processes. The Z19 protein oligomers population distribution depends on the conditions of temperature. The secondary structure content strongly depends on pH.

Acknowledgements

This work was partially funded by CONACyT project No. 35948-B. Authors thanks Arancia Corn Products, S.A. de C. V. for the materials provided. Sandra Pérez Munguía and Idalia Flores provided technical assistance. We thank Gabriela Saldeño-Monroy for her English revision. VC would was a recipient of a CONACyT scholarship 159981 and of funds from UNAM (PAEP 203304).

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3) DESAMIDACIÓN: EVALUACION DE LAS PROPIEDADES EMULSIFICANTES DE LA Z19 NATIVA Y MODIFICADA.

Las características determinadas para la α-zeína Z19 indicaban que los siguientes factores pueden contribuir a considerarla como posible emulsificante, pues coincidían con los de otras proteínas usadas con este propósito:

a. .periodicidad de sus patrones de hidrofobicidad
b. elevado contenido de residuos de aminoácidos hidrofóbicos
c. sensibilidad frente a la desnaturalización térmica
d. capacidad para formar agregados fácilmente mediante la formación de puentes disulfuro

por lo tanto, en la siguiente etapa del trabajo se evaluaron las propiedades emulsificantes de la proteína, tanto en su forma nativa como modificada químicamente.

Los resultados se muestran en el artículo III (página 53):

Cabra, V., Arreguín, Vázquez-Duhalt, R. and Farrés, A. (2006) Effect of alkaline deamidation on the estructure, surface hydrophobicity and emulsifying properties of 19 kDa alpha-zein. *J. Agric. Food Chem.* En prensa.:

La α-zeína Z19 en estado nativo desarrolla pobres propiedades emulsificantes y su hidrofobicidad superficial (S_o) es muy elevada. Con el objetivo de mejorar sus propiedades emulsificantes y saber cómo cambiaría la S_o de la proteína al verificarse esta mejora, se procedió a realizar el proceso de desamidación alcalina de la proteína, con lo que se pretendía introducir cargas negativas, aumentar el número de interacciones electrostáticas y con ello cambiar el estado electrostático y la hidrofobicidad superficial iniciales de la proteína.

Bajo las condiciones experimentales con las que se llevó a cabo la desamidación alcalina se desamidaron el 60.6% del total de los residuos de Asn y Gln susceptibles a esta modificación y se verificó 5% de hidrólisis proteica. Las propiedades emulsificantes mejoraron y se registró un S_o muy pequeño en comparación con el verificado antes de la modificación.

Con la finalidad de comparar los resultados de las propiedades emulsificantes y de hidrofobicidad superficial de la mezcla de α-zeínas, de la Z19 nativa y de la desamidada

con las de otras proteínas de fuentes diferentes pero medidas bajo las mismas condiciones experimentales (las indicadas en el artículo anterior), se extrajeron fracciones proteicas vegetales de granos de soya (*G. max*), trigo (*Triticum aestivum*), arroz (*Oryza sativa*) y cebada (*Hordeum vulgare*). Las proteínas de origen animal utilizadas para la comparación fueron albúmina sérica bovina (ASB, SIGMA), gelatina de piel de cerdo (SIGMA) y caseína (Research Organics).

Fraccionamiento y extracción a partir de las harinas de cada grano

Las proteínas se extrajeron de forma secuencial a partir de las harinas desgrasadas con hexano de acuerdo a los métodos reportados por diversos autores (Gorinstein et al., 2001; Lookhart y Bean, 1995; Osborne, 1924). La extracción de cada grupo de proteínas fue repetida tres veces. El proceso de extracción llevado a cabo se muestra en la **Figura 12**:

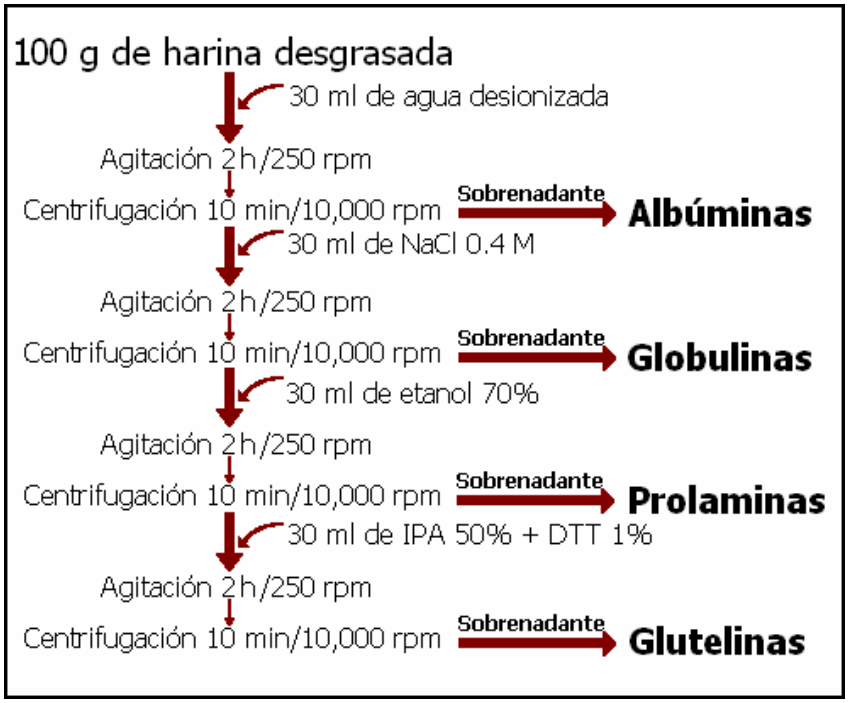

Figura 12. Extracción secuencial de los grupos de proteínas con base en su solubilidad a partir de las harinas desgrasadas con hexano. Las extracciones se llevaron a cabo a 25ºC.

Perfil electroforético

Se elaboraron geles desnaturalizantes de acrilamida para identificar con base en su peso molecular, a las proteínas extraídas de las diferentes fuentes y para determinar la pureza de las proteínas animales. Todas las muestras de proteínas se resuspendieron en volúmenes iguales de agua y buffer (0.125 M Tris-Cl, 4% SDS, 20% glycerol, 10% BME, 5 M urea y azul de bromofenol 0.01%, pH 6.8) y se sometieron a ebullición por 10 min. La electroforesis se llevó a cabo de acuerdo a Laemmli, 1970, en geles al 15% (p/v) de poliacrilamida que posteriormente fueron teñidos con azul de Coomasie.

Fracciones proteicas obtenidas de la soya, el trigo, el arroz y la cebada.

Las condiciones de extracción utilizadas fueron las descritas por Osborne (1924), cuya clasificación sigue utilizándose en la actualidad y divide a las proteínas en 4 grupos con base en su solubilidad: albúminas, globulinas, prolaminas y glutelinas. Es importante señalar que las condiciones de extracción son muy importantes ya que pueden generar diferencias significativas en las características conformacionales, de solubilidad e hidrofobicidad, las cuales afectarán a las propiedades funcionales de las fracciones proteicas (Abugoch et al., 2003; Zhang y Liu, 2005; DuPont et al., 2005).

Los patrones electroforéticos de todas las fracciones extraídas así como de las proteínas animales utilizadas se muestran en la **Figura 13**. Las bandas de las globulinas de soya corresponden a la β-conglycinina (α, α' y β), las sub-unidades ácida (A_x y A_3) y básica (A5, B_x y B_3) (Home-Jer and Kow-Ching (2004)).

Las albúminas de trigo de bajo peso molécular (14 klDa a 22 kDa) incluyeron a diversos inhibidores de amilasas. Las albúminas de alto peso molecular comprenden enzimas como la β-amilasa (DuPont et al., 2005) y se encuentran en grandes cantidades. Las gliadinas (las prolaminas del trigo) presentes en el extracto fueron las alfa (35-38 kDa), beta (37-43 kDa), gama (37-46 kDa) y omega gliadinas (48-63 kDa) (Woychick y col., 1961., Lookhart y Bean, 1995; DuPont y col., 2005). Debido a la reducción con BME, en el gel se observaron las sub-unidades tanto de alto como de bajo peso molecular de las glutelinas. Estas últimas, son muy semejantes a las alfa gliadinas en composición de residuos de aminoácidos y peso molecular. Las sub-unidades de alto peso molecular presentaron pesos de 80 a 160 kDa.

Las fracciones extraídas del arroz presentaron las siguientes bandas: 15, 27, 39, 50 y 56 kDa para las albúminas, la prolamina de 13 kDa y la glutelina de 15 kDa (Agboola y col., 2005).

La hordeína de 38 kDa fue la proteína que se extrajo en mayor proporción de los granos de cebada (Kanerva y col., 2005)

Se utilizaron proteínas de origen animal en un estado semi-purificado. La ASB se encontró ligeramente hidrolizada. La gelatina presentó una sola banda de alto peso molecular. En la muestra de caseína se detectaron bandas cercanas a los 35 kDa correspondientes a las α, β y γ-caseínas (Souza y col., 2000).

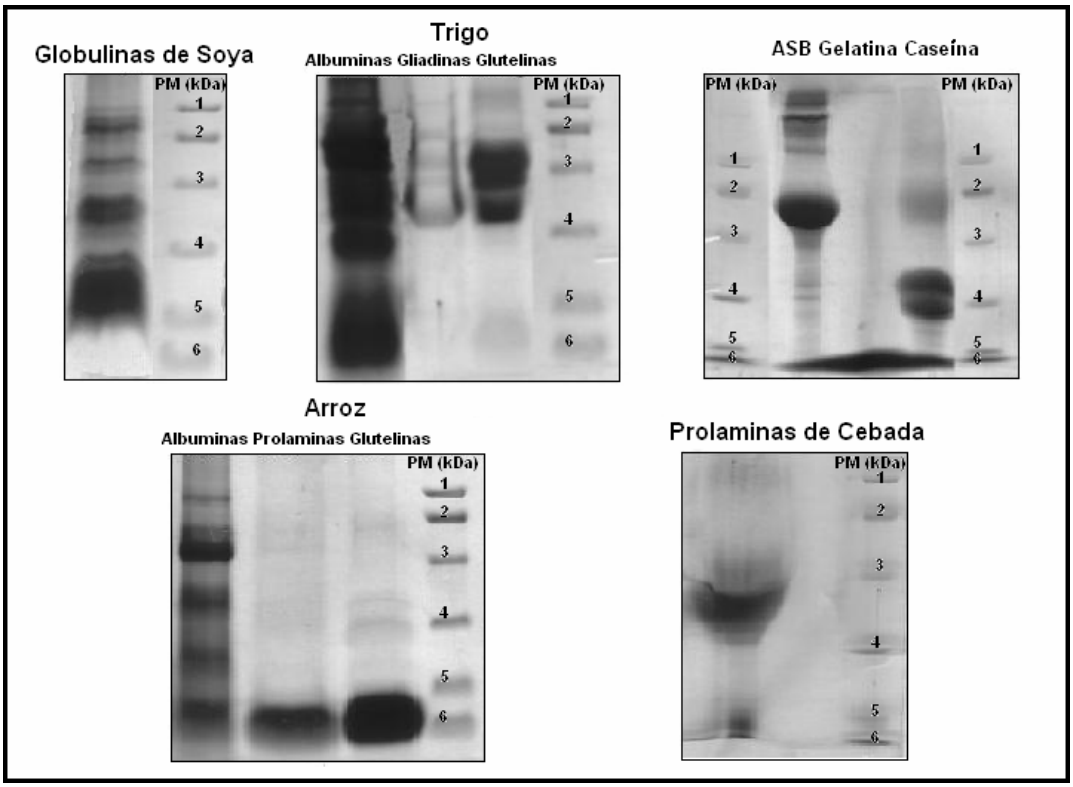

Figura 13. Perfiles electroforéticos de las fracciones proteicas de las diferentes fuentes utilizadas para la comparación de las propiedades emulsificantes y de hidrofobicidad superficial: Los marcadores de peso molecular utilizados fueron: 1.- Phosphorilasa b (97.4 kDa), 1.- Albúmina sérica bovina (66.2 kDa), 3.- Ovoalbúmina (45 kDa), 4.- Anhidrasa Carbonica (31 kDa), 5.- Inhibidor de tripsina de soya (21.5 kDa) y 6.- Lisozima (14.4 kDa).

Resultados de la comparación de los índices de hidrofobicidad superficial (S_o) y de las propiedades emulsificantes de las diferentes fracciones proteicas con los resultados obtenidos para las α-zeínas.

La mezcla de α-zeínas presentó un S_o muy similar al obtenido para las prolaminas de trigo y cebada (**Figura 14)**. En comparación con el resto de las fracciones, la hidrofobicidad obtenida por las prolaminas fue mayor. Sin embargo, el proceso de purificación al que fue sometida la mezcla de α-zeínas para la obtención de la Z19 y la desamidación de esta redujeron notablemente el S_o llegando a valores similares a los presentados por las globulinas de soya, la gelatina y la caseína, proteínas que son frecuentemente utilizadas por sus buenas propiedades emulsificantes en la industria alimentaria.

El valor de So obtenido para la prolamina del arroz fue muy similar al registrado para la Z19. Ambas proteínas presentaron un alto grado de pureza, lo que sugiere que en las mezclas de proteínas la asociación de las mismas promueve incrementos en la hidrofobicidad superficial, pero también de la hidrofilicidad superficial, condición que les permite mantenerse solubles en soluciones acuosas.

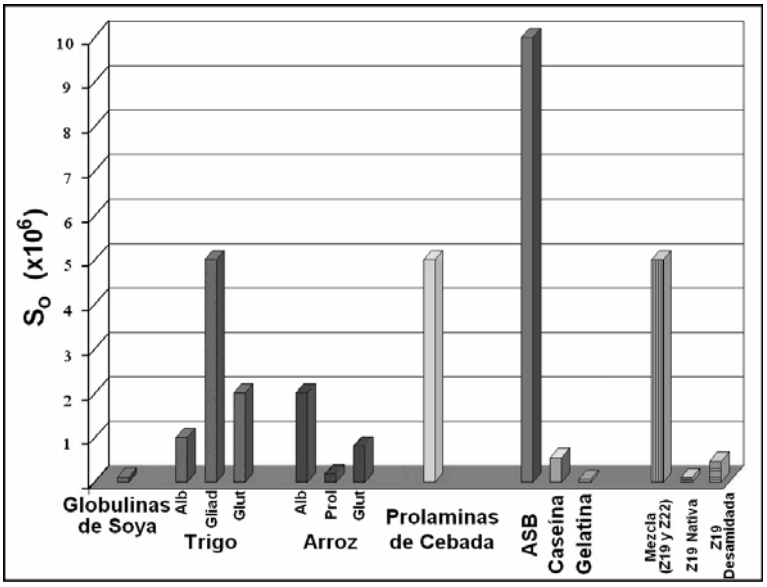

Figura 14. Comparación del Ìndice de Hidrofobicidad Superficial (S_o) obtenido de las diferentes fuentes proteicas con los resultados obtenidos para las α-zeínas. Las soluciones proteicas se hicieron al 0.1% (p/v) de proteína en buffer de fosfato de sodio 0.01 M (pH 7). El S_o se determinó con ácido 1-anilinonaphthalene-8-sulfónico (ANS).

A pesar de que las prolaminas comparten características comunes, presentan algunas diferencias en sus propiedades emulsificantes y de hidrofobicidad superficial. Las estructuras de las prolaminas de reserva del maíz, la cebada, el trigo y el arroz varían entre ellas, pero tienen en común dos características importantes: la presencia de dominios estructurales distintos que difieren en la composición de residuos de aminoácidos, y las repeticiones dentro des estos mismos dominios (Kreis y Shewry, 1989). Es importante mencionar que la solubilidad de todas las fracciones de proteínas es diferente y dependiente del solvente de extracción, razón por la cual la conformación adoptada por cada proteína en el buffer de fosfatos será diferente. Esto es particularmente relevante en el caso de las prolaminas, cuyos solventes ideales son los alcoholes y los cuales no pueden ser utilizados en esta prueba debido a que provocan sobreestimación de los valores (Matulis y Lovrien, 1998).

La emulsión elaborada con la mezcla de α-zeínas registró baja estabilidad de emulsión (EE), incluso menor que la presentado por las emulsiones elaboradas con las otras prolaminas. (**Figura 15**). La purificación de la Z19 permitió mejorar la estabilidad de la emulsión a valores similares a los alcanzados por las emulsiones hechas con las albúminas de trigo, las glutelinas de arroz y la hordeína, esta última la más parecida a la Z19 en cuanto a solubilidad y estado de pureza.

La desamidación de la Z19 permitió mejorar notablemente la EE, ya que se obtuvo un valor similar al obtenido por la emulsión con caseína y superior al obtenido con las globulinas de soya y la gelatina, proteínas que, como ya se había mencionado, son muy buenas emulsificantes.

Al analizar el tamaño de partícula registrado para cada una de las emulsiones elaboradas (**Tabla 1**), vemos que a pesar de que el diámetro establecido para los glóbulos de grasa estabilizados en una emulsión es de 1000 μm como máximo para considerarla como adecuado para emulsiones alimentarias, proteínas empleadas cotidianamente como las globulinas de soya y la gelatina sobrepasan ese límite.

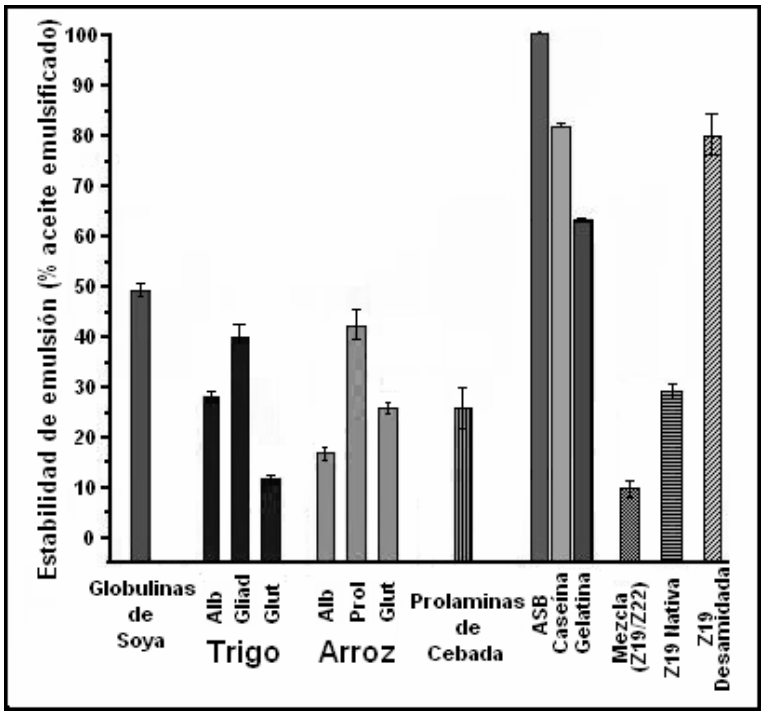

Figura 15. Comparación de los resultados de EE obtenidos de las α-zeínas con los obtenidos de las fracciones de otras fuentes proteicas. La mezcla de α-zeínas es la obtenida antes de la purificación de la Z19.

Los glóbulos de grasa estabilizados en la emulsión elaborada con la mezcla de α-zeínas son muy grandes, lo que coincide con la baja EE presentada. Los pasos de purificación y desamidación además de disminuir la hidrofobicidad superficial aumentaron la EE y disminuyeron el tamaño de partícula hasta un valor similar al obtenido para las albúminas de trigo y en general, menor al registrado por la mayoría de las fracciones proteicas probadas. Las mejores emulsiones en general fueron las elaboradas con las proteínas de origen animal, sin embargo estas presentaron tamaños de partícula mucho más grandes que el obtenido para la Z19 desamidada.

El análisis comparativo de los resultados obtenidos de la mezcla de α-zeínas para la hidrofobicidad superficial y las propiedades emulsificantes permitió ubicarlas dentro de este grupo de proteínas como malas emulsificantes. Sin embargo, la purificación de una de ellas, la Z19 y su posterior desamidación, colocan a esta proteína como buena emulsificante en comparación con el resto de las fracciones de proteínas evaluadas.

Tabla 1. Comparación de los tamaños de partícula promedio obtenidos de las emulsiones elaboradas utilizando las proteínas de diferentes orígenes con los obtenidos de las emulsiones elaboradas con α-zeínas.

Proteína	**Diámetro promedio del glóbulo graso[a] (nm)**
α-zeínas:	
- Mezcla	2380±8.4
- Z19 no desamidada	1488±4
- Z19 desamidada (60.6 GD)	58±6.7
Globulinas de soya	1040±2.5
Aislado de trigo:	
-albúminas	79±8
-gliadinas	>6000
-glutelinas	394±44
Aislado de arroz:	
-albúminas	117±15
-prolaminas	1610±3
-glutelinas	33±3
Prolaminas de cebada	>6000
ASB	788±2
Caseína	269±6

Gelatina	>6000

[a]Promedio de la determinación por triplicado

En general no se observó un patrón claro en la relación de la hidrofobicidad superficial – EE– tamaño del glóbulo graso. Para la ASB se tuvo un elevado valor de S_o, muy buena EE y un diámetro de glóbulo ligeramente menor a 1000 μm. Para las globulinas de soya se registraron un S_o muy bajo, una estabilidad de emulsión cercana al 50% y un tamaño de glóbulo graso similar al obtenido con la ASB

En el caso particular de las α-zeínas, se determinó que los procesos de purificación y desamidación provocaron la disminución de la hidrofobicidad superficial, la mejora de la EE y un diámetro menor del tamaño del glóbulo graso estabilizado en las emulsiones elaboradas con ellas.

Carta de aceptación del artículo:

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Dear Dr. Farres:

We are pleased to inform you that your manuscript entitled "Effect of alkaline deamidation on the structure, surface hydrophobicity and emulsifying properties of 19 kDa alpha-zein" (manuscript ID jf061002r) has been accepted for publication in Journal of Agricultural and Food Chemistry. Your manuscript has been forwarded to the ACS Publications office.

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Kind regards,

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Associate Editor, Journal of Agricultural and Food Chemistry

jagfoodchem@comcast.net

Effect of alkaline deamidation on the structure, surface hydrophobicity and emulsifying properties of the Z19 α-zein.

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Different deamidation conditions for the Z19 α-zein were studied in order to find the best conditions for the development of the emulsifying properties. Alkaline deamidation was chosen and the effects on the peptide bond cleavage, secondary structure, emulsifying properties and surface hydrophobicity were studied. The Z19 α-zein was deamidated by using 0.5 N NaOH containing 70% ethanol at 70ºC for 12 h, a deamidation degree (DD) of 60.6±0.5% and a degree of hydrolysis (DH) of 5±0.5% were achieved. Analysis by far-UV circular dichroism showed the denaturation was mainly promoted by the high temperature used during the incubation. The adequate balance between the DD and the DH results in an effective emulsifying property improvement for the Z19 α-zein. Thus, after the deamidation treatment, the surface hydrophobicity (S_o) decreased from $9.5x10^4$±$6.8x10^3$ to $46x10^4$±$2.1x10^3$, and the emulsion stability (ES) increased from 18±0.7% to 80±4.7 % since the oil globules stabilized by the modified protein were smaller (57.7±5.73 nm) and more resistant to coalescence than those present in the native protein emulsions (1488±3.92 nm).

KEY WORDS: α-Zein, protein emulsifying properties, protein deamidation, protein hydrolysis, surface hydrophobicity, circular dichroism, dynamic light scattering.

INTRODUCTION

Zeins, maize (*Zea mayz)* prolamins, are synthesized during the endosperm development and stored in proteic bodies (*1*). α-zeins are the most abundant (75 - 85% of the total) prolamins and are divided in two types: Z19 and Z22, with approximate weights of 22 and 25 kDa respectively (*2*). The Z19 protein is rich in hydrophobic amino acids (more than 50%), especially aliphatic amino acids, with the highest levels corresponding to alanine, leucine and proline (*3*,*4*). This structure yields high aliphatic indexes and high surface hydrophobicity. The low amounts of polar-charged amino acids explain their high insolubility in water and its tendency to aggregate. The amount of aromatic residues is low and there are no tryptophan residues (*3*). Another of its characteristics is that almost all the β- and γ-carboxyl residues of aspartic and glutamic acids are amidated (asparagines and glutamines, respectively). α-zeins posses a high α-helical structure content. The Z19 protein is 40% α-helical and 14.5% β-sheet (*4*). Diverse authors (*5*,*6*,*7*) propose that the Z19 protein is divided in two distinct regions, a hydrophobic hydrocarbon portion is thought to dominate one end of the zein molecule while the other end is weakly polar resulting from an abundance of hydroxyl, ethoxyl and keto groups. These features promote poor functional properties and consequently, the α-zeins are not utilized as food ingredients in human food and are destined to animal consumption (*4*). However, due to all the features mentioned before, Z19 is suitable for modifications to improve its functional properties, particularly, its emulsifying properties. Proteins are commonly used as emulsifiers of oil/water mixtures in food products, but the relationship between protein structure and emulsifying properties has not been clearly established (*8*). The amphipatic α-helix is a structural feature which has previously been proposed as favoring good emulsifying properties and contributing to the surface activity proteins (*9*, *10* ,*11*). Furthermore, *in vitro* studies have shown that the presence of an interface can induce or increase the degree of α-helix formation (*12*, *13*, *14*). The structure-function relationship of food proteins can be studied by modifying the amino acid side chains (*15*).

The modification of the protein usually refers to physical, chemical or enzymatic treatments which change its conformation and structure and, consequently, its physicochemical and functional properties (*16*). Deamidation, the hydrolysis of amino acid side-chains of the protein, is one of such modifications, which is considered necessary for the development of desirable food-use functionality. Deamidation could be performed by chemical or enzymatic methods. The chemical reactions by mild acid hydrolysis and/or basic solutions have been used to modify many cereal proteins, such as wheat, corn, and oat proteins, which have high levels of the amide-containing

amino acids: glutamine and asparagine. These side chains are susceptible of hydrolysis on the amide bond, which leads to the release of ammonia and the transformation of acidic groups. The conversion of the amide groups into acid groups may partially unfold the protein, resulting in an amphiphilic molecule that can be used as a surface active agent or emulsifier for food processors (*17*). The deamidation process may indirectly lead to protein hydrolysis by cleavage of the peptidic bond; its extent depends on the reaction conditions. Thus, a moderate hydrolysis will increase flexibility and exposure of different fragments in proteins containing a high proportion of secondary structures (*18*). It has been shown that these changes improve the functional properties of different proteins, making them useful products, especially for the food, pharmaceutical, and related industries. (*19*,*20*). However, excessive peptide bond cleavage during hydrolysis could affect the polymeric structure of the protein, resulting, often, in undesirable properties such as bitter taste and reduced functionality (*18*).

The use of enzymes in protein modification is desirable due to their speed, mild reaction conditions, and their high specificity. Transglutaminase, protease, peptidoglutaminase (PGase), glutaminase (*21*,*22*) and recently protein glutaminase (PG) (*23*) are the only enzymes reported in literature for protein deamidation. In both types of deamidation, the changes in structure and physicochemical properties, as well as the effect on the functional properties, are unclear. A partial protein unfolding with hydrophobic amino acids residue exposure could be verified as a consequence of chemical and physical modifications of proteins.

MATERIALS AND METHODS

Biological material. Waxy yellow dent from corn hybrid grains (*Zea mays* L.) were grounded in a disk mill until a particle size of 40 μm was obtained (Weber BROS. & White, Metal Works Inc., U.S.).

Z19α-Zein extraction and purification. The extraction was performed according to Dickey, *et al*. (*25*) with a slight modification. Lipids from maize flour were extracted with hexane. Carotenes and xanthophylls were removed using a chloroform-methanol mixture (2:1). Defatted, decolorized flour was mixed with 95% ethanol (5:1 solvent-flour (v/w)) through agitation for 12 h at 25°C. This mixture was then centrifuged at 12000 g for 30 min at 4ºC and the supernatant was

Hydrophobic interactions are extremely important in the protein folding as well as protein interactions. The changes in the hydrophobic surface of the protein can result in important changes in the functionality of the protein (*24*). A significant correlation has been obtained between the emulsifying activity and the protein hydrophobicity determined fluorometrically (*24*). These results suggest the emulsification of oil by protein could be explained based on protein hydrophobicity. In addition to protein hydrophobicity, the protein flexibility is another important feature to consider for emulsifying capacity of a protein, since they could facilitate its interaction with the oil surface. An increment of surface hydrophobicity could decrease the interfacial tension between the oil and water. Thus, the increment in emulsifying capacity could be debt to a better hydrophobicity-hydrophilicity balance, which is more effective in the modified protein (*15*).

In this paper, we compared different methods for the deamidation of the Z19 α-zein. Alkaline deamidation was chosen in order to study the effect of deamidation on the structural characteristics (peptide bond cleavage, secondary structure content and surface hydrophobicity changes) of the protein and relate them with the emulsifying properties.

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recovered. The protein was purified using the previously described protocol (*3*): a cationic exchange column was used (SP-Sepharose 2.6 x 11 cm Amersham Biotech, Uppsala, Sweden). The mobile phase was a citrate buffer, 0.02M with 70% methanol, pH 3.5. The sample was solubilized in this phase to reach a final concentration of 0.5 mg/ml and injected into a high pressure liquid chromatograph (AKTA prime, Amersham Pharmacia Biotech, Uppsala, Sweden). The elution buffer had 0.7M NaCl, and a gradient from 0 to 1.2 mM NaCl was performed. The purified Z19 □-zein preparation was dialyzed against 70% ethanol, concentrated by ultrafiltration using YM10 membranes (Amicon, Millipore, U. S.) and dried under N_2 flux.

Deamidation of Z19 α-zein. Three forms of deamidation were carried out: a) Enzymatic deamidation was carried out in a 50 mM sodium citrate buffer pH 4.9 containing 70% ethanol (such ethanol concentration was determined by preliminary experiments to assure enzyme activity and solubility), 2 mg/mL purified Z19 protein, and 2 unit/mL glutaminase (catalogue G-5894, SIGMA ALDRICH, Steinheim, Germany). The deamidation was performed at 25ºC in 24 hour intervals from 0 to 144 h. After the reaction, the protein solution was adjusted with NaOH to pH 7
b) For the acidic deamidation, Z19 protein (2 mg/mL) was solved in 70% ethanol 0.5 N HCl and incubated at 25ºC in 24 hour intervals from 0 to 144 h. After the reaction, the reaction mixture was adjusted with 0.5 NaOH to pH 7.
c) Basic deamidation was carried out dissolving Z19 protein (2 mg/mL) in 70% ethanol 0.5N NaOH. The protein solution was incubated at 25ºC in 24 hour intervals from 0 to 144 h. After the reaction, the reaction mixture was adjusted with 0.5 HCl to pH 7. For the second part of the experiments, the deamidation conditions were 70% ethanol 0.5, 1, 1.5 and 2N NaOH, the incubation temperature was 70ºC and the reaction times were 12, 18, 24 and 30 hours.
The final protein solutions were dialyzed toward 70% ethanol, concentrated by ultrafiltration using YM10 membranes (Microcon, Millipore, U. S.), dried under N_2 flux and lyophilized.

Determination of the deamidation degree. After incubation, the amounts of ammonia released were determined for all the deamidated Z19 protein solutions by using an Ammonia Enzymatic BioAnalysis Test Kit according to the manufacturer's instruction (Boehringer Mannheim, Darmstadt, Germany). The deamidation degree was expressed as the ratio of the amount of released ammonia by deamidation reactions and the total released ammonia when the Z19 protein solution was treated with 3N sulfuric acid.

Measurement of the degree of hydrolysis. The degree of hydrolysis is expressed as the percentage of the dissolved protein in the hydrolysate after precipitation in 0.2 N aqueous trichloroacetic acid; refered to the total dissolved protein (100%) obtained after complete hydrolysis with 3 N sulfuric acid.

Circular Dichroism (CD) Spectroscopy. The CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a Peltier thermostatic cell holder, using 1 mm path length quartz cell in the far-UV, base line and solvent corrected. The non modified as well as the enzymatical, acid and alkaline deamidated Z19 protein solutions containing a final concentration of 0.5 mg/mL in 70% ethanol were filtered with 0.22 □m membranes (Millipore, Ireland). the non deamidated Z19 □-zein used as control was incubated at 25ºC and CD spectra were monitored between 200 and 260 nm at 24 h intervals from 0 to 72 h. After the deamidation treatments, the Z19 protein solutions CD spectra were measured at 25ºC between 200 and 260 nm. Three scanning acquisitions were gathered and averaged to obtain the final spectra in all cases. The results are expressed as mean of the residue ellipticity, [θ]mrw, using a value of 109.867 g/mol for the molecular weight of the mean residue. Secondary-structure estimation from the far-UV CD spectra was calculated using CDNN (*26*).

Isoelectric point calculation. A prepared IEF acrylamide gel (Amersham Pharmacia, Uppsala, Sweden) was used, with a 3-9 pH gradient. The non modified and deamidated Z19 protein lyophilized samples were solubilized in a 0.5% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate (CHAPS) solution and 1 μL of each sample was used per lane. Bands were visualized using a silver staining kit (Amersham Biosciences, Uppsala, Sweden).

SDS-Polyacrilamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was used to examine the purity of the purified Z19 protein. All protein samples were resuspended in equal volumes of deionized water and buffer (0.125M Tris-Cl, 4% SDS, 20% Glycerol, 10% BME, 5 M Urea and bromphenol blue 0.01%, pH 6.8) and then submitted to heating at 100°C for 10 min. SDS-PAGE was performed according to Laemmli (*27*). Electrophoresis was carried out on 15% (w/v) polyacrilamide gels for all the samples except for

the zeins which were carried out on a PhastGel 20% (w/v) polyacrylamide gels (Amersham Biosciences, Uppsala, Sweden). The gel was stained with silver (Amersham Biosciences, Uppsala, Sweden) for band visualization. The molecular weight standards (Bio-Rad, Hercules California, USA) were: Phosphorylase b (97.4 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (31 kDa), Soybean trypsin inhibitor (21.5 kDa) and Lysozyme (14.4 kDa).

Surface Hydrophobicity Index (S_o). Surface hydrophobicity of protein solutions 0.1% (w/v) in 0.01 M sodium phosphate buffer (pH 7) was determined using a fluorescence probe, 1-anilinonaphthalene-8-sulfonic acid (ANS), according to the method of Kato and Nakai (*28*). Fluorescence intensity (FI) was measured at wavelengths of 340-380 nm (excitation) and 505-515 nm (emission) using a Versafluor™ Fluorometer System (Bio-Rad, Hercules, CA., U. S. A.). The surface hydrophobicity index was calculated by linear regression analysis using the slope of the straight line obtained by plotting the FI as a function of the protein concentration.

Evaluation of emulsifying properties. The emulsifying properties were measured as previously described (*29, 30, 31*). To prepare the emulsions, 0.2 mL of tricaprilin and 1.8 mL of protein solutions (0.1% w/v in 0.01M sodium phosphate buffer pH 7) were homogenized for 3 min at 22000 rpm with a high-speed homogenizer Ultraturrax (Tekmar, Mod. TYPE STD 1810, Cincinnati, OH, U. S. A.).

a) Emulsion stability. The stability of the emulsions was verified by filling microcentrifuge tubes with emulsion and centrifugating at 3000 rpm for 3 min to observe the cream phase formation and quantify the amount of oil separated.

b) Particle size measurement. The particle size distribution of the emulsions was measured using dynamic light scattering (DLS). Light scattering measurements were performed in a multi-angle light scattering instrument (Zetasizer Nano series Malvern Instruments, Ireland, U. K.). Emulsion sample aliquots of 25 μL were diluted in 225 μL 0.01M sodium phosphate buffer (pH 7) and filtered in 0.22 μm membranes (Millipore, Ireland). The measurements were carried out at 25°C. Three replicate measurements for each sample were collected and averaged. The results were analyzed with the Zetasizer Nano series Software (2004).

RESULTS AND DISCUSSION:

Enzymatic, Alkaline and Acid deamidation of Z19 α-zein

The three different types of deamidation were compared and their effects on the secondary structure of the Z19 α-zein were determined. **Figure 1** shows the deamidation degrees (DD) obtained as a function of the enzymatic, alkaline and basic deamidation reaction time. From our previous experiments and in agreement with data reported by other authors (32, 23), the α-zein emulsifying properties improves when deamidation degree (DD) reaches values higher than 15-20%.

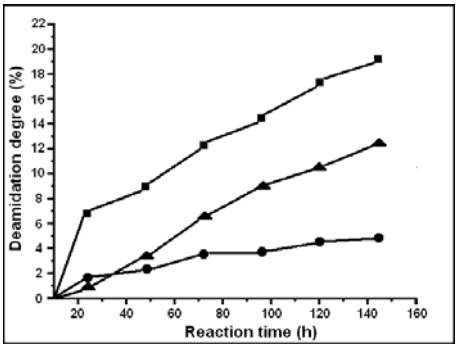

Figure 1. Deamidation degree as a function of time obtained by the (■) enzymatic, (▲) alkaline and (●) acid deamidation treatments. In all cases, the Z19 protein solution concentration was 2 mg/mL containing 70% ethanol pH adjusted. The incubation was carried out at 25ºC for 144 h.

Thus, the deamidation reactions were monitored until reach these values. The deamidation reactions catalyzed by the PG reached higher DD values, faster than the obtained by the chemical modifications. Among the chemical modifications, the alkaline diamidation was the most effective.

In order to compare the secondary structure changes obtained by the three different deamidation treatments, the far-UV CD spectra (200-260 nm) of the alkaline, acid and enzymatically deamidated samples were recorded. To distinguish between the effects of the deamidation versus protein unfolding, a control non-deamidated Z19 α-zein sample was also monitored during its incubation in ethanol 70% at the same temperature and times as the deamidated samples (**Figure 2**). The Z19 α-zein native spectra before the incubation at 25ºC shows two strong ellipticity values around 210 and 222 nm (characteristic of α-helix structures) with average molar ellipticity values similar to those reported previously for the Z19 α-zein (*3, 6*). As expected, no signals were obtained for the chemically

deamidated samples (data not shown) indicating a total protein unfolding and denaturation promoted by the high temperature and the strong acidic and alkaline incubation conditions. Signals at 210 and 221 nm were minimal for the enzymatically modified Z19 α-zein. The control non-deamidated Z19 α-zein sample was incubated under the same conditions and it was totally unfolded. Incubation conditions were the only reason for changes in the secondary structure content of the non deamidated control.

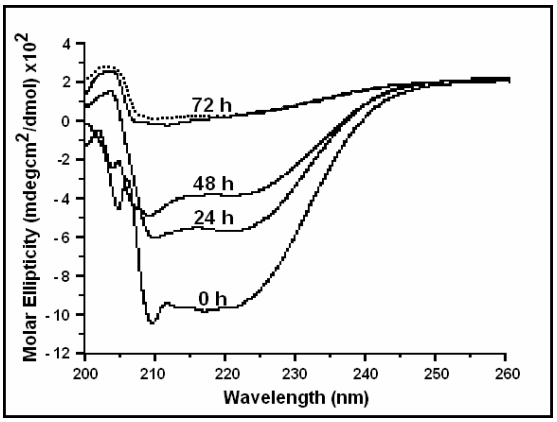

Figure 2. Far UV-CD spectra of the control non-deamidated (solid lines) and enzymatically deamidated (72 h/25ºC, DD = 19%) (dotted line) Z19 α-zein. The control non-modified Z19 zein sample was incubated for 72 h at 25ºC and measured at this temperature. In both cases, the lyophilized samples were solubilized in ethanol 70% with a final protein concentration of 0.5 mg/mL .

Thus, the Z19 α-zein proved highly thermolabile, as a result of its tendency to oligomerization, avoiding a reversible thermal renaturation(*33*). Yong et al, (*23*) reported the effects of enzymatic deamidation with PG on the conformation of an α-zeins mixture in 70% ethanol at a higher temperature and a longer reaction time. They reported α-helix content of non-modified α-zeins of 46%, on the other hand, for the deamidated sample (64 DD, the incubation was carried out at 40ºC for 137 h), this value decreased to 36%. In contrast, our results show that, at lower temperature (25ºC) and shorter incubation time (72 h), the purified Z19 α-zein looses all the α-helix content. The thermal stability seems to be lower in the purified preparation than in the α-zeins mixture.

The enzymatic, alkaline and acid deamidation resulted in a water (pH 7) solubility increment of 22±1.7%, 34±2.3% and 9±2.1% respectively. The results are in agreement with the reported by previous studies on α-zeins for the enzymatic (*23*) and chemical deamidation (*32*). The only deamidation conditions where the Z19 protein did not present precipitates were the alkaline ones. This preparation was easily dispersed in water at pH 7. For this reason, we chose the alkaline deamidation to continue with the surface and emulsifying properties.

Evaluation of alkaline deamidation

In order to find the best alkaline deamidation conditions to verify an improvement in the emulsifying properties, 4 different NaOH concentrations and 4 reaction times were tested. We decided to raise the temperature to 70ºC to reduce incubation time. This temperature was chosen based on previous studies, in which the high temperature effects on the protein structure are the irreversible unfolding and protein aggregation (33). Disulphide bonded polymers of zeins are also formed when maize samples are heated. The electrophoretic analysis has shown that during the heating of maize at 100ºC for 3 hours, a disulphide-mediated polymerization occurs (*34, 35*) without significant protein hydrolysis. In addition, heating promotes hydrophobic interactions and the aggregation of the denatured protein molecules, originated by the exposed hydrophobic core of the unfolded protein (*36, 37*). The protein hydrolysis occurs under the conditions used for alkaline deamidation in this work. Due to protein hydrolysis, molecular properties of proteins change as follows: molecular weight decreased, charge increased, hydrophobic groups are exposed and reactive amino acid side chains are disclosed (*38, 39*). As a result, the functional properties are affected. It has been determined that in general, protein solutions with a low degree of hydrolysis (1-10%) show improved functional properties (mainly foaming and emulsifying capacity) (40-45). Thus, the measurement of the degree of hydrolysis (DH) was carried out for the samples (**Table I)**. The DH incremented in function of the incubation time and NaOH concentration. Protein hydrolysis degrees, higher than 10%, were detected in almost all the deamidated protein samples, except in those incubated in NaOH 0.5 N for 12, 18 and 24 h.

Table I. Degree of hydrolysis (DH) obtained using different NaOH concentrations for the Z19 α-zein deamidation[a].

Incubation time (h)	DH (%)[b]			
	0.5 N	**1 N**	**1.5 N**	**2 N**
12	5±0.5	18±0.6	40±3.1	81±7.8
18	7±0.3	45±1.1	45±2.7	85±7.9
24	8±0.4	57±3.2	64±4.9	86±5.7
30	11±0.5	60±4.2	73±5.1	91±8.3

[a]The protein solutions were incubated at 70ºC in ethanol 70%.
[b]Data are expressed as the average and standard deviation of three independent replicates

The increment of DD of the Z19 α-zein obtained by the different NaOH concentrations in function of reaction time is presented in **Figure 3**. DD higher than 50% were reached after 12 h at NaOH concentrations of 0.5, 1 and 1.5 N, during the next 18 h the DD increment was slower. The use of NaOH 2 N caused protein insolubilization since the beginning of the incubation which delayed the DD increase.

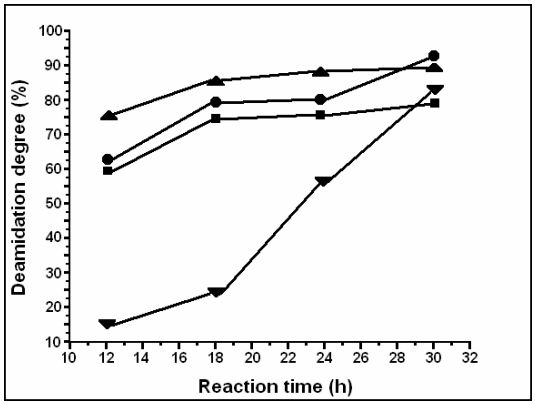

Figure 3. Deamidation degree as a function of incubation time obtained by the alkaline deamidation using different NaOH concentrations: (■) 0.5 N, (●) 1 N, (▲) 1.5 N and (▼) 2 N. Z19 α-zein solutions (2 mg/mL) contained 70% ethanol. The incubations were carried out at 70ºC for 30 h.

After the reaction time reached 30 h, the four protein samples had DD higher than 80%.
Only 5 conditions promoted emulsion stabilities (ES) higher than 60%. (**Table II**). The protein sample incubated in NaOH 0.5N for 12 h showed the best ES, the minor DH and a DD of 60.6 ±0.5%.

Table II. Results of the emulsion stability from emulsions obtained from the Z19 α-zein alkaline deamidation using different NaOH concentrations[a]

Incubation time (h)	Emulsion stability (% of emulsified oil)[b]			
	0.5 N	1 N	1.5 N	2 N
12	80±4.7	40±3.2	10±0.8	0
18	69±3.5	74±4.8	0	0
24	65±3.9	62±4.3	0	0
30	59±4.5	29±1.3	0	0

[a]The protein solutions were incubated at 70ºC. All the protein dried samples were dispersed in 0.01M sodium phosphate buffer pH 7 (0.1 % w/v). 1.8 mL of protein solutions was homogenized with 0.2 mL of tricaprilin and then they were homogenized and centrifuged.
[b]Data are expressed as the average and standard deviation of three independent replicates

The samples with DD near 60% but a higher DH showed poor emulsion stability. It is important to point out that a high DD does not necessarily promote an improvement in the emulsion stability. These results are evidence of the importance of the balance between the DD and the DH in an effective emulsifying property. These results show that when deamidation of the Z19 α-zein is accompanied by a limited protein hydrolysis the emulsifying properties are improved. As it was mentioned before, α-zeins tend to aggregate when they are heated. We suggest that the hydrolysis could reduce the formation of large aggregates, allowing the formation of a proteic web that covers the oil drop. DH results indicate that peptides generated by an extensive Z19 protein hydrolysis tend to destabilize the emulsion, possibly as a consequence of inadequate hydrophobicity-hydrophilicity balances. Conde et al. (*46*) suggested this misbalance causes the decrease of the monolayer thickness as the degree of hydrolysis increases. These phenomena explained the poor functional properties for the formation and stabilization of an emulsion by protein hydrolysates from sunflower flour isolates at high degrees of hydrolysis.
There was an increment in the negative charge density of the deamidated Z19 α-zein (DD=60.6%; DH=5% and ES=80%) (**Figure 4**). Before the deamidation reaction the isoelectric point diminished from 6.8 to 5.9. This caused the increment in the protein solubility at pH 7. In order to compare the electrophoretic pattern of the α-zeins, the SDS-PAGE of the mixture, composed by the Z19 and the Z22 α-zeins, the non-deamidated and deamidated Z19 α-zeins is shown in **Figure 5**. The deamidation reaction did not affect the Z19 α-zein electrophoretic pattern.

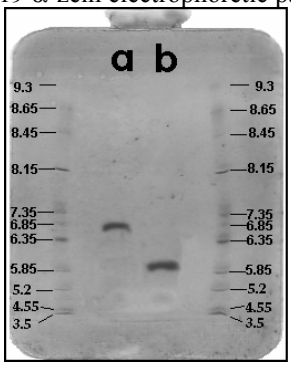

Figure 4. Isoelectric point determination for: a) native and b) deamidated (incubation in 0.5 N NaOH/12h/70ºC, deamidation degree 60.6%) Z19 α-zein. The IEF acrylamide gel pH gradient was 3-9.

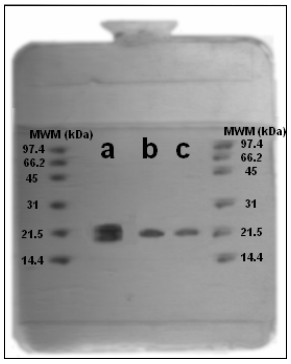

Figure 5. SDS-PAGE analysis of: a) α-Zeins mixture obtained before Z19 α-zein purification (Z19 and Z22 mixture), b) Native Z19 α-zein and c) Deamidated Z19 protein (incubation in 0.5 N NaOH/12 h/70ºC, deamidation degree 60.6%). The amounts of protein loaded were 500 ng, 200 ng and 100ng respectively. The gel was silver stained.

Evaluation of surface hydrophobic and emulsifying properties

Due to the propensity of non polar amino acid residues to position themselves in the interior of protein molecules in solutions, thus avoiding contact with the aqueous surroundings, only a portion of them participate in the emulsification of oil into aqueous phase. Hydrophobicity thus measured would be "surface" or "effective hydrophobicity" which does not directly correlate with the "total hydrophobicity" (*47*). The α-zein mixture presented a high surface hydrophobicity index (S_o) (**Table III)**. The S_o of the non-deamidated Z19 α-zein was smaller than that obtained for the α-zein mixture. Proteins minimize their energy by folding and associating into structures of low energy. In corn seed, the two α-zeins are bonded by electrostatic and hydrophobic, not covalent, interactions, so, this suggests that the more stable arrangement for these proteins are the large and highly hydrophobic arrangements. When the native Z19 α-zein was purified, rearrangements in the protein structure took place and many of the nonpolar amino acid residues were hidden. As a consequence of the deamidation treatment and unfolding of the protein, some of these amino acid residues were exposed to the aqueous surroundings and the S_o of the deamidated Z19 α-zein was higher than in the native protein.

Table III. Surface Hydrophobicity Index (S_o) of the α-zeins samples[a]

	Surface Hydrophobicity Index (S_o)[a]
α-zeins mixture[b]	500 (±7) x 10^4
Non deamidated Z19 α-zein[c]	9.6 (±0.7) x 10^4
Deamidated Z19 α-zein[d]	46.2 (±0.2) x 10^4

[a]Data are expressed as the average and standard deviation of three independent replicates.
[b]α-Zeins mixture was obtained before Z19 α-zein purification (Z19 and Z22 mixture)
[c]Native Z19 protein
[d]Deamidated Z19 α-zein (incubation in 0.5 N NaOH/12h/70ºC, deamidation degree 60.6%).
The S_o was determined using 1-anilinonaphthalene-8-sulfonic acid (ANS) with protein solutions 0.1% (w/v) in 0.01 M sodium phosphate buffer (pH 7).

The deamidation reaction promoted the Z19 α-zein S_o increment due to hydrophobic zones exposure which before the deamidation were hidden inside the protein. As a consequence of protein unfolding and electrostatic and hydrophobic forces rearrangement, the hydrophobic regions reoriented to the surface and the fluorescence signal increased. Surface hydrophobicity is an important factor in determining the emulsifying properties. As it was previously reported by other workers (*47*, *48*) the hydrophobicity exposed by the protein would allow a better molecular anchorage to be established in the oil-water interface, giving more stable emulsions. They reported that the surface hydrophobicity of the soy protein increased at an early stage of the mild acid treatment, then gradually increased until a deamidation degree of approximately 10% was reached. However, it slightly decreased after that. In the case of the deamidated Z19 α-zein, when compared with that of the non-deamidated, the higher emulsifying capacities might be due to its lower hydrophobicity value. The deamidation treatment significantly improved the emulsion stability of the Z19 α-zein from 18 ±0.7% to 80 ±4.7% of emulsified oil (**Figure 6**), since smaller oil

globules, more resistant to coalescence are formed in the deamidated protein (**Table IV**).

The main factors of the improvement of emulsifying properties of the Z19 α-zein are the charge increment and the protein unfolding which promotes the formation of protein arrangements with better hydrophilic-lipophilic balances to interact in the water-oil interface, stabilizing the oil drop. However, a discrete protein hydrolysis may play an important roll, because it could diminish the large aggregates. The generated small peptides, with higher solubility, may facilitate the diffusion at oil-water interfaces and enhance the interaction between the protein and the oil.

Table IV. Results of the average particle sizes obtained from α-zeins emulsions[a].

	Mean droplet diameter (nm)
α-zeins mixture[b]	2380 (±8.4)
Non deamidated Z19 α-zein[c]	1488 (±3.9)
Deamidated Z19 α-zein[d]	57.7 (±5.7)

[a] Data are expressed as the average and standard deviation of three independent replicates.
[b]α-Zeins mixture was obtained before Z19 protein purification (Z19 and Z22 mixture)
[c]Native Z19 α-zein
[d]Deamidated Z19 α-zein (incubation in 0.5 N NaOH/12 h/70ºC, deamidation degree 60.6%).
Protein solutions were 0.1% (w/v) in 0.01 M sodium phosphate buffer (pH 7).

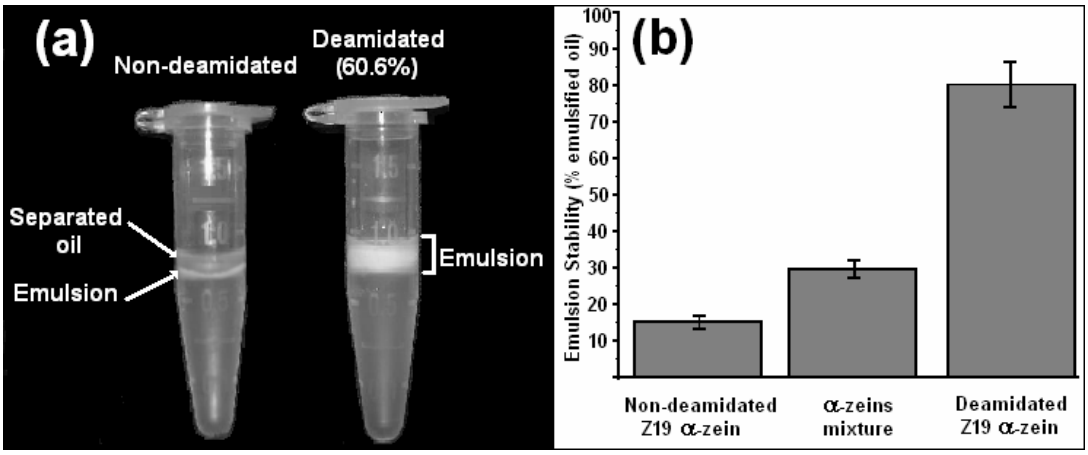

Figure 6. (**a**) Z19 α-zein emulsions obtained from the non-deamidated and deamidated (incubation in 0.5 N NaOH/12 h/70ºC, deamidation degree 60.6%) %) proteins. Dried samples were dispersed in 0.01M sodium phosphate buffer pH 7 (0.1 % w/v). 1.8 mL of protein solutions was homogenized with 0.2 mL of tricaprilin and then they were homogenized and centrifuged. The cream as well as the separated oil amount indicates the stability of liquid emulsions to creaming. (**b**) Emulsion stability results obtained from the non-deamidated Z19 protein, the α-zeins mixture obtained before Z19 α-zein purification (Z19 and Z22 mixture) and the deamidated Z19 α-zein.

ACKNOWLEDGEMENTS

This work was partially funded by CONACyT project No. 35948-B. Authors thanks Arancia Corn Products, S.A. de C.V. for the materials provided. Sandra Pérez Munguía and Idalia Flores provided technical assistance. We thank Gabriela Salcedo-Monroy for her english revision. VC was a recipient of a CONACyT scholarship 159981 and of funds from UNAM (PAEP 203304).

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XI. DISCUSIÓN GENERAL

La α-zeína de maíz Z19 no había sido purificada debido a sus características de solubilidad, pero principalmente a su similitud con la Z22. Del análisis del alineamiento de las secuencias de estas proteínas (**Figura 16**), se sabe que su tamaño difiere solo en 27 residuos y que se tiene un porcentaje de identidad del 45.4% lo que representa secuencias que son homólogas cercanas y que por lo tanto tienen estructuras similares. El que sean tan parecidas aunado a sus características de hidrofobicidad y a su tendencia a la oligomerización y agregación, explica el por qué era un reto separarlas y purificarlas.

Figura 16. Alineamiento de las secuencias de residuos de aminoácidos de las α-zeínas de 19 y 22 kDa hecho con el programa CLUSTAL W (1.83)

Esto provoca que cuando están juntas se comporten de manera similar a la de la Z19 pura. Tanto individual como conjuntamente tienden a formar agregados, posen un alto contenido de estructura en hélice α y presentan propiedades emulsificantes. Para entender la importancia de estudiar individualmente a cada una de estas proteínas, es importante resaltar las sutiles diferencias que se encontraron en su comportamiento en cuanto a los tamaños de los agregados y los valores obtenidos para las distintas propiedades emulsificantes evaluadas.

Enfocándonos exclusivamente en los datos obtenidos de la Z19, su alto contenido de estructura en α-hélice sugiere un cierto grado de anfipaticidad, aspecto considerado de gran importancia por diversos autores para el desarrollo de propiedades emulsificantes (Shimizu y Saito, 1984; Krebs y Phillips, 1984; Le Visage et al., 1997; Poon et al., 2001). Las propiedades emulsificantes de esta proteína se manifiestan mejor a valores

de pH por arriba de su punto isoeléctrico. Aunado a esto, la gráfica de hidropaticidad generada a partir del análisis de la secuencia de residuos de aminoácidos nos muestra un patrón de 3 amplias zonas hidrofóbicas intercaladas con 3 pequeñas regiones hidrofílicas, aspectos importantes para que una proteína se pueda anclar fuerte y adecuadamente en una interfase aceite-agua y con ello mejorar la estabilidad de la emulsión. Los resultados mostraron la gran hidrofobicidad que presenta esta proteína, esto es, su gran insolubilidad en agua, tendencia a la agregación, composición y secuencia de residuos de aminoácidos que resultan en altos índices hidrofóbicos. Sus propiedades emulsificantes mejoraron cuando se desamidó, se generó un mayor número de cargas negativas y con ello un mejor balance hidrofobicidad-hidrofilicidad para la emulsificación. Por lo anterior, podemos decir que una elevada hidrofobicidad es importante para la emulsificación, sin embargo, tiene que estar equilibrada con la polaridad que presente la proteína.

La flexibilidad es un aspecto importante en el desarrollo de las propiedades emulsificantes de una proteína (Kato et al., 1985, 1988). La Z19 mostró ser muy flexible ya que, tras someterla al calentamiento y posteriormente al enfriamiento hasta la temperatura inicial, no regresó a su estado inicial, el espectro de dicroismo circular reveló un patrón de plegamiento semejante al nativo. Es decir, se desnaturaliza térmicamente de manera irreversible, sin embargo, la estructura a la que llega tras tratar de renaturalizarla es muy similar a la nativa. La formación de puentes disulfuro, posibles responsables de la irreversibilidad de la desnaturalización, si bien afectan la flexibilidad de la proteína también pueden ser factores benéficos en la emulsión, generando mayor rigidez y con ello estabilidad de la malla proteica que rodea al glóbulo graso.

Se ha demostrado que los procesos de agregación constituyen un factor importante en el desarrollo de las propiedades emulsificantes de muchas proteínas (Dumay et al 2006; Pommet et al, 2005; Roesch y Corredig, 2005; Lefevre y Subirade, 2003). La Z19 presenta una elevada tendencia a la oligomerización y agregación, pero para confirmar que este también es un factor importante en la estabilización de las emulsiones se sugiere para estudios futuros evaluar la presencia de estos agregados tras haber elaborado la emulsión.

XII. CONCLUSIONES

 El protocolo de purificación desarrollado permitió obtener a la Z19 con un alto grado de pureza y homogeneidad. Éste consistió en la separación a partir de la mezcla de α-zeínas extraida del grano de maíz con etanol al 95% y purificada mediante cromatografía de intercambio catiónico utilizando SP-sefarosa como fase estacionaria y amortiguador de citratos 0.02 M con metanol al 70%, pH 3.5.

 La Z19 posee las siguientes características bioquímicas y estructurales:

Secuencia de residuos de aminoácidos	TIFPQCSQAPIASLLPPYLPSIIASICENPALQPYRLQQ AIAASNIPSSPLLFQQSPALSLVQSLVQTIRAQQLQQ LVLPLINQVVLANLSPYSQQQQFLPFNQLSTLNPAAY LQQQLLPSSQLATAYCQQQQLLPFNQLAALNPAAYL QQQILLPFSQLAAANRASFLTQQQLLLFYQQFAANP ATLLQLQQLLPFVQLALTDPAASYQQHIIGGALF
Peso molecular	24.5 kDa
Punto isoeléctrico	6.8
Absortividad molar	12415 M^{-1} cm^{-1}
Contenido de estructura secundaria	Hélice α: 40% Lámina β:19.5% Giros: 15.4%
Contenido de SHs y puentes disulfuro	SHs superficiales: 0 SH internos: 0 SH libres totales: 0 No. de Cys formando S-S: 3

- Es muy hidrofóbica, como lo muestran su alto contenido de residuos de aminoácidos hidrofòbicos (76.25%), su valor de energía libre de hidratación (-131.228 kcal/mol), sus índices GRAVY y alifático (-0.273 y 119.13 y su insolubilidad en soluciones acuosas.
- Tiene una elevada tendencia a la oligomerización y agregación. Forma dímeros, trímeros y oligómeros de alto peso molecular que son muy estables frente a condiciones desnaturalizantes de alta temperatura, desnaturalizantes y agentes reductores. La formación de estos oligómeros se favorece al incrementar la temperatura. A temperaturas superiores a 25ºC la proporción de los oligómeros aumenta y la forma

monomérica desaparece después de los 50ºC. Se atribuye a los procesos de oligomerización y posterior agregación la pérdida de estructura en α-hélice a medida que la temperatura aumenta, lo que provoca también que la Z19 sufra una desnaturalización térmica irreversible.

- Presenta un plegamiento muy inestable que le permite regresar a una estructura muy similar a la nativa tras ser calentada a 90ºC y posteriormente enfriada a 25ºC, lo que sugiere una gran flexibilidad en la estructura.
- A valores de pH superiores al punto isoeléctrico, la Z19 posee una estructura predominantemente helicoidal, con contenidos de hélice α mayores al 40%.
- La Z19 nativa estabiliza emulsiones aceite en agua (O/W), en las cuales se presentan tamaños de glóbulos grasos estabilizados de 1488±3.9 nm.

 Las condiciones de desamidación alcalina determinadas para la verificación de mejores propiedades emulsificantes fueron las siguientes: [Proteína]= 9.87 mg/ml, Tiempo de reacción = 12 h/NaOH 0.5 N, Temperatura de reacción = 70 ºC, condiciones con las cuales se lograron 60.6 GD.

 Las condiciones de desamidación con las cuales se mejoran las propiedades emulsificantes de la Z19 requieren temperaturas superiores a los 25 ºC con incubaciones prolongadas que provocan la pérdida total de la estructura secundaria de la proteína. La desamidación alcalina provocó:

- El aumento de la carga total negativa de la proteína ya que su punto isoeléctrico cambió a 5.9.
- El índice de hidrofobicidad superficial aumentó de 9.6 (±0.7) x 10^4 a 46.2 (±0.2) x 10^4 debido a la exposición de grupos hifrofóbicos que se encontraban escondidos en el interior de la proteína.
- La estabilidad de emulsión aumentó en un 80% y el tamaño de los glóbulos estabilizados disminuyó 25 veces.
- Se conservó el 18.5% del índice de actividad emulsificante que se tenía antes de la desamidación.

 Se demostró que la relación entre las interacciones electrostáticas y la hidrofobicidad superficial es importante en la estabilización de las emulsiones a

partir de α-zeínas, la relación adecuada entre estos dos factores estructurales permite la verificación de mejores propiedades emulsificantes.

Cabe señalar que la formación de puentes disulfuro juega también un papel importante en las propiedades emulsficantes de estas proteínas.

XIII. PERSPECTIVAS

La Z19 es una proteína que por sus características resulta ser un modelo adecuado para el estudio de la relación estructura-función en cuanto a propiedades emulsificantes se refiere. Su conocimiento general así como la posibilidad de tenerla pura y en cantidades suficientes para los estudios fisicoquímicos necesarios permitirá profundizar en su estudio mediante la aplicación de técnicas moleculares y por medio de ellas se sugieren las siguientes estrategias:

- La sustitución de residuos de aminoácidos hidrofóbicos por residuos con carga.
- La sustitución de los tres residuos de cisteínas para la evaluación del efecto de la eliminación de los puentes disulfuro en la agregación.
- La inserción de residuos de aminoácidos con carga entre los segmentos de proteína con estructura en hélice α.

Estas modificaciones permitirían variar el balance hidrofobicidad-hidrofilicidad y el estado electrostático en la proteína y por consiguiente la estructura general de la Z19 y con ello lograr determinar un rango en el cual se obtengan las mejores propiedades emulsificantes. Esto también llevaría a evaluar de una forma más precisa el papel que juegan los puentes disulfuro en la agregación, estabilidad y emulsificación de la Z19.

Por otra parte, durante el presente trabajo de investigación se fracasó en el intento de cristalización de esta proteína debido principalmente a la insolubilidad y agregación de la proteína en soluciones acuosas, por lo que la sustitución de residuos de aminoácidos podría abrir la posibilidad de lograr la cristalización y a partir de esto, la generación de un modelo más cercano al real que los que tenemos hasta el momento.

También se podría realizar la alquilación de los SH de la proteína nativa para determinar el efecto de la formación de los puentes disulfuro tanto en la agregación y emulsificación, pero además se podría realizar la desamidación química para saber si sus efectos se ven afectados por la ausencia de estos enlaces.

Le elección de una proteína con características potenciales de emulsificante en general se hace mediante el proceso de ensayo y error. La experiencia adquirida en la realización del presente trabajo permite sugerir el análisis previo de la secuencia de residuos de aminoácidos de la siguiente manera:

- Contenido de residuos hidrofóbicos e hidrofílicos.

- Cálculo de índices como el GRAVY y el alifático para saber más sobre su hidrofobicidad
- Gráficas de hidropaticidad para ver la distribución de las zonas hidrofóbicas e hidrofílicas a lo largo de la secuencia
- Cálculo de la energía libre de hidratación

La comparación de secuencias de proteínas con propiedades emulsificantes comprobadas experimentalmente con sus correspondientes índices y gráficas, permitiría en un futuro contar con una base de datos que llevaría a determinar los valores entre los que se deben encontrar las proteínas emulsificantes.

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ANEXO

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México D. F., a 17 de agosto de 2006.

Dr. Roberto Arreguín,

Informo a usted que su artículo:
Emulsifying properties of proteins ha sido aceptado para su publicación en el Boletín de la Sociedad Química de México.
Anexo encontrará usted los comentarios de los árbitros. Le agradecería elaborara un nuevo manuscrito tomando en cuenta sus sugerencias y me lo reenvíe.

Atentamente,
Dr. Jesús Valdés Martínez
Editor del *Boletín de la Sociedad Química de México*

Emulsifying properties of proteins

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Abstract

Emulsions form the basis of a huge range of food products, where those stabilized by proteins are of great interest. The interfacial properties of proteins have been extensively studied in the field of food colloid research. Emulsifying properties of proteins basically depend on two effects: (1) a substantial decrease in the interfacial tension due to the adsorption of the protein at the oil-water interface and (2) the electrostatic, structural and mechanical energy barrier caused by the interfacial layer that opposes the destabilization processes. The knowledge on the biochemical and physicochemical characteristics, interfacial behaviors and emulsifying properties of proteins will help us understand the structure-function relationship of the emulsifying proteins.

Key Words

Proteins, Food Emulsions, emulsifying properties, protein functionality, interface.

Resumen

Muchos productos alimenticios son emulsiones, de las cuales aquellas estabilizadas por proteínas son de gran interés. Las propiedades interfaciales de las proteínas han sido ampliamente estudiadas en el campo de la investigación de los coloides alimentarios. Las propiedades emulsificantes de las proteínas dependen básicamente de dos efectos: (1) un decremento sustancial de la tesión interfacial debido a la adsorción de la proteína en la interfase aceite-agua y (2) la barrera de energía electrostática, estructural y mecánica causada por la capa interfacial que se opone a los procesos de desestabilización. El conocimiento de las propiedades bioquímicas y fisicoquímicas, del comportamiento interfacial y de las propiedades emulsificantes de las proteínas ayudará a entender la relación estructura-función de las proteínas emulsificantes.

Palabras clave

Proteínas, emulsiones alimenticias, propiedades emulsificantes, funcionalidad de proteínas, interfase.

1.- Introduction

Proteins are essential ingredients in the food industry, not only due to their nutritive value, but because of all the other properties, known as "functional properties", [1]. These properties result form the physicochemical interactions among the food system components; the most widely used are the interfacial ones [2] such as foaming and emulsifying properties, which are used in the food industry. Proteins are the single most commonly used ingredients since they are natural, non toxic, cheap and widely available, thus making them ideal ingredients.

2.- Food Emulsions

Food emulsions are defined on a molecular level as complex colloidal systems comprising two immiscible phases, one dispersed in the other. The study of emulsions is complicated by the interactions that can occur when multiple components are present; the fact is that the systems are easier to study in dilute solutions, under conditions that may not apply to those likely to be found in foods. It is possible to explain how proteins work in emulsions from the knowledge of the forces that operate during emulsion formation and from information about protein structure [2, 3, 4].

The intrusion of a non-polar molecule interferes with the normal structure of water increasing its order [5]. When a liquid of low polarity such as fat is mixed with water there is a strong driving force to limit the contact between the two liquids. This happens when phase separation occurs. When two immiscible liquids are forced into contact by the application of work, the result will be the formation of a number of spherical droplets within the dispersed phase. Given enough time this leads to the situation of minimum contact and phase separation [6].

Recently, the interest in microemulsions and nanoemulsions has grown. Microemulsions are thermodynamically stable, and transparent, with a low viscosity and isotropic dispersions consisting of oil and water stabilized by an interfacial film of surfactant molecules, typically in conjunction with a cosurfactant. Microemulsions (so-called due to their small particle size; 50-1000 nm) can be applied in a wide variety of systems, such as pharmaceutical processes[7, 8] and oil recovery, but their application in food systems has been hindered by the types of surfactant permissible in the food industry. Nanoemulsions can be defined as oil-in-water (o/w) emulsions with mean droplet diameters ranging from 5 to 100 nm. Usually, the average droplet size is between 100 and 500 nm. The terms sub-micron emulsion (SME) and mini-emulsion are used as synonyms. Emulsions which match this definition have been used in parenteral nutrition for a long time. Usually, SMEs contain 10 to 20 per cent oil stabilized with 0.5 to 2 per cent emulsifying protein or peptide [9].

a) Mechanisms of Emulsion Stability

The disperse system can be stabilized against coalescence and phase separation if another component, partially soluble in both phases, is added. Molecules that are composed of portions that are soluble in water and portions that are soluble in lipids can serve as emulsifiers. An emulsion formed from a mixture of oil, water and emulsifier is at a higher energy level than the non emulsified system. The goal of the food scientist is to elevate the energy of activation in order to give the emulsion a reasonable lifetime [6].

In order to form an emulsion, energy must be provided in excess, due to the creation of the new interfacial area of the emulsion. The size of the droplets and thus their interfacial energy depends on the amount of work done on the system. The rate of coalescence depends on the energy barrier and the rate of droplet collision

b) Emulsion destabilization

Once an emulsion is formed, it can undergo several changes. With protein stabilized emulsions, phase inversion is generally not a problem because when fat globules approach each other, the proteins usually provide an effective barrier to coalescence. The removal of proteins from the surface of a fat globule is energetically unfavourable and does not occur at any appreciable rate. In food products, fluctuations in temperature are a common cause of emulsion destabilization. As the temperature is lowered, water attains more and more structure. As the water becomes more ordered, there is a lower energy difference between the hydrophobic groups exposed to the aqueous phase and those buried in the oil phase. When the system is thawed, coalescence occurs when the physical damage has been extensive. One of the best ways

to minimize this type of damage is by adding substances that will modify the size and extent of water crystal formation [11].

A more common defect in food emulsions results from the phenomenon known as creaming. If enough time is given or a centrifuge force is applied a depletion of the lipid from the bulk aqueous phase occurs with the formation of a compact cream layer containing the majority of the lipid (**Figure 1**). The extent of the emulsion shelf life depends on the fact that the density of the fat globules must be made identical to that of the continuous phase or the viscosity must be high enough so that the yield value is greater than the acceleration due to buoyant differences.

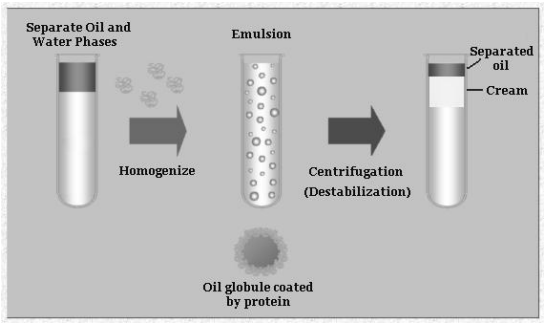

Fig. 1 Emulsion destabilization to measure the emulsion stability by appliance of centrifuge force.

Low-molecular weight emulsifiers are often more surface-active than proteins, and will therefore compete for interfacial area (competitive adsorption). It is known that adding surfactants to protein-stabilised emulsions will have a detrimental effect on stability [12-15-16]. In fact, surfactants are sometimes used as the anti-emulsifying agents [12]. Surfactants are known to displace proteins from emulsion droplets [17-18]. It has been shown that the addition of polysaccharide stabilizers to emulsions has little effect on the stability of the systems unless they increased the viscosity to the point of imparting a yield value. [19]. Thus, while Stoke's law is important in predicting the rate of emulsion creaming, for most products with any appreciable shelf life, other factors, especially viscosity, pseudoplasticity and yield stress, must also be considered [20, 21].

The amount of damage done to a product by the formation of a cream layer depends on the product type and the tenacity of the formed layer [22].

c) Surfactants classification

Emulsifiers can be divided into two categories:

* Small molecules: Mono and diglicerides, Sucrose Esters, Sorbitan Esters (SPAN), Polysorbates (TWEEN), Stearoyl Lactylates, Lecithin and Derivatives

* Macromolecules: Proteins such as bovine serum albumin, β-lactoglobulin, lysozyme, and ovalbumin

Only food emulsifiers defined as food additives are usable by law. Those emulsifiers are shown in **Table 1** which had been used ordinarily in food systems.

Table 1. Food Emulsifiers utilized in Food Systems [a]

Name	Common Name
Glycerin Fatty Acid Esters	Monoglyceride (MG)
Acetic Acid Esters of Monoglycerides	Acetylated Monoglyceride (AMG)
Lactic Acid Esters of Monoglycerides	Lactylated Monoglyceride (LMG)
Citric Acid Esters of Monoglycerides	CMG
Succinic Acid Esters of Monoglycerides	SMG
Diacetyl Tartaric Acid Esters of Monoglycerides	DATEM
Polyglycerol Esters of Fatty Acids	PolyGlycerol Ester (PGE)
Polyglycerol Polyricinoleate	PGPR
Sorbitan Esters of Fatty Acids	Sorbitan Ester (SOE)
Propylene Glycol of Fatty Acids	PG Ester (PGME)
Sucrose Esters of Fatty Acids	Sugar Ester (SE)
Calcium Stearoyl Di Laciate	CSL
Lecithin	Lecithin (LC)
Enzyme Digested/Treated Lecithin	EDL or ETL
Proteins	

[a] http://www.rike-vita.co.jp/int/emulsifier/basic/LMG#LMG accessed in May, 2006.

3. –Proteins as Emulsifiers

Protein functionality has been defined as: "any property of a protein, exception being its nutritional ones that affects its utilization" [23]. Proteins show a large number of functions and functional properties and some of the most important are shown on **Table 2**.

Table 2. Typical Functional Properties performed by proteins in Food Systems [a]

Functional Property	*Mode of action*	*Food system*
Solubility	Protein solvation, pH dependent	Beverages
Water absorption and binding	Hydrogen-bonding of HOH, entrapment of HOH (no drip)	Meats, sausages, breads, cakes
Viscosity	Thickening, HOH binding	Soups, gravies
Gelation	Protein matrix formation and setting	Meats, curds, cheese
Cohesion-adhesion	Protein acts as adhesive material	Meats, sausages, baked goods, pasta products
Elasticity	Hydrophobic bonding in gluten, disulfide links in gels (deformable)	Meats, bakery
Emulsification	Formation and stabilization of fat emulsions	Sausages, bologna, soup, cakes
Fat adsorption	Binding of free fat	Meats, sausages, donuts
Flavor binding	Adsorption, entrapment, release	Simulated meats, bakery, etc.
Foaming	Forms stable films to entrap gas	Whipped toppings, chiffon desserts, angel cakes

[a] From Kinsella and Srinivasan, [44].

Protein functionality is evident by its interaction with other components within the food or chemical system. These interactions may involve solvent molecules, solute molecules, other protein molecules or substances that are dispersed in the solvent such as oil or air [24-26].

When proteins are used to generate emulsions, the system becomes highly complex. They are responsible for the creation of a new surface area. The high energy state is relieved by rapid coalescence of fat globules. For prevention of coalescence, protein molecules need to diffuse to the fat/ water interface and then unfold and coat the surface. When enough of the new surface is covered, coalescence ceases. With proteins, the rate of diffusion to the interface is a significant variable in the amount of protein that absorbs to the interface during the emulsion formation. If something tends to decrease the rate of diffusion of the protein molecules, the protein load decreases [6].

Emulsions are thermodynamically unstable mixtures of immiscible liquids. If energy is applied the systems may be dispersed, but an increment on the surface energy causes the phases to coalesce unless an energy barrier that would prevent coalescence is established. Emulsified droplets can be stabilized by the addition of molecules that are partially soluble in both phases. In foods a number of small emulsifier molecules can serve this function. Proteins capable of unfolding at the interface may also serve this function. Protein coats the lipid droplet and provides an energy barrier to particle association and phase separation [27-29].

Proteins are included in emulsions to aid in their formation and to increase their stability. They are much larger and more complex than other simple emulsifier molecules and the formation of a protein stabilized emulsion requires that the protein molecule must first reach the water/ lipid interface and then unfold so that its hydrophobic groups can contact the lipid phase. To illustrate the forces involved, the situation of a protein molecule approaching a static water/lipid interface will first be considered. In native proteins most of the non-polar amino acid side chains are located in the interior of the molecules. Proteins have charged groups at the surface of the molecule which are in contact with water molecules. The favourable interaction of water with surface charge lowers the total energy of the protein molecule. The hydrophobic groups are removed

from contact with the aqueous phase while charged groups maximize solvent contacts [6, 11].

As a protein molecule approaches the interface, there is less opportunity for the charged groups to interact with the solvent. In the extreme case, charged groups are removed from the aqueous phase and enter the lipid phase. This is energetically unfavourable and these groups are repelled from the interfacial area. If the groups closer to the interface are in a region of the protein molecule that contains some flexibility, the molecule may begin to unfold. This unfolding causes the exposure of hydrophobic groups to the surface. If these groups are exposed to the aqueous environment, there is an increase in total energy and random fluctuations in protein structure cause these groups to return to the inner part of the molecule. If the exposure occurs at an interface, the state of lowest free energy depends on the nature of the interface. In the case of a protein un-folding near lipid, the hydrophobic groups are inserted into the lipid phase. This insertion has a very low energy of activation and proceeds spontaneously. For proteins such as soybean glycinin [30-32], tryptophan syntase [33] and lysozyme [3, 34-35], the size of the hydrophobic region inserted is about 6 to 8 amino acid residues. The enthalpy for this step is positive so that the driving force must be an increment in the entropy of the system. This increase in entropy has two components, one due to the conformational entropy of the protein and one due to the structure of water near hydrophobic groups. There is an increment in the conformational entropy of the protein as the hydrophobic groups are removed from the interior of the molecule and placed into another non-polar environment. The original protein had a limited number of ways of arranging its components to attain a low energy state. The partially unfolded molecule has many ways of inserting a hydrophobic group into a non-polar environment and once there the group can assume more conformations than before. The solvent molecules at the interface are arranged in highly ordered structures. The protein with hydrophobic groups inserted will coat the non-polar material and will release the solvent from the surface. The release of this water is responsible for a significant increase in the entropy of the system [11].

While the original insertion of a hydrophobic group proceeds spontaneously with a small energy of activation, the reaction is not readily reversible. In time other sections of the protein molecule approach the surface and if these occur in flexible portions of the protein they too may be inserted into the lipid phase. As this continues the protein will unfold at the interface [6].

Proteins that become attached by more than one hydrophobic group desorb very slowly from the surface, if at all. Langmuir and Schaeffer [36] calculated that if absorption were completely reversible and the Gibb's absorption equation is applied, the changes in the magnitude of surface pressure they observed, in ovalbumin stabilized emulsions, resulted in an essentially complete desorption of protein form the interface. This does not occur for protein stabilized emulsions suggesting that a significant energy barrier to protein desorption exists. Removal of hydrophobic groups from the lipid exposes the lipid to the aqueous phase as well as the hydrophobic groups that are being removed. Even if the removed hydrophobic groups could be buried in the protein interior, the protein would remain attached to the fat globule at other points and reattachment would be likely. If other hydrophobic molecules are available to cover the exposed lipid area, desorption is easier to achieve. It has been shown, for instance, that gelatin molecules can be replaced by more hydrophobic casein molecules from the water/ lipid interface [29, 37].

Once a layer of protein has been adsorbed additional protein layers cannot be added in the same way since an energy barrier to absorption arises. In order for more protein to be absorbed, the protein already at the surface must be compressed to make room. The amount of compression that is possible depends on the rigidity of the protein and also on the amount of residual charge near the surface. At some level of compression, the absorption of more protein will require more energy, which can be gained by the insertion of hydrophobic groups into the lipid layer. Further interaction involves the interaction of protein molecules in the bulk phase with those already adsorbed to the lipid and the formation of multilayer [37].

a) Classification of Protein Based Surfactants

The three types of protein-based surfactants are: (1) Amino Acids, (2) Peptides, both of them derived from synthesis and hydrolysis of the (3) Proteins. [38].

The amino acid-based surfactants are composed of an amino acid as the hydrophilic part and a long

hydrocarbon chain as the hydrophobic part. The hydrophobic chain can be introduced through acyl, ester, amide, or alkyl linkage [39]. Examples of these kind of surfactants are the long-chain N^{α}-acyl amino acid derivatives from pure amino acids or protein hydrolysated, which have been extensively used in the cotton chemical industry [40]. N-Acylsarcosinate salts are suitable for cosmetics, toothpaste, wound cleaners, personal care items, shampoo, bubble-bath pastes, aerosols and synthetic bars [41]. The many kinds of amino acid-based surfactants have a potential wide application in the cosmetic, personal care, food, and drug industries.

The peptide surfactants are derived from the condensation of dipeptides or tripeptides and hydrophobic chains such as fatty acids. Most of the surfactants in literature have been chemically synthesized, although some have been biosynthetically produced [42]. Examples of these surfactants are the diethanolamides (DEA) of N-lauroyl dipeptides of various molecular structures [43].

b) Molecular basis of protein surfactants

It is essential that the forces and energies involved in the achievement and maintenance of native protein structure be described. While a complete discussion of the forces involved is beyond the scope of this revision, some observations on the nature of protein structure will be useful.

Hydrophobic Interactions. One of the main mechanisms by which proteins diminish their free energy involves the removal of hydrophobic groups from the aqueous environment. This may provide the greatest single decrease in free energy of all the types of binding that occur within proteins [2, 5, 33, 44]. The strength of hydrophobic binding is, however, very sensitive to changes in temperature and the dielectric constant, thus, the changes in these parameters strongly influences protein structure [45].

Once a protein begins to unfold, there must be hydrophobic groups present to insert into the non-polar phase. In theory, a measure of the relative hydrophobicity of a protein should be related to its ability to function as an emulsifying agent [3, 46]. In practice, relative hydrophobicity measurements have been difficult to obtain [47]. The early methods generally assigned some relative value to each amino acid and then the value for the protein is calculated from its composition [48-49]. These procedures have rarely correlated well with functionality because they measure the total potential of hydrophobicity of the protein rather than those of the hydrophobic groups which can actually reach the surface upon unfolding. Recently a number of procedures have been developed which measure what is termed the "effective hydrophobicity" of proteins, which means obtaining a quantitative measure of those hydrophobic groups that are capable of binding to a selected probe molecule. The groups that are deeply buried in a portion of the protein that does not unfold are not measured, while those accessible to the protein surface are the ones detected by the probes (fluorescent) and the ones able to interact in emulsions or foams. The quantum yields of fluoresce and wavelength of maximum fluorescence emission of these compounds depend on the polarity of their environment [50]. Due to high sensitivity, non-invasiveness, and availability of imaging techniques, fluorescence spectroscopy has been considered to be one of the most promising and potentially widely used techniques in medicine, biology, biochemistry, and molecular biophysics for the 21st century [51-52]. Fluorescent probes used include 1-anilinonaphthalene-8-sulfonic acid (ANS), *cis*-parinaric acid (CPA) and 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN) have been widely used to measure protein hydrophobicity. These probes have a low quantum yield of fluorescence in aqueous solution. Upon binding of the probes to accessible hydrophobic regions of proteins, an increase in fluorescence is observed, which is used as a measure of protein surface hydrophobicity. However, due to the possible contribution of both electrostatic and hydrophobic interactions to the binding of these anionic probes, the interpretation based on these probes has not been easy. Therefore, an uncharged probe is needed to circumvent this problem [53].

The distribution of hydrophobic groups is also important. In proteins such as ß-lactoglobulin [54], the hydrophobic groups are evenly distributed throughout the molecule. There are no large portions of the molecule where hydrophobic amino acids are grouped, nor are there large sections of the molecule that do not contain charged amino acids [55]. This makes it difficult to find portions of the molecule that are sufficiently hydrophobic or to find residues that do not contain amino acids with charged groups that would resist their removal from the aqueous phase. In molecules such as β casein there are large sections of the protein that contain hydrophobic amino acids without the presence of charged groups. The molecule has such an uneven distribution of charge and hydrophobic groups that

it is amphipathic. It is easy to find portions of this molecule that contain at least six non-polar amino acids and no charged groups [56].

Electrostatic Interactions.
Electrostatic interactions play a major role in the determination of the molecular structure of a protein [57]. Proteins contain a number of amino acids that can ionize to form either positively charged ions (e. g., arginine, lysine, proline, histidine and the terminal amino group) or negatively charged ions (e. g. glutamic and aspartic acids and the terminal carboxyl group) [57-58]. If the protein contains many similarly charged groups, it is more likely to adopt an extended configuration because this increases the average distance between the charges and therefore minimizes the unfavourable electrostatic repulsions. If, on the other hand, the protein contains many oppositely charged groups, it is more likely to fold up into a compact structure that maximizes the favourable electrostatic attractions. As a result, proteins are often extremely compact at their isoelectric point and unfold as the pH is either increased or decreased. Electrostatic interactions also play an important role in determining the aggregation of proteins in solution. Similarly charged proteins repel each other and therefore tend to exist as individual molecules, whereas oppositely charged proteins attract each other and therefore tend to aggregate (depending of the strength of the various other types of interactions involved). The binding of low-molecular weight ions, such as Na+ and Ca 2+, is also governed by electrostatic interactions and may influence the strength of the hydration repulsion between proteins in solution [59].

Hydrogen Bonding.
Proteins contain monomers that are capable of forming hydrogen bonds. Hydrogen bonds are a relatively strong type of molecular interaction, and therefore a system attempts to maximize the number and strength of the hydrogen bonds formed. The protein may adopt an arrangement that enables it to maximize the number of hydrogen bonds which are formed between the monomers within it, which leads to the formation of ordered regions such as helixes, sheets and turns [58]. Alternatively, a protein may adopt a less ordered structure where the monomers form hydrogen bonds with the surrounding water molecules. Thus, a part or all of the protein may be found in either a highly ordered conformation (which is entropically unfavourable) with extensive intramolecular hydrogen bonding or in a more random-coil conformation (which is entropically more favourable) with extensive intermolecular hydrogen bonding. The type of structure formed by a protein under certain sets of environmental conditions is governed by the relative magnitude of the hydrogen bonds compared to the various other types of interactions, most notably hydrophobic, electrostatic and configurational entropy [59].

Configurational Entropy. Proteins exist in the lowest kinetically attainable state of free energy. The free energy of the protein may not be the global minimum, but it will be the lowest that the protein can achieve in a reasonable period of time. Protein structure is highly dependent upon the environment and the protein will assume different conformations as the environmental conditions change. Factors of importance include pH, temperature, dielectric constant, ionic strength and the presence of other molecules including air, fat, denaturants, etc.
The structures attained by proteins are not rigid but very dynamic. There is rotational freedom around many of the bonds within the protein molecule and the entropy gain of this freedom lowers the total free energy of the native structure. There are also portions of the protein structure that are stabilized by rather weak secondary forces and these are often free to assume different conformations. These alternate conformations lead to structures of higher free energy and thus are not stable or long lived. A protein may be envisioned as a dynamic entity that is constantly trying a variety of structures. These new structures are usually only slightly different from the native conformation and almost always lead to a situation where the free energy of this system increases. The increment in free energy causes the protein to spontaneously refold into the state of lowest free energy. Thus, the native structure of a protein is not the only structure it can assume, but rather the one with the lowest free energy and hence the greatest probability [59-62]. Slight changes in the environment can cause alternate structures to be of a lower free energy and thus lead to protein denaturation. In order for a protein to exhibit functionality, it must interact with other components of the food system. These interactions may often require that the protein be free to either move throughout the system or to alter its structure in such a way to allow interactions with other components. In some cases the simple

presence of other molecules in the protein solution will allow interaction to occur, but more commonly, the interactions require an input of energy into the system to insure adequate mixing. This energy may alter the physical nature of the molecules being mixed, e.g. decrease in average fat globule size, and also alter the conformation of the protein molecule [21, 63-64].

Disulfide Bonds and Protein Flexibility

Flexibility is an important feature affecting the emulsifying properties of proteins [33, 65]. In aqueous solution, the hydrophobic domains of a protein are generally buried in the interior of the molecule. To stabilize an emulsion, the hydrophobic domains of the protein should ideally be oriented toward the oil phase. The ease with which a protein is able to unfold (i. e., denature) to expose its hydrophobic domains, therefore, affects its emulsifying properties. The three-dimensional structure of proteins can be stabilized by both covalent and non-covalent interactions. Covalent interactions consist of disulfide bonds, both intra- and intermolecular. Several approaches have been used to modify disulfide bonds and to test whether the resulting protein has enhanced emulsifying properties [66-67]. The improvement in functionality may be attributed to increased conformational mobility. Other attempts were the elimination of the cysteine residues using recombinant DNA technology [68-69], but the results not were conclusive. Molecules that contain crosslinks such as disulfide bonds are more rigid and less able to unfold. Such molecules are less effective in emulsion formation. The reduction of disulfide bonds enhances the emulsifying ability of some proteins as long as the molecules do not unfold to the point where there is a large increase in viscosity [70]. The content of disulfide bonds has been related to the emulsion capacity of complex mixtures of proteins such as whey protein concentrates. Small highly crosslinked protein molecules tend to perform poor emulsifiers. Protein flexibility is also affected by non-covalent interactions such as hydrogen bonding, van der Waal's forces, electrostatic links, and hydrophobic interactions [71]**.**

Protein molecules may contain crosslinks of a non-covalent nature, e.g. salt bridges, or a covalent nature, e.g. disulfide bonds. These crosslinks lower the conformational entropy of the molecule which must be compensated by a decrease in binding energy. The presence of crosslinks adds greatly to the stability of the native protein structure and makes the molecules resistant to unfolding or denaturation. For example, simple unfolding is inhibited sterically by the presence of crosslinking because portions of the molecules are held in place by the crosslinks. Denaturation is also less likely because one of the driving forces of denaturation, an increment in conformational entropy, is greatly reduced [66-67]. In a non-crosslinked protein, if unfolding to a random coil structure can be induced, there is a very large gain in the number of conformations the molecule can assume. This gain in conformational entropy is a large driving force for the maintenance of the denatured state when the denaturing agents are removed. In contrast, a highly crosslinked protein cannot assume the same degree of random conformations and thus the increase in entropy is much smaller. This helps explain why molecules that contain large numbers of disulfide bonds are often resistant to denaturation.

Molecular conformation and aggregation.

The conformation and aggregation of proteins depend on the relative magnitude of the various attractive and repulsive interactions which occur within and between molecules, as well as their configurational entropy [72].

It has shown the protein aggregation processes is an important factor in the emulsifying properties of proteins like wheat glutenins [73-74] and soy globulins [75-76]. Segments of one protein may be capable of forming strong hydrogen bonds with segments on another proteic molecule, which causes the molecules to aggregate. These junction zones usually involve hydrogen-bonded helical or sheet-like structures. Hydrogen-bonded junction zones tend to be stable, which stabilizes the oil globule covering web at low temperatures but dissociate as the temperature is raised above a certain value because the configurational entropy term dominates [59].

The role of secondary structure.

Once a protein molecule reaches the surface it must be able to unfold enough to expose hydrophobic groups if it is to function as an emulsifier. At this point, it is important to highlight the relevance of the secondary structure content [70, 77] and the flexibility of the protein [65]. It is widely recognized that the interaction of the amino acid side chains with water is a major factor in determining the native structure of proteins [78-81]. The side chains of hydrophilic residues seek contact with water, whereas the side chains of hydrophobic residues avoid this contact. Most α-helixes in proteins consist of both

hydrophobic and hydrophilic residues, except for proteins with transmembrane α-helixes, whose residues are basically hydrophobic [82-84]. The amphipatic α-helix is a structural feature which has previously been proposed as favouring good emulsifying properties [85] and which contributes to their surface activity[71, 77, 86]. This motif is also common to many proteins in physiological systems where binding to an interface or a non-polar ligand is involved (e. g., apolipoproteins [87]. Furthermore, *in vitro* studies have shown that the presence of an interface can induce or increase the degree of α-helix formation [88]. The average hydrophobicity and hydrophobic moment over 60 helixes have been compared by Eisenberg *et al.* [78] who found that helixes could be classified into three groups according to their helix parameters. Each group represented an *in vivo* location: (1) globular proteins; (2) transmembrane helixes; and (3) helixes which were believed to seek the surface between aqueous and non-aqueous phases ("surface-seeking helices"). The surface-seeking helixes form a subset of globular helixes, which is not surprising, given the diversity of structures within globular proteins. **Figure 2** is based on the data from Eisenberg *et al.* [70, 78] and data from additional peptides [70].

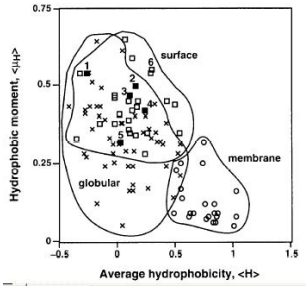

Fig. 2 Hydrophobic Moment Plot of α-Helices showing the separation between Globular, Membrane and Surface-seeking/Emulsifier Helices. Globular helices (x) are those derived from globular proteins; membrane helices (○) are those which have tendency to align at the interface between polar and non-polar phases. Emulsifier helices (■) are those from molecules which have good emulsifying activity. The *y*-axis is the average hydrophobic moment per residue ‹μ_H›, and the *x*-axis is the average hydrophobicity per residue ‹H›. Where segments are greater than 18 residues, two points have benn plotted, one representing the average value ant the other representing the 18-residue segment (theoretically five complete turns of an α-helix) having the highest Hydrophobic moment. (From Poon et al., [70])

c) Characterization of protein emulsifying properties

The determination of meaningful emulsion data with complex food products is difficult. Much of the experimental work with model systems has been done in very dilute solutions. The surface pressure or interfacial tension is often the quantity measured. With a food product the relevant information is concerned with the question: How much lipid can be emulsified and how long will it be stable to coalescence and / or creaming? The situation in food products is also complicated by the presence of other surface active molecules in addition to the proteins present [89-92].

A variety of tests has been applied to indicate the value of a protein in an emulsion. A range of experimentally controllable parameters will alter the measured emulsification properties of a protein being evaluated. These include: type of equipment used to produce the emulsion, energy input into the system, amount o protein used, phase volumes used, ionic strength, pH and type of oil used. In **Table 3** some of the properties and tests used in the evaluation of the protein emulsifying properties are shown.

d) Emulsifying properties improvement

Proteins are widely utilized as food emulsifiers However, in recent years, food materials with new functionality have become strongly desired by the increasing variety of demands. Much attention has been paid to the preparation of proteins with new functions, and in particular, protein modification has captured wide interest. The modification of a protein usually refers to physical, chemical or enzymatic treatment which change its conformation and structure and consequently its physicochemical and functional properties [93-94]. To improve the functional properties, and particularly, the emulsifying properties of food proteins, various chemical and enzymatic modifications have been carried out such as alkylation, glycosilation [95-96], esterification [97], phosphorylation [94], amidation, deamidation [98-101], lipophilization [102], hydrolysis [103], disulfide-mediated polymerization [104], covalent attachment of gluconic acid, cross-linking with transglutaminase, and conjugation with polymers [105] and Polyethylene Glycol [106] or Carboxymethyl cyclodextrins [107]. In particular, multiple improvements to protein functions can be expected by conjugating with a charged polymer due to the difference in molecular weight or charge of the chemical species conjugated [108-110]. However, chemical modifications of

proteins generally suffer from a lack of control in the extent of derivatization attainable, often yielding polydisperse sub-products. Recent advances in recombinant deoxyribonucleic acid technology offer the opportunity to relate systematically well-defined alterations in the primary sequence to changes in protein functionality. Using oligonucleotide-directed mutagenesis, one can now use a synthetic sequence of proteins. Incorporation of the altered genes into an appropriate host can lead to the production of the modified protein for structure-function relationship studies. Site directed mutagenesis can also be achieved by using PCR**.** The recombinant deoxyribonucleic acid techniques may eventually provide the means to engineer proteins and enzymes with improved functional properties [111-112].

Table 3. Tests utilized in the evaluation of proteins emulsifying properties [a]

Property	Test
Emulsifier Efficiency	Emulsifying Capacity Emulsion Stability Index Interfacial Tension Interfacial Rheology
Microestructure and Droplet Size Distribution	Microscopy Static Light Scattering Dynamic Light Scattering Electrical Pulse Counting Sedimentation Techniques Ultrasonic Spectrometry Nuclear Magnetic Resonance Neutron Scattering Dielectric Spectroscopy Electroacoustics
Dispersed-Phase Volume Fraction	Proximate Analysis Density Measurement Electrical Conductivity Alternative Techniques
Droplet Crystallinity	Dilatometry Nuclear Magnetic Resonance Thermal Analysis Ultrasonics
Droplet Charge	Electrophoresis Setasizer© Electroacoustics

[a] From McClements, D. J.[57]

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