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POSGRADO EN CIENCIA Y TECNOLOGÍA AGROALIMENTARIA

MODIFICACIÓN DE PROTEÍNA DE HUAUZONTLE: EFECTO EN PROPIEDADES TECNO-FUNCIONALES, ESTRUCTURALES Y EN COMPLEJOS SOLUBLES

TESIS

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Presenta

ADRIANA HERRERO GALINDO

Bajo la supervisión de: CONSUELO S.O. LOBATO CALLLEROS, DRA.

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MODIFICACIÓN DE PROTEÍNA DE HUAUZONTLE: EFECTO EN PROPIEDADES TECNO-FUNCIONALES, ESTRUCTURALES Y EN COMPLEJOS SOLUBLES

Tesis realizada por **Adriana Herrero Galindo** bajo la supervisión del Comité Asesor indicado, aprobada por el mismo y aceptada como requisito parcial para obtener el grado de:

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RESUMEN GENERAL

Modificación de proteína de huauzontle: efecto en propiedades tecno-funcionales, estructurales y en complejos solubles

La proteína de huauzontle (*Chenopodium nuttalliae* Saff.) se sometió a pH alcalino, ultrasonido o calentamiento para determinar el efecto sobre las propiedades funcionales y estructurales. Adicionalmente, se estudió la formación de complejos solubles (CS) con almidón (S) modificado con Anhidrido Octenil Succínico (OSA) y se realizó su caracterización. Para determinar el pH y la relación para la formación de SC se realizaron curvas de turbidez para cada tratamiento a diferentes relaciones (1:0.50, 1:0.25, 1:1, 1:2, 1:4), en un rango de pH de 2 a 6. La relación a la que se formaron los CS entre la proteína de huauzontle y el almidón modificado en cada tratamiento fue de 1:1 proteína nativa (P_{nativa}-S); 1:2 para las modificadas (sonicada (P_{US}-S); calentada (P_{calor}-S); P_{pH11}-S). La turbidez de los complejos se mantuvo estable después de 24 h de almacenamiento a pH por encima del punto isoeléctrico de la proteína (4.3), lo que indicó la formación de complejos solubles. El pH óptimo para la formación de complejos fue alrededor de 5. La espectroscopia infrarroja por transformada de Fourier (FT-IR) mostró picos característicos de la proteína (amida I, II y III), en la proteína nativa, modificada y en los complejos solubles, picos que desaparecieron o disminuyeron de tamaño, demostrando los cambios estructurales en la proteína y en complejos proteína-polisacárido. Las micrografías revelaron estructuras diferentes en forma y tamaño en la proteína nativa y modificada, mientras que en los complejos solubles se observó una aglomeración proteína-almidón. En cuanto a las propiedades tecno-funcionales de la proteína se encontraron cambios significativos. De acuerdo con los resultados obtenidos es posible la aplicación de la proteína de huauzontle en alimentos que busquen mejorar las propiedades nutricionales y estructurales. Los métodos de modificación estructural de proteínas, empleados en este trabajo de investigación nos permiten evaluar diferentes enfoques del uso de la proteína de huauzontle.

Palabras clave: proteína vegetal, almidón modificado, complejos solubles interacciones electrostáticas, propiedades funcionales

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GENERAL ABSTRACT

Huauzontle protein modification: effect on techno-functional and structural properties and soluble complexes

The huauzontle protein was subjected to alkaline pH, ultrasound, or heating to determine the effect on the functional and structural properties. Additionally, the formation of soluble complexes (SC) with starch (S) modified with Octenyl Succinic Anhydride (OSA) was studied and characterized. To determine the pH and ratio for SC formation, turbidity curves were performed for each treatment at different ratios (1:0.50, 1:0.25, 1:1, 1:1, 1:2, 1:4), in a pH range from 2 to 6. The ratio at which the SC_S were formed between huauzontle protein and modified starch in each treatment was 1:1 for native protein (P_{native}-S); 1:2 for modified (sonicated (P_{US}-S); heated (P_{heat}-S); P_{pH11}-S). The turbidity of the complexes was stable after 24 h of storage at pH above the isoelectric point of the protein (4.3), indicating the formation of soluble complexes. The optimum pH for complex formation was around 5. Fourier transform infrared spectroscopy (FT-IR) showed characteristic peaks of the protein (amide I, II and III), in the native, modified protein and in the soluble complexes, peaks that disappeared or decreased in size, demonstrating structural changes in the protein and protein-polysaccharide complexes. Micrographs revealed different structures in shape and size in the native and modified protein, while protein-starch agglomeration was observed in the soluble complexes. As for the techno-functional properties of the protein, significant changes were found. According to the results, the huauzontle protein application in foods that seek to improve their nutritional and structural properties is possible. The protein structural modification methods used in this research work allow us to evaluate different approaches to the use of huauzontle protein.

Keywords: plant protein, modified starch, soluble complexes, electrostatic interactions, functional properties

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1 INTRODUCCIÓN GENERAL

Recientemente, los hábitos alimentarios se han orientado hacia fuentes de naturales, ecológicas y sostenibles, debido al aumento del conocimiento y la concientización de los consumidores (Akharume et al., 2021). En particular, las proteínas son ampliamente utilizadas en la industria alimentaria debido a sus múltiples propiedades tecno-funcionales, como la solubilidad, la capacidad de formación de geles, espumas y emulsiones, así como la capacidad de absorción de agua y aceite, las cuales confieren estabilidad, textura y sabor a los productos alimentarios procesados (Kamani et al., 2022). En este sentido, las proteínas vegetales presentan múltiples ventajas frente a las proteínas animales, por ejemplo, sus bajos costos de producción, mayor disponibilidad sostenibilidad climática. La fuente principal de proteína vegetal la constituyen los cereales, las legumbres, las oleaginosas y recientemente los pseudocereales; estos últimos incluyen al amaranto, quinoa, trigo sarraceno y el huauzontle, del cual la información es escasa. Como se indicó, el huauzontle es un pseudocereal, perteneciente a la familia Chenopodiaceae, este cultivo es de origen mexicano y ha sido poco valorado. En trabajos previos (Álvarez-Jubete et al., 2010 y López-Monterrubio et al., 2020), se han evaluado las propiedades fisicoquímicas y tecno-funcionales de la proteína de huauzontle, reportando un contenido aminoácidos esenciales (fenilalanina y leucina) superior a 10 $g_{aminoácido}$/100 $g_{proteína}$, el cual es mayor con respecto al amaranto y la quinoa. También reportaron una digestibilidad del 83 %, así como el estudio de propiedades tecno-funcionales como capacidad de espumante, estabilidad de la espuma, capacidad de gelificación y retención de agua. Sin embargo, su uso en estado nativo está limitado, debido su baja solubilidad y escasa capacidad emulsionante (Li et al., 2020).

Las propiedades tecno-funcionales de las proteínas están directamente relacionadas con sus características estructurales, por lo que se ha recurrido a la modificación del estado nativo mediante métodos físicos, químicos y biológicos (Jiang et al., 2017). Diversas investigaciones han demostrado la ventaja de las proteínas modificadas sobre las nativas en la solubilidad, capacidad emulsionante y propiedades espumantes, debido principalmente a cambios en los grupos sulfhidrilo libres, tamaño de partícula, hidrofobicidad superficial y propiedades reológicas (Jiang et al., 2014).

La selección del método más adecuado para promover cambios estructurales dependerá del tipo de proteína, viabilidad, costos y aplicación (Kamani et al., 2022). En este sentido, el cambio de pH es un método en el cual, la proteína es expuesta a un pH extremo, provocando el desdoblamiento de su estructura. Posteriormente, el medio se neutraliza favoreciendo el plegado de la cadena polipeptídica. Este tipo de modificación ha demostrado ser eficiente para aumentar la solubilidad y la capacidad emulsionante (Wang et al., 2018). Por su parte, la modificación con ultrasonido se ha utilizado para mejorar las propiedades tecno-funcionales de las proteínas vegetales, como la solubilidad, gelificación, capacidad emulsionante y espumante (Martínez-Velasco et al., 2018). Con este método, los cambios que se producen en la estructura de la proteína se deben al efecto del fenómeno de cavitación generado durante la sonicación (Ren et al., 2020). Adicionalmente, el tratamiento térmico constituye otro método físico que induce la desnaturalización de las proteínas debido a los cambios en las interacciones intra e intermoleculares (puentes de hidrógeno, interacciones hidrofóbicas y electrostáticas (Van de Vondel et al., 2021).

Las proteínas y los polisacáridos se han utilizado ampliamente de forma conjunta en la industria alimentaria, ya que contribuyen a la estructura, textura y estabilidad de los alimentos (Muhoza et al., 2022). El uso combinado de estos biopolímeros ha demostrado tener múltiples ventajas sobre su uso individual, ya que, además de preservar las propiedades individuales de los componentes, se desarrollan

nuevas propiedades estructurales y funcionales, lo que los hace ideales para su aplicación en diversos alimentos (Zhao et al., 2023).

1.1 Objetivo general

Evaluar el efecto del procesamiento del aislado de proteína de huauzontle sobre las propiedades tecno-funcionales, estructurales y la formación de complejos solubles entre la proteína de huauzontle - nativa y modificada - y el almidón modificado con anhídrido octenil succínico.

1.2 Objetivos específicos

1) Evaluar el efecto del calor, cambio de pH y ultrasonido sobre las propiedades estructurales y tecno-funcionales (solubilidad, capacidad emulsionante y capacidad espumante) de la proteína de huauzontle.
2) Establecer las condiciones óptimas (pH, densidad de carga relativa y relación de masas de los biopolímeros) que promueven la formación de complejos solubles entre un almidón de maíz comercial modificado con anhídrido octenil succínico y el aislado proteico de huauzontle nativo y modificado mediante tratamientos térmicos, ultrasónicos y cambio de pH.
3) Determinar el efecto del tratamiento de modificación estructural de la proteína de huauzontle sobre la estructura y la morfología de los complejos solubles de proteína-almidón.
4) Evaluar la digestibilidad del almidón, proteína nativa y proteína modificada, mediante técnicas *in vitro* para comparar este parámetro nutricional entre biopolímeros individuales y en complejos solubles.

1.3 Hipótesis

La aplicación de tratamientos térmicos, ultrasonido y pH para modificar la estructura de la proteína del huauzontle, promoverá una mejora en las propiedades tecno-funcionales (solubilidad, capacidad emulsionante y capacidad espumante) de la proteína de huauzontle. Además, será determinante en la

formación, morfología y estructura en complejos solubles estables (proteína-almidón), así como en la digestibilidad de sus componentes.

1.4 Organización de la tesis

El **Capítulo 1**, contiene la introducción general de la tesis. El **Capítulo 2** corresponde al estado del arte de los principales temas que conforman el trabajo de investigación como los pseudocereales, proteínas vegetales, métodos de modificación estructural, que mejoran las propiedades tecno-funcionales en proteínas de origen vegetal, cuya aplicación en la industria alimentaria se ve limitada debido a su baja solubilidad a pH neutro en medios acuosos, así como la importancia del uso de proteínas y polisacáridos en la formación de complejos solubles, los principales factores e interacciones que intervienen en su formación. El **Capítulo 3,** presenta el proceso de extracción y modificación estructural de la proteína, así como los resultados obtenidos en las propiedades tecno-funcionales y estructurales después de aplicar los tratamientos pH alcalino, ultrasonido y calentamiento. El **Capítulo 4**, explica las condiciones establecidas para determinar el pH, la relación y concentración de aislado de proteína de huauzontle y almidón modificado con OSA (anhídrido octenil succínico), para la formación de complejos solubles. Finalmente, se presentan los resultados obtenidos de las características estructurales y morfológicas de los complejos, así como de los biopolímeros individuales.

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2 REVISIÓN DE LITERATURA

2.1 Pseudocereales

Los pseudocereales poco a poco se han ido posicionando en el mercado como cultivos prometedores debido a su alto contenido de proteína comparado al de otros granos, por el contenido de aminoácidos esenciales, así como por su alta variabilidad genética permitiendo que sean capaces de adaptarse a diferentes ambientes, desde condiciones climáticas tropicales hasta templadas (Joshi et al., 2019; Mustafa et al., 2011). Por otro lado, se caracterizan por su bajo o nulo contenido de gluten lo que los hace ser una opción adecuada para agregar a la dieta de personas con enfermedad celíaca (Rastogi y Shukla, 2013). Actualmente, el amaranto, la quinoa y el trigo sarraceno (Fig. 1) son los pseudocereales más estudiados, sus semillas son comestibles, pertenecen a la familia Chenopodiaceae y se asemejan a los cereales, sin embargo, por sus características morfológicas se consideran plantas dicotiledóneas (Jubete-Álvarez et al., 2010; Malik et al., 2022).

La característica para agrupar a los pseudocereales radica en similitud de sus frutos y composición en materiales de almacenamiento, haciéndolos adecuados para sustituir a los cereales. El interés sobre el consumo de los pseudocereales se ha incrementado significativamente debido a los contenidos de micronutrientes como el almidón, proteínas de alta calidad, minerales, vitaminas y compuestos bioactivos (Haros & Schoenlechner, 2017).

Fig. 1. Pseudocereales: quinoa (*Chenopodium quinoa* Willdenow); huauzontle (*Chenopodium nuttalliae* saff.); trigo sarraceno (*Fagopyrum esculentum*); amaranto (*Amaranthus* spp.) (elaboración propia).

2.2 Huauzontle

Dentro de la familia Chenopodiaceae, se encuentra el huauzontle, pseudocereal que no se ha explorado, por lo que resulta interesante conocer las propiedades tecno-funcionales de sus componentes para su aplicación en la industria alimentaria. El huauzontle (Fig. 2) (*Chenopodium nuttalliae* spp. es originario de México (Pereira 2019). Bhargava et al. (2006) lo describe como una verdura parecida al brócoli por su apariencia y sabor, se consumen sus hojas en estado tierno, las flores, semillas o inflorescencia en estado maduro, su embrión se caracteriza por tener forma circular (Fig. 3) y está combinado con la cubierta de la semilla para formar la fracción del salvado, la cual es rica en lípidos, fibra,

proteínas y cenizas, rodeada por el pericarpio rico en almidón (Scanlin y Luis 2017).

El huauzontle presenta un contenido de proteína de aproximadamente 20 %, almidón 60 % base seca, es rico en antioxidantes, no contiene gluten, y se caracteriza por su alto contenido de aminoácidos esenciales. Las principales fracciones proteicas presentes en el huauzontle son globulinas P, detectadas entre 20 y 22 kDa, albumina-1 alrededor de 28 kDa y a 50 kDa se atribuye la presencia de la globulina (López-Monterrubio et al., 2020).

Fig. 2. Semilla de huauzontle.

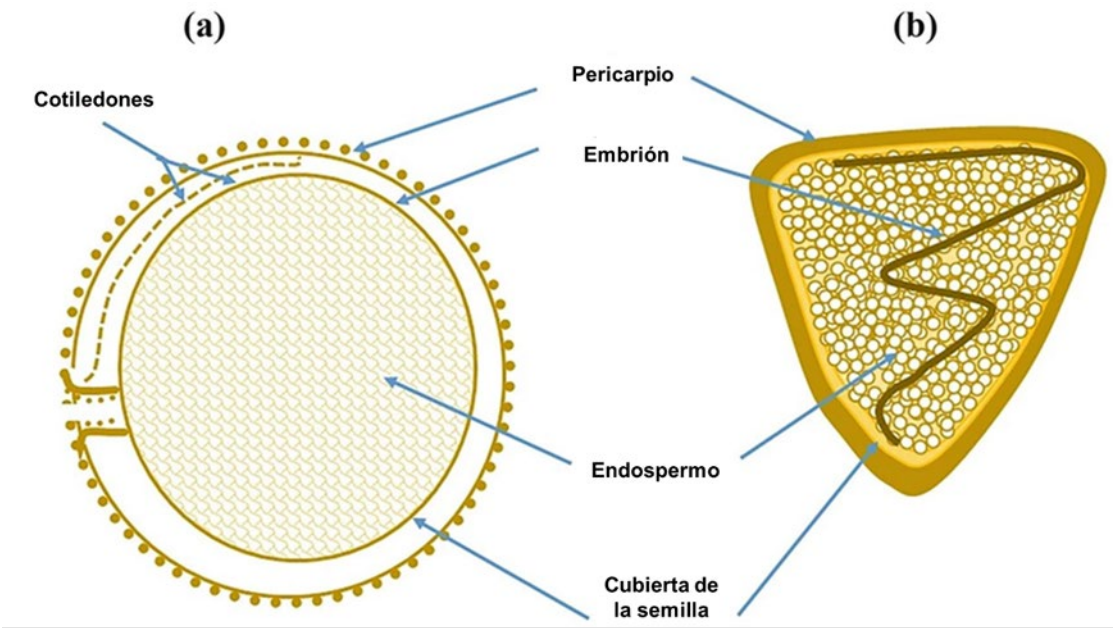

Fig. 3. Estructura común del grano en pseudocereales como a) quinoa y amaranto; b) trigo sarraceno. (Adaptado por Rolandelli et al., 2023)

2.3 Proteínas

Las proteínas son utilizadas como ingredientes tecno-funcionales en la industria alimentaria y farmacéutica para la formación de emulsiones, espumas y geles, así como para aumentar la viscosidad de soluciones. Las propiedades de la proteína se deben a la estructura primaria (Walstra y VanVliet, 2003; O´Sullivan et al., 2016). La aplicación de las proteínas en procesos alimentarios como agentes emulsionantes radica en su capacidad para adsorberse y formar películas viscoelásticas en la interfase aceite-agua (O´Connell y Flynn, 2007).

2.4 Proteínas vegetales

Las proteínas vegetales se han empleado como agentes espesantes y gelificantes, estabilizadores de emulsiones y espumas, así como aglutinantes de grasa y agua, por otro lado, se ha demostrado que algunas proteínas tienen actividades biológicas como antioxidantes o antimicrobianas (Nasrabadi et al., 2022). Por otro lado, las proteínas pueden clasificarse en función de su solubilidad en cuatro fracciones: prolaminas, albúminas, glutelinas y globulinas

(Kumar et al., 2021). Thakur et al. (2021) describen a las albúminas como solubles en agua; las globulinas solubles en solución salina; las prolaminas en soluciones de etanol y las glutelinas parcialmente solubles en ácidos o bases diluidas. Sin embargo, las proteínas de origen vegetal presentan algunas limitaciones como su baja solubilidad a pH neutro en soluciones acuosas y al ser una mezcla de diferentes fracciones de proteínas presentan diversos puntos isoeléctricos (pI) (Usman et al., 2022). Así también, se debe mencionar la presencia de antinutrientes como ácido fítico, taninos condensados y saponinas, haciendo que la biodisponibilidad de la proteína en el cuerpo humano sea limitada. Por lo anterior, se han utilizado métodos de modificación como tratamientos físicos, químicos y enzimáticos, que permiten mejorar la funcionalidad de la proteína, y así puedan ser empleadas en la industria alimentaria (Uluko et al., 2016; Nasrabadi et al., 2021).

2.5 Métodos de modificación en proteínas vegetales

2.5.1 Tratamiento térmico

El calentamiento es una operación unitaria común para el procesamiento de alimentos, en proteínas induce la desnaturalización, es decir, se lleva a cabo el desplegado de la cadena polipeptídica (Fig. 4), dando como resultado cambios en los puentes de hidrógeno, interacciones hidrofóbicas y electrostáticas y enlaces cruzados no covalentes (Belitz et al., 2009). El principal efecto de la desnaturalización de la proteína es que los grupos funcionales de los aminoácidos que estaban ocultos quedan expuestos y accesibles para formar agregados proteicos mediante interacciones intermoleculares no covalentes. O bien, enlaces covalentes cruzados que se dan mediante puentes disulfuro (S-S) formados por la reacción de oxidación del sulfhidrilo (SH) (Van de Vonde et al., 2021). A temperaturas suaves, las propiedades estructurales y tecno-funcionales mejoran. En cambio, la temperatura extrema puede provocar cambios irreversibles en la estructura, dando lugar a la disminución de sus propiedades tecno-funcionales (Nasrabadi et al 2021).

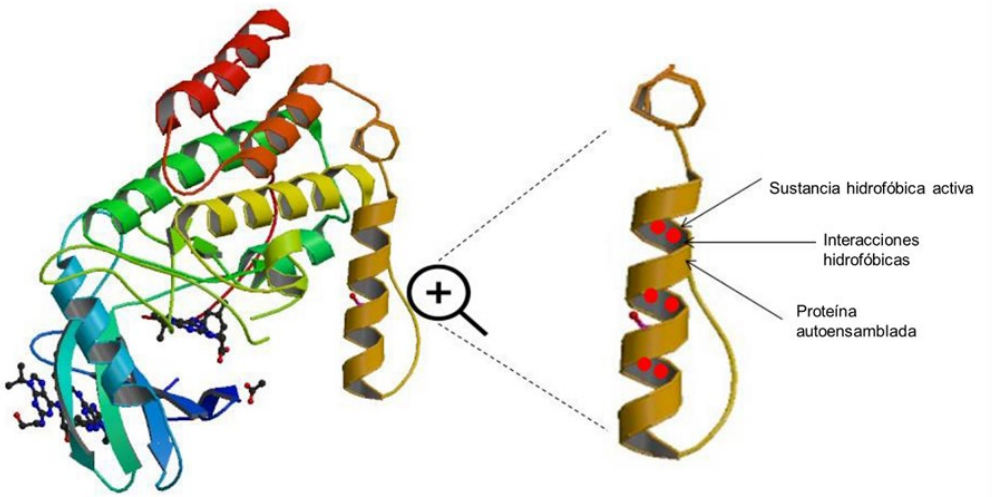

Fig. 4. Representación de una estructura nativa de una proteína (Tomadoni et al., 2020).

2.6 Ultrasonido

El ultrasonido es ampliamente utilizado para mejorar las propiedades tecno-funcionales de las proteínas. El efecto que provoca en la estructura de la proteína es la ruptura de los enlaces peptídicos, interacciones no covalentes e induce la disociación y agregación de subunidades (Jin et al., 2015; Huang et al., 2017; Higuera-Barraza et al., 2016). Zhu et al. (2018) aplicaron ultrasonido a diferentes niveles de potencia durante 15 y 30 min y evidenciaron cambios en las propiedades tecno-funcionales como el índice de actividad emulsionante, la solubilidad y el índice de estabilidad emulsionante. Este tratamiento se ha aplicado en aislados de proteína de arroz, girasol y soya, entre otros (Morales et al., 2015; Malik et al., 2017). Hu et al. (2013) reportaron que el tratamiento con ultrasonido produce un desdoblamiento parcial y mejora la solubilidad en soya (Hu et al., 2013); mientras que Morales et al. (2015) en aislado de proteína de soya se mejoró la capacidad espumante, al modificar el tamaño de partícula.

2.7 Cambio de pH

El tratamiento con cambio de pH consiste en modificar el pH a ácido o alcalino y volver a ajustar a pH neutro. Estos cambios de pH dan una estructura más

flexible**,** mejor conocida como glóbulo fundido (Jiang et al., 2018; Tian et al., 2020). Con el despliegue de la proteína se da un aumento de las repulsiones entre las cadenas laterales haciendo posible el desdoblamiento parcial de las estructuras secundaria y terciaria, con el repliegue de la proteína no regresa a su conformación original, si no que se obtiene una nueva estructura conocida como "glóbulo molten" (Xu et al., 2023). Por otro lado, el pH alcalino ayuda a potenciar la reactividad de algunos aminoácidos como la cisteína, favoreciendo las reacciones de intercambio tiol-disulfuro con enlaces disulfuro vecinos y polimerización de proteínas (Van de Vondel et al., 2020).

2.8 Interacción proteína-polisacárido

La coacervación compleja entre proteínas y polisacáridos surge de la atracción electrostática entre cargas positivas ($-NH_3^+$) y negativas ($-COO^-$). La magnitud de la ionización del grupo amino de la proteína ($-NH_3^+$) y del grupo carboxilo del polisacárido ($-COO^-$) depende del pH y de la fuerza iónica del medio (Timilsena et al., 2019). El equilibrio de carga entre las proteínas y los polisacáridos depende de su proporción, que afecta esencialmente a la magnitud de la coacervación compleja. Los mayores rendimientos de coacervados se consiguen con una relación de biopolímeros y pH donde la carga está equilibrada y la atracción electrostática es alta (Muhoza et al., 2019).

La interacción entre las proteínas y los grupos carboxilo libres cargados negativamente en los polisacáridos puede tener lugar mediante atracción electrostática, interacciones hidrofóbicas y puentes de hidrógeno, mejorando las características texturales y la estabilidad fisicoquímica de muchos sistemas alimentarios (Gentile, 2020; Telis, 2019). Comert y Dubin (2017); Eghbal y Choudhary (2018), se refieren a la coacervación como la separación de un sistema acuoso coloidal en dos fases líquidas, una rica en ambos biopolímeros y otro está compuesta por el disolvente. Las interacciones en los coacervados son no covalentes (interacciones electrostáticas principalmente, aunque también

pueden estar los puentes de hidrógeno y las interacciones hidrofóbicas) (Pathak et al., 2017).

Existe un gran número de características que se deben considerar en la formación de complejos como, la estructura química de los biopolímeros implicados, concentración total del biopolímero y la relación proteína-polisacárido, características del disolvente, así como factores ambientales (Schmitt y Turgeon, 2011). Uno de los parámetros más importantes a considerar en la coacervación de proteínas y polisacáridos es la turbidez, ya que es el resultado de la formación de complejos solubles (Amine et al., 2019).

2.8.1 Características de los complejos

De la interacción proteínas-polisacáridos se obtienen nuevas estructuras alimentarias las cuales pueden funcionar como sistemas de encapsulación y administración, así como agentes gelificantes, formadores de películas y sustitutos de grasa (Salminem et al., 2021). La formación de complejos depende en gran medida de las características moleculares de los biopolímeros, como su conformación, peso molecular y densidad de carga, así como de diversas condiciones externas, como el pH, la fuerza iónica, la temperatura, el tiempo de reacción y las proporciones de mezcla de los biopolímeros (Warnakulasuriya et al., 2018; D.; Wu et al., 2020; You et al., 2018).

En la formación de complejos entre proteínas y polisacáridos se involucran diversos factores entre los que se encuentran las interacciones electrostáticas repulsivas y se dan entre mezclas de proteínas y polisacáridos aniónicos, también se encuentran las interacciones atractivas entre las que se identifican las electrostáticas, de Van der Waals, puentes de hidrógeno e hidrofóbicas también conocidas como interacciones débiles (Fig. 5). Las interacciones electrostáticas son las principales fuerzas que intervienen en la formación de complejos proteína cargadas positivamente/polisacáridos cargados negativamente, provocando la disminución de la energía libre electrostática en el sistema. De esta interacción

se forman complejos solubles que se obtienen cuando las cargas opuestas que llevan los dos biopolímeros no son iguales en número y los insolubles se dan cuando la carga neta del complejo es cercana a cero (Goh et al., 2019).

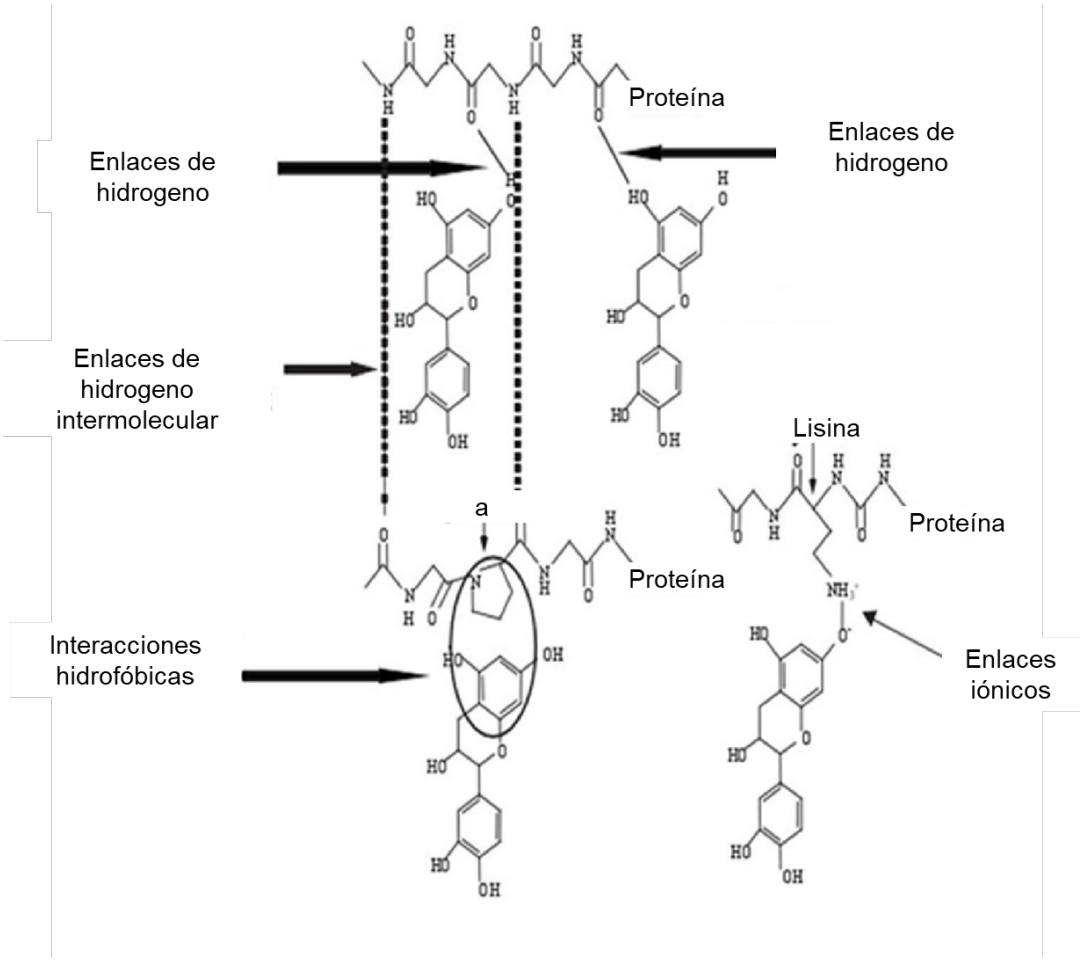

Fig. 5. Interacciones presentes en la formación de complejos solubles e insolubles (Nicolás-García et al., 2021)

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3 MODIFICATION OF HUAUZONTLE PROTEIN BY ULTRASONICATION, PH SHIFTING OR HEAT: EFFECT OF SECONDARY STRUCTURE, EMULSIFYING ACTIVITY AND FOAMING CAPACITY[3]

Abstract

BACKGROUND: Huauzontle (*Chenopodium nuttalliae* Saff.) is an underutilized pseudocereal native from Mexico, with a high protein content (~ 17%). In this work, huauzontle protein isolate (P_{native}) was subjected to ultrasonication (P_{US}), pH-shifting (P_{pH}) or heat (P_{heat}) treatments, evaluating their effect on protein structure, emulsifying and foaming properties. induced by these treatments and evaluating their effect on emulsifying activity and foaming capacity.

RESULTS: For all treatments FTIR showed that the intensity of the amide I bands decreased, and deconvolution of the secondary structure showed a decrease in β-sheet and β-turn, but an increase in α-helix structures. The latter are associated with increased surface activity and stable interfacial stability. Both, the emulsifying activity and emulsion stability, and the foaming capacity and foam stability was enhanced with the modified proteins compared to the native protein.

CONCLUSION: The structural changes produced on the native huauzontle protein by ultrasonication, pH-shifting or heat treatments, significantly enhanced the emulsifying and foaming properties. This confirms that modification of

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vegetable proteins enhances their properties and extends their potential applications.

Keywords: huauzontle, protein modification, emulsifying and foaming properties

3.1 INTRODUCTION

Pseudocereals resemble in function and composition true cereals, are rich sources of starch, high quality protein, fiber, vitamins, minerals, but do not contain gluten.[1] Pseudocereals are considered underutilized crop species that are closely knitted to the population of local, regional, national or even international communities since ancient times, but have the potential to contribute global food security.[2] Examples of pseudocereals native from the American continent are amaranth, quinoa, chia and huauzontle, while buckwheat and chenopod are native from Asia. An ongoing research topic is to investigate the suitability of underutilized crops for producing healthy functional foods or ingredients which may contribute to meet the food needs of a growing worldwide population. Most studies regarding pseudocereals in recent years have focused on the substitution of carbohydrate-rich cereal grains by pseudocereals, seeking to produce foods that may deter the incidence of disorders in the population related to the metabolic syndromer.[4] However, lately interest has driven the search for specific ingredients such as proteins which are of high quality in pseudocereals.

The main sources of dietary proteins are animal and plants, but they exhibit differences in molecular structure, amino acid profile, digestibility, and on their ability to impart functional properties to food. These inherent differences require the undertaking of in depth-research that allow to design plant-based foods or ingredients which are able mimic animal food or ingredients successfully.[5] This cannot be achieved by simple substitution. Proteins of amaranth (AP), quinoa (QP) and buckwheat (BP) have a high nutritional quality which makes them very attractive for their incorporation in processed foods. However, the native proteins of these pseudocereals exhibit low solubility in water which limits their techno-

functional applications in food industry. Fortunately, there are several methods available for modifying the structural and physicochemical properties of vegetable native proteins, which may allow to extend their industrial application. Constantino and Garcia-Rojas[6] reported that high-intensity ultrasound (US) improved the solubility of AP and QP, while heating allowed QP to form aggregates. Combined HIUS and heat treatment improved the solubility, digestibility, and foaming properties of AP. BP and QP interfacial properties were by protein conjugation via Maillard reaction. The physicochemical and functional properties of huauzontle protein isolates (HPI) were studied by López-Monterrubio et al.[7] These authors found that huauzontle contained a higher protein (~ 17%) content and higher digestibility (~ 86%) than that reported for amaranth and quinoa. The relative percentage of protein secondary structures varied with pH and affected the HPI functional properties. Thus, maximum solubility was achieved at pH 11, self-supporting gels were formed between pH 7-9, foaming capacity increased with HPI concentration, and its stability was of ~ 91% at HPI concentration of 0.01 mg. g^{-1}.

Therefore, the objective of this work was to evaluate the impact of ultrasonication, pH-shifting or heating treatments on the structural and functional properties of the huauzontle protein isolate, in comparison to the properties of the native huauzontle protein isolate.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Huauzontle seeds (*Chenopodium nuttalliae* Saff.) were obtained from local producers in Tepoztlan (18°59´ 7´ N, 99°5´59´´ W, elevation above sea level of 1706 m, State of Mexico). The seeds were washed and dried in an oven at 35 °C for 24 h and ground to obtain the flour, which was defatted in a Soxhlet with hexane for 3 h. Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from J.T. Baker (Xalostoc, State of Mexico, Mexico). β-

mercaptoethanol (β-ME) and protein marker (10–170 kDa) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Distilled and deionized water (DDW) was used in all of experiments.

3.2.2 Protein modification

The huauzontle protein isolate (HPI) was obtained by the alkaline extraction and isoelectric precipitation method and coded as P_{native}.[7] HPI was dispersed in deionized water, pH was adjusted 7 with 1 M HCl and 1 M NaOH, with continuous stirring for 1 h. The solution was subjected to three different treatments for HPI structure modification: *i*) Heat (P_{heat}), it was maintained at pH 7 and heated at 70 °C for 20 min in a water bath. The solution was then cooled in an ice bath; *ii*) High intensity ultrasound (P_{US}), using a frequency of 20 kHz, 50% amplitude for 10 min (pulse duration: 5 min on, 5 min off) with an ultrasound processor (Sonics and Materials, Inc, Newtown, CT, USA) equipped with a flat tip probe of 8 mm diameter. An ice bath was placed to avoid sample heating; *iii*) pH-shifting (P_{pH11}), it was adjusted to pH 11 using 1M NaOH, stirred for 2 h at room temperature, pH was lowered to 7 with 0.5 M HCl and stirred for 1 h. No adjustment was made to P_{native}, which was maintained at pH 7. The solutions were allowed to stand for 12 h for complete hydration at 4 °C. To eliminate the insoluble protein present, they were centrifuged at 1238 ×g for 30 min, and then lyophilized.

3.2.3 Particle size

The particle size (d_h: hydrodynamic diameter) of the native and modified huauzontle protein solutions (1 mg. mL^{-1}) were determined using a Zetasizer Nano ZS90 particle size analyzer (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Stock solutions were diluted with DDW to a concentration of 0.005 mg. mL^{-1}, pH 7 at 25 °C. Stock solutions were kept refrigerated (4 °C) for 24 h before testing.

3.2.4 Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR spectra of the native and modified huauzontle protein, S and SC were obtained with a Frontier FT-IR/FIR spectrophotometer (Perkin Elmer, Waltham, MA, USA) following the methodology proposed by Trujillo-Ramírez et al.[8]. The deconvoluted spectra were adjusted to Gaussian band profiles and curve fitting was performed with the OriginPro 2024 program version 9.9.5 program.

3.2.5 Molecular weight profile

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed.[9] Staining of the protein bands was performed by immersion in Coomassie Brilliant Blue R-250 gel (Cat. 161–0400, Bio-Rad) in a solution containing 400 mL of methanol, 70 mL of acetic acid, and 530 mL of deionized water. MW standards of proteins in the range of 17.4-102.6 kDa were used to (Cat. 161-0305, Bio-Rad®).

3.2.6 Protein solubility

The solubility in the protein samples was determined.[11] Aqueous protein solutions (0.01 $g.mL^{-1}$) were prepared in 0.01 M phosphate buffer at pH 3, 5, 7, 9, and 11. The protein content was determined from the supernatants.[12] The percentage of solubility of the samples was calculated with the following equation:

$$Soluble\ protein\ (\%) = (PCS/PCD) * 100 \quad (1)$$

where PCS and PCD is the protein concentration in the supernatant and solution.

3.2.7 Gel formation and rheological properties of protein solutions

For gel formation, protein dispersions (15 g/mL) at pH 3, 5, 7, 9 and 11 were heated in a water bath at 90 ± 1 °C for 20 minutes and allowed to cool to room temperature[7]. The formation of the gel was determined by visual observation. Considering that if the dispersion did not move when rotating the tube, the gel had

formed. For the rheological properties, pH 7 was chosen. The determination was conducted in a rheometer Physica MCR 301 (Anton Poor, Messtechnik, Stuttgart, Alemania) with a plate-plate geometry (25 mm diameter). The edge of the geometry was covered with a thin layer of paraffin to prevent evaporation, while to mimic the gelation process, the protein solutions were subjected to a temperature ramp from 20 to 90 °C with a heating rate of 3 °C/min, maintained at 90 °C for 5 min and cooling was carried out at 20 °C at a rate of 5 °C/min applying a strain amplitude of 1 % at a frequency of 0.1 Hz. Subsequently a strain sweep (0.01-100 %) was applied with a constant frequency of 0.1 Hz. The values of the storage modules (G') and loss modules (G'') were obtained by controlling the equipment software.

3.2.8 Free sulfhydryl (SH) content

Sulfhydryl groups were determined by Mäkinen et al.[12] diluting 62 µL of protein sample in 1312 mL of phosphate buffer (0.05 M sodium phosphate and sodium chloride at pH 8, containing 2 mmol.L^{-1} ethylenediaminetetraacetic acid (EDTA)), 25 µL, 0.004 g.mL^{-1} of Ellman DTNN reagent (5,50-dithio-bis-(2-nitrobenzoic acid). The solution was allowed to stand for 15 min at room temperature and centrifuged at 4000 ×g at 15 °C for 15 min. To obtain the SH content, the DNTB extinction coefficient (73.53 M^{-1} cm^{-1}) was used; the values were expressed in µmol. g^{-1} protein.

$$SH = (73.53 \times A \times DF)/C \quad (2)$$

Where A is the absorbance at 412 nm, DF is the dilution factor and C is the protein concentration.

3.2.9 Emulsifying activity (EA) and emulsion stability (ES)

Protein (0.005 g. mL^{-1}) was dissolved in DDW at pH 7 and stirred for 1 h. 50 mL of the protein solution was taken and mixed with 10 mL of soybean oil, in a homogenizer (Ultra-Turrax IKA T50 Works, Inc., Wilmington, USA) at 8000 rpm

for 3 min. Then, an aliquot of 50 µL of each emulsion was taken at 0 min and 30 min and transferred to a tube containing 10 mL of 0.001 g.mL^{-1}sodium dodecyl sulfate (SDS) solution. Absorbance was measured at 500 nm. SDS solution was taken as blank. EA and ES were determined as follows:[15]

$$EA\ (m^2/g) = 2 \times 2.303 \times A_0 \times DF/C \times \varphi \times 10000 \quad (3)$$

$$ES\ (min) = (A_0/A_0 - A_{30}) * 10 \quad (4)$$

where A_0 and A_{30} are the absorbance at 0 and 30 min; DF the dilution factor; C is the concentration of test sample (g.L^{-1}); and φ is the volumetric fraction of oil ($\varphi = 0.2$).

3.2.10 Foaming capacity (FC) and foam stability (FS)

20 mL of huauzontle protein dispersions (0.5 g. L^{-1}, pH 7.0) were whipped (10000 rpm, 1 min) with an Ultra Turrax® homogenizer for air incorporation, and subsequently transferred to 50 mL test tubes. The total foam volume after the 1 min whipping was measured at 0, 0.5, 5, 10, 40 and 60 min. The FC was expressed as the foam expansion achieved immediately after the 1 min homogenization respect that of the unhomogenized sample, while foaming stability was considered as the relative foam expansion measured at a given time after whipping and that achieved immediately after whipping.[7]

3.2.11 Statistical analysis

All variables were analyzed in triplicate from three independent experiments. The results were expressed as the mean ± standard deviation. Statistical analysis was performed with the statistical package (SAS version 9.4, SAS Institute Inc., NC, USA). ANOVA analysis of variance was performed to determine significant differences using the Tukey test with a significance level of $p \leq 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Particle size

The treatments P_{US} (119.86 ± 2.95 nm) and P_{pH11} (143.70 ± 4.22 nm) had significantly smaller particle size than P_{native} (204.23 ± 6.12 nm) and P_{heat} (194.10 ± 4.62 nm) which were non-significantly different between themselves. It has been observed that when proteins are modified by heat at neutral pH they tend to exhibit similar particle sizes as the native proteins, as protein-protein attraction forces dominate over breakdown forces.[14] Whereas it has been reported that quinoa[15] and album seed[16] proteins treated by ultrasound had significantly smaller particle sizes than their native counterparts. Cavitation, turbulence, and shear forces generated during sonication are responsible for the breakdown of non-covalent, hydrogen bonds, hydrophobic and electrostatic interactions between protein aggregates, leading to the reduction of particle size. The reduction in the particle size of P_{pH11} could be attributed to the partial unfolding of the protein structure at alkaline pH, causing changes in the interactions between the protein molecules and their state of aggregation. In addition to this, the disulfide bonds could have collapsed under these conditions, favoring the reduction in particle size.[17]

3.3.2 Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra of native and modified huauzontle proteins are shown in Fig. 6. All the spectra showed similar bands, but the intensity of the amide I bands decreased for P_{US}, P_{pH11} and $P_{heat,}$ respect that of P_{native}. The absorption peak at 3272 cm^{-1} is attributed to the amide group A, to the NH stretching vibration. The presence of β-sheets is confirmed with the absorption peak present at 1636 cm^{-1} which corresponds to the amide I group, while the amide II group and α-helix are attributed to the absorption peaks from 1520 to 1461 cm^{-1}, these stretching vibrations correspond to NH, CN and C-C.[18] The peak at 1231 cm^{-1} corresponds to amide III (CN stretching vibration and NH deformation).

The deconvolution of the secondary structure of the protein was conducted in the region of 1700-1600 cm^{-1} corresponding to amide I. The main structures identified were β-sheet (1640-1610 cm^{-1}), α-helix (1660-1650 cm^{-1}) and β-turn (1700-1660 cm^{-1}) (Table 1). In P_{native} the proportion of β-sheet was about double that of α-helix and β-turn, which had equal proportions. All the treatments produced decrease in β-sheet and β-turn, and an increase in α-helix proportions. Zheng et al.[19] reported an increase in α-helix structures in soy protein isolate when heated at 90 °C for 1 h, which produced a decrease in aggregated and ordered structures. Vera et al.[20] found that the functional properties of ultrasound-modified quinoa proteins improved and related this to increases in α-helix and formation of protein aggregates. Alkaline treatment (pH= 12) on chickpea protein isolate, produced an increase in the α-helix content and a decrease in β-sheet.[21] While a high proportion of β-sheet structures is associated with a rigid structure and low solubility,[22] a high proportion of α-helix structures, which are highly flexible structures, are deemed to enhance the solubility and techno-functional properties of proteins.[23]

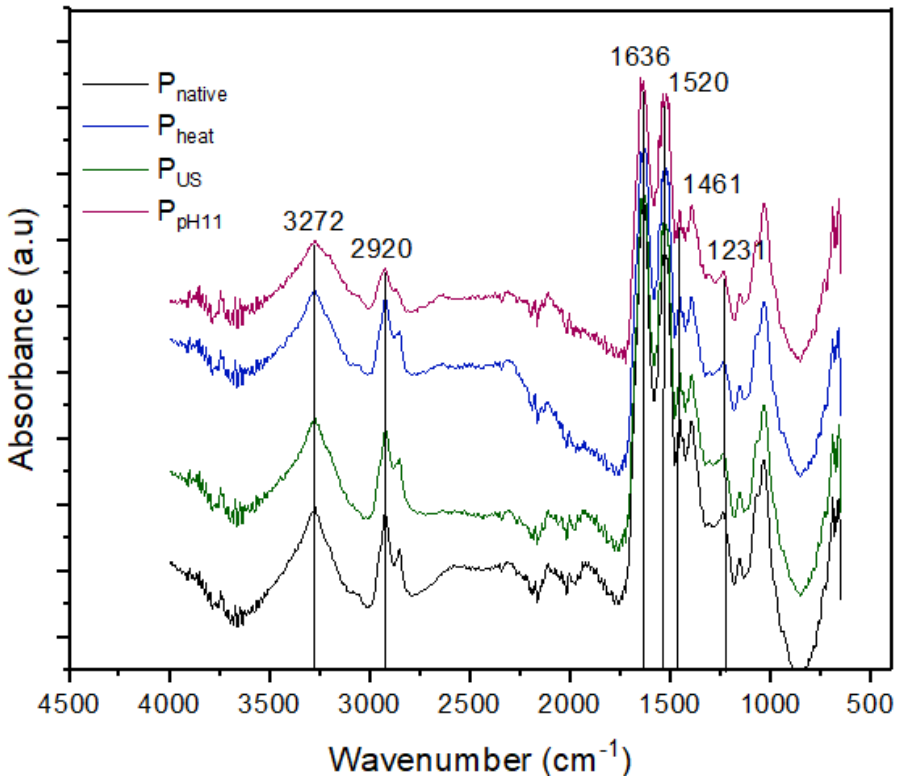

Fig. 6 FT-IR analysis in native and modified huauzontle protein.

3.3.3 Molecular weight profile

Fig. 7 shows the molecular weight profile of the native and modified huauzontle protein isolates. Five intense bands were observed for all samples, with molecular weight ranging from 17 to 46 kDa. The bands around 20 to 22 kDa correspond to the P globulin subunits, those around 31 to 33 kDa to the 11S globulin subunits, and the bands between 35 to 50 kDa to the 8S globulin subunit.[24] Furthermore, in the P_{pH11} treatment, a band below 17 kDa was observed, which can be attributed to the presence of albumin-S.[25]

No change in the intensity or shift of the main bands was observed for any of the treatments compared to P_{native}, indicative that there was no cleavage of the peptide bonds due to the treatments. Comparable results have been reported for sonicated quinoa protein,[26] alkaline treated mung bean protein[24] and in heat-modified coconut protein.[27] Thus, protein fragmentation depends on the type of

protein, the solution conditions, and the type of treatment to which it is subjected for modification.

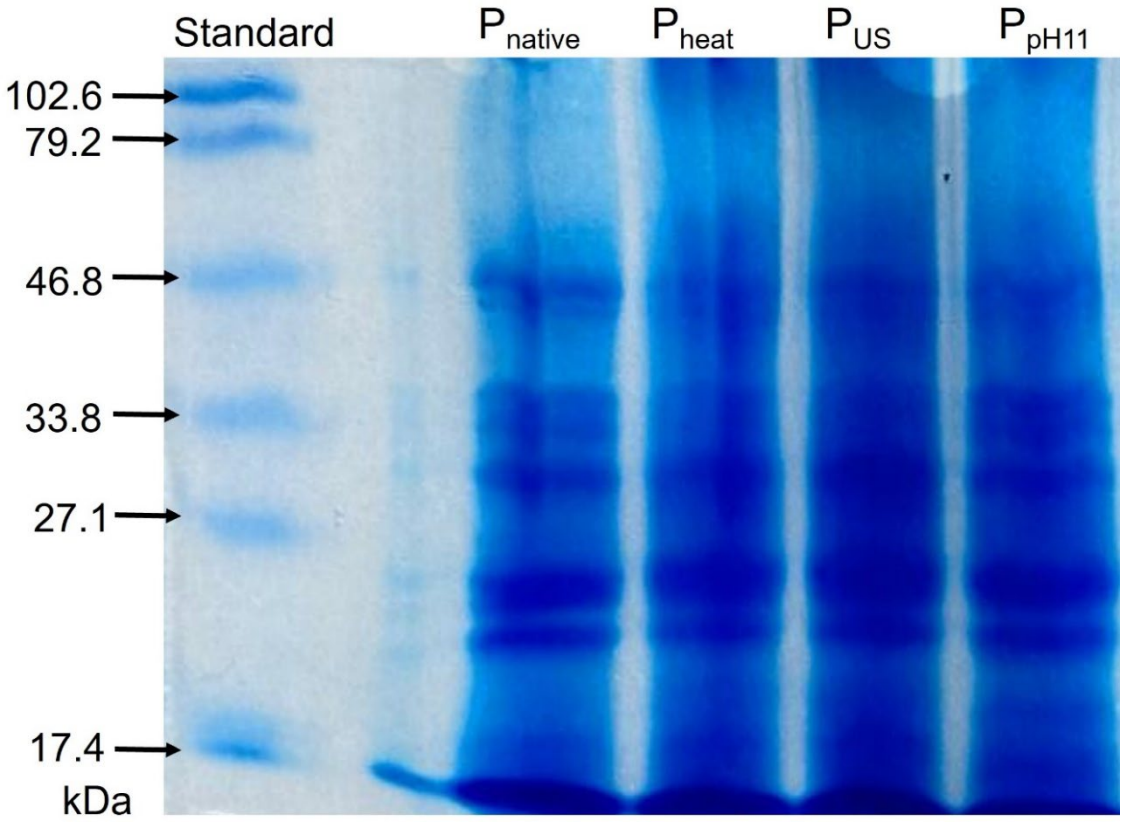

Fig. 7. SDS-PAGE on native and modified huauzontle protein samples. MW standard (kDa).

3.3.4 Protein solubility

The solubility of the native and modified huauzontle protein isolates at pH 7 is given in Table 1. The solubility from highest to lowest was $P_{heat} = P_{US} > P_{pH11} > P_{native}$. Native proteins have limited solubility under neutral pH conditions, which is why they are treated seeking to break hydrophobic interactions and protein-protein aggregates.[28] The improved solubility in heat-treated proteins has been associated with partial unfolding of the protein molecule structure leading to a molten globule state with increased functionality.[29] During unfolding the

polypeptide chains, internal sulfhydryl groups and hydrophobic side chains, which were originally buried in the core of the native protein structure, are exposed.[30] Ultrasound interrupts the secondary, tertiary and quaternary structure of the protein, causing changes in the hydrophobic/hydrophilic regions; decreases particle size increasing the contact surface with water; and dissociates protein aggregates by disrupting non-covalent interactions.[31] pH alkaline conditions lead to the loss of side chain interactions, an increase in the flexibility of the protein molecule, a decrease in particle size and redistribution of the electron cloud, favoring protein solubility.[17]

Table 1. Functional and structural properties of the huauzontle protein isolates native and modified.

	P_{native}	P_{heat}	P_{US}	P_{pH11}
Solubility (%)	36.73 ± 1.00^{c}	87.22 ± 2.19^{a}	86.33 ± 0.44^{a}	68.85 ± 2.68^{b}
β-sheet	49.27 ± 2.27	43.62 ± 2.19	36.23 ± 2.02	27.96 ± 1.91
α-helix	25.15 ± 1.31	46.81 ± 1.89	52.22 ± 3.29	55.37 ± 4.37
β-turn	25.56 ± 1.04	9.55 ± 1.02	11.54 ± 1.16	16.65 ± 1.08
Free sulfhydryl (µmol/g)	3.471 ± 0.06^{c}	3.804 ± 0.18^{bc}	4.395 ± 0.18^{a}	4.190 ± 0.25^{ab}
Emulsifying activity (m^2/g)	11.17 ± 0.08^{c}	13.22 ± 0.18^{a}	12.56 ± 0.09^{b}	12.38 ± 0.06^{b}
Emulsifying stability (min)	61.90 ± 3.03^{c}	90.03 ± 5.46^{a}	74.31 ± 1.35^{b}	77.94 ± 6.03^{b}
Foaming capacity (%)	151.33 ± 4.16^{b}	196.66 ± 3.055^{a}	197.33 ± 8.32^{a}	206.66 ± 8.32^{a}

±Standard deviation. Different letters indicate significant statistical differences ($p \leq 0.05$)

3.3.5 Rheological profile of the heat-induced gelation

The heating-cooling behavior of G' and G'' of the native and modified huauzontle protein isolate are shown in Fig. 8a and b, respectively. The heating (20-90 °C)-

cooling (90-20 °C) profiles for P_{heat} and P_{US} were similar, but different from those of P_{native} and P_{pH11}. With P_{heat} and P_{US}, a decrease in G' and G'' occurred upon initial heating, reaching minimum values at ~ 50-70 °C, due to the weakening of the hydrogen bonds with warming.[7] Subsequently, the values of both moduli increased steadily, due to the unfolding and aggregation of the proteins with further heating.[32] At the minimum, G'' > G', but with G' regained a higher value than G'' at ~ 70 °C. This point can be considered as the gelation point,[32] where a transition from a viscous-like gel to a solid-like gel occurred.[33] On the other hand, P_{native} and P_{pH11} were characterized for having higher values of G' than for G'' over the whole heating range, i.e., these systems were dominated by elastic characteristics from the onset.[34]

Finally, all the protein treatments showed a constant increase in the viscoelastic moduli with cooling, with G' > G'', indicative that the gel structure was strengthened because of van der Waals attraction forces and intra- and inter-molecular hydrogen bonds.[35]

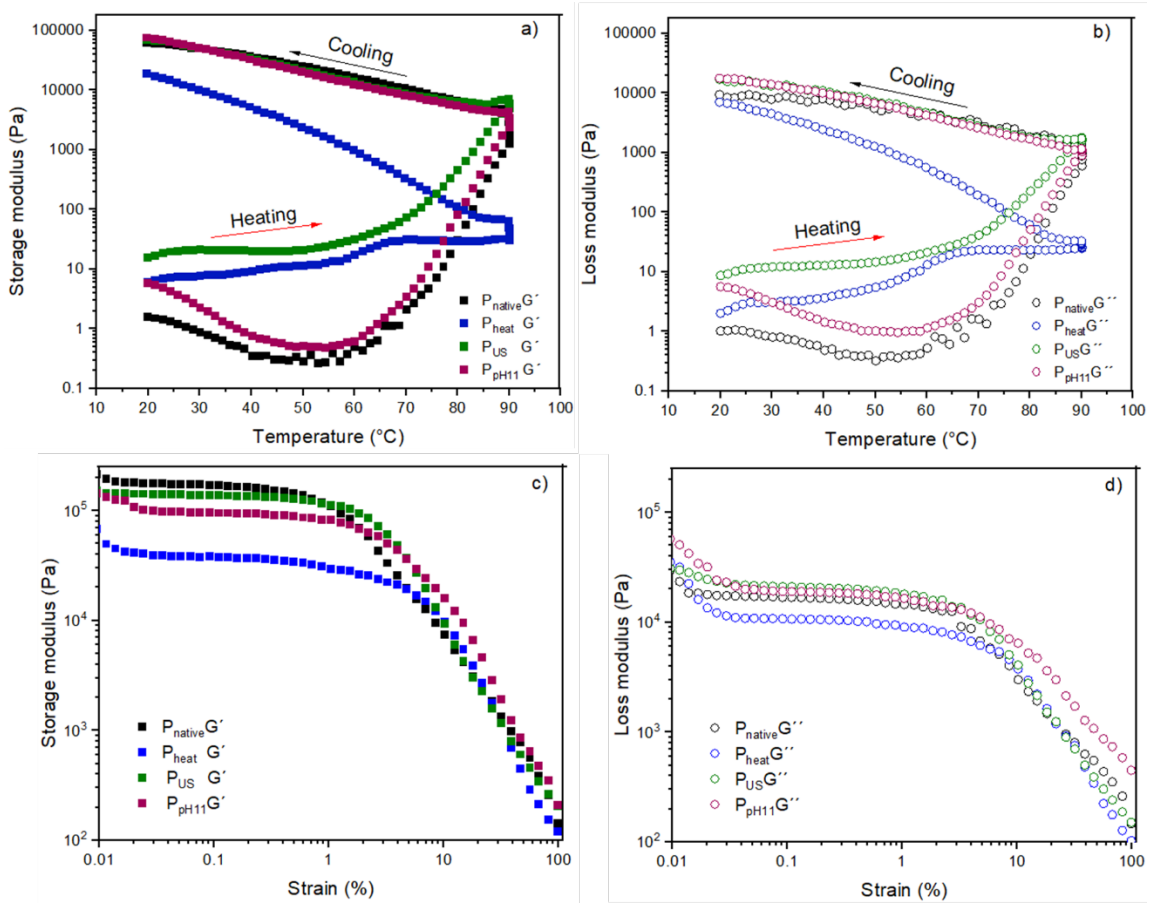

Fig. 8. Effect of a) and b) temperature and strain sweep c) and d) on storage and loss modules in native and modified huauzontle protein at 15 % concentration at pH 7.

3.6 Amplitude sweep tests

The behavior of G' (Fig. 8 c) and G'' (Fig. 8d) as a function of strain for the native and modified huauzontle proteins are shown in Fig. 8c and d, respectively. as a function of strain (%). All the treatments were characterized by a linear viscoelastic region (LVR) at strains below ~ 1%, with G' predominating over G''. The LVR was followed by a nonlinear region characterized by decreasing moduli values. In the LVR the elastic nature dominated over the viscous nature of the proteins.[33] Furthermore, the long LVR is indicative that they were capable of withstanding relative high strain values before their structure was ruptured. Higher G' than G'' values may be attributed to the formation of a more continuous gel network.[32] P_{heat}

presented lower G' values than P_{US} and P_{pH11}, and this behavior could be related to the higher content of α-helix structures that promote the formation of more elastic gels in the latter two than in the former.[36]

3.3.6 Free sulfhydryl groups

The SH content is presented in Table 1. All the treatments tended to increase the content of the free sulfhydryl groups respect that of P_{native}. The content of free sulfhydryl groups was from higher to lower as follows: $P_{US} \geq P_{pH11} \geq P_{heat} \geq P_{native}$. These results are indicative that the three treatments broke-down to a greater or lesser degree the SS bonds of the proteins, which produced an unfolding of the protein structure, exposing buried hydrophobic groups to the surface,[37] and increasing the free sulfhydryl content. The result of this is an increased solubility and improved techno-functionality.[38] These results are in agreement with those found for the molecular weight profile of the huauzontle proteins (Fig. 7), where non-significant changes in the bands were found, indicative that only partial unfolding of the proteins occurred with the different treatments.

3.3.7 Emulsifying ability (EA) and stability (ES)

Proteins are widely used as emulsifiers in food systems, since they can interact at the water-oil interface, forming films that stabilize emulsions. The emulsifying properties of proteins are influenced by the rate at which they adsorb at the interface, the amount of protein present, their ability to rearrange at the interface, and to reduce the interfacial tension.[14] Table 1 shows the EA and ES of the huauzontle protein treatments. EA was from higher to lower as follows: $P_{heat} > P_{US} = P_{pH11} > P_{native}$. Heat treatment favored the exposure of hydrophobic groups, increasing the surface activity of the protein, which allowed its rapid adsorption at the oil droplet surface.[39] pH-shifting caused protein unfolding and refolding, resulting in more soluble, flexible structures with higher surface hydrophobicity, which favored protein adsorption at the interface.[40] Ultrasonication produced

protein unfolding, altered the protein secondary structure, producing an increase in their flexibility, and rearrangement capability at the oil-water interface, increasing the emulsion activity and stability.[41]

ES followed the same trend as EA. Emulsion stability is a complex interrelationship between the intermolecular forces, the intrinsic nature of the molecules, the effect of environmental conditions, the relative concentrations, all of which have bearing on their conformation in solution and at interfaces, affecting emulsion stability.

3.3.8 Foaming capacity (FC) and stability (FS)

The FC of the modified proteins was significantly higher than for P_{native} (Table 1), but non-significantly different among themselves. FC is associated with the ability of the protein to rapidly reach the water-air interface, decrease the interfacial tension, and form a rigid film that protects air bubbles from destabilization processes.[43] pH-shifting causes protein structure to dissociate into lower order structures at extreme alkaline pH, unfolding into relatively flexible individual polypeptide chains with more balanced amphiphilicity, which on return to neutral pH causes protein refolding. This new structural order has been associated with improved FC.[44] Ultrasonication decreases particle size, produces an increased surface hydrophobicity and solubility of the protein molecules, making possible the rapid adsorption of the protein molecules at the water-air interface, thus reducing the interfacial energy and thereby increasing the foamability.[45]

FS values are presented in Table 2. All the foams immediately after formation exhibited 100% stability. After 1 h aging, their stability higher than 90%. High FS is due to structural changes in the protein which foment higher flexibility and solubility, enhancing adsorption at the air-water interface, entrapping rapidly the air molecules.[34] On the other hand, P_{native} and P_{heat} showed significant differences after 40 min of aging. This could be due to gravitational and to Ostwald ripening drainage processes.[46] These results coincide with those reported for foam made

with native huauzontle protein isolate.[7] The highest overall FS with aging was displayed by P_{pH11}, it possessed the highest ability to protect bubbles against coalescence and disproportionation.[34] Higher foam stability is attributed to the formation of a thick, cohesive layer around the air bubbles, which strong viscoelasticity.[45]

Finally, Fig. 9 shows micrographs of the foam's morphology with aging time. P_{native} and P_{heat} presented a more marked foam coarsening than P_{pH11} and P_{US} after 1 h of aging.

Table 2. Foam stability at different times in native and modified protein.

	Time (min)					
	0	1	5	10	40	60
P_{native}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	94.71 ± 0.14^{b}	92.51 ± 0.74^{c}
P_{heat}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	98.98 ± 0.1^{ab}	96.94 ± 0.4^{bc}	94.57 ± 0.60^{c}	91.20 ± 2.26^{d}
P_{US}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	99.67 ± 0.56^{a}	97.66 ± 1.10^{b}
P_{pH11}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	100.00 ± 0.1^{a}	98.10 ± 1.85^{a}

±Standard deviation. Different letters indicate significant statistical differences ($p \leq 0.05$)

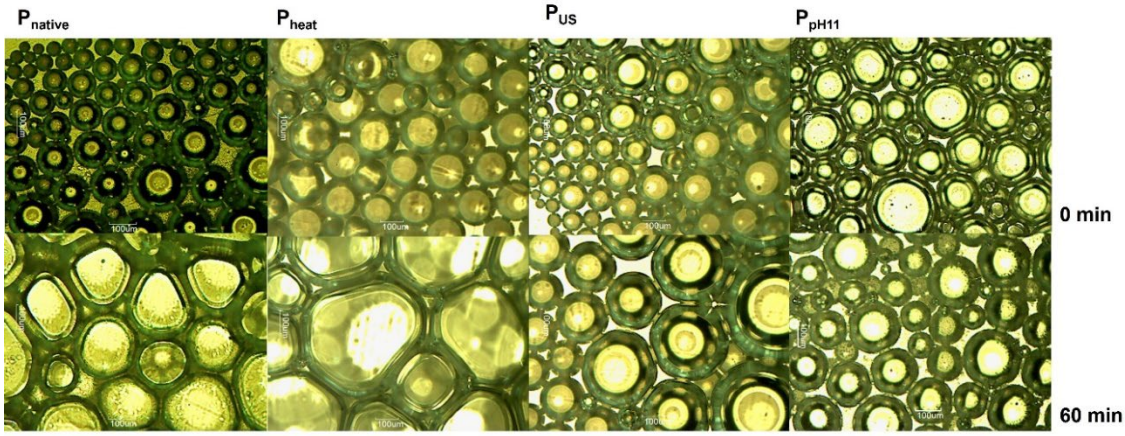

Fig. 9. Micrographs of foam stability at time 0 and after 60 min, in native and modified huauzontle protein.

3.4 Conclusions

This work focused modifying huauzontle native protein using heat, ultrasonication and pH-shifting techniques, and evaluating the effect on the physicochemical, structural, and functional properties. All the treatments induced changes to a different extent in the secondary structure of the proteins, and consequently, had different effect on properties. All the treatments produced an increase in the relative proportion of α-helix structures at the expense of β-sheet and β-turn structures, on the unfolding of proteins, the surfacing of buried functional groups, which promoted new protein-protein interactions, contributing to an improvement of their functional properties, which included smaller particle size, higher solubility, enhanced rheological properties, increased emulsifying and foaming properties and stability. This work expands the knowledge of huauzontle protein and promotes its utilization as a novel food ingredient.

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4 MODIFIED HUAUZONTLE (*Chenopodium nuttalliae* Saff.) PROTEIN-OCTENYL SUCCINIC ANHYDRIDE CORN STARCH SOLUBLE COMPLEXES: STRUCTURAL FEATURES AND *IN VITRO* PROTEIN AND STARCH DIGESTIBILITY

Abstract

Native huauzontle protein (P_{native}) structure was modified by thermal (P_{heat}), ultrasound (P_{US}) and pH shifting (P_{pH11}). Soluble complexes (SC) were formed between the huauzontle proteins and a commercial octenyl succinic anhydride modified corn starch (S). The secondary structure of the native and the modified huauzontle proteins differed in terms of abundance of β-sheet, α-helix, and β-turn structures. The pH at which maximum presence of soluble complexes was observed varied from 5.1 for P_{native}-S to 5.8 for P_{us}-S, and being the required protein: polysaccharide weight ratio of 1:1 for P_{native}-S and of 1:2 for the modified proteins-S. FTIR confirmed the formation of SC, as the characteristic carboxylate group in S disappeared from their spectra. Also, the absorption peaks of the proteins in the Amide I and II bands shifted slightly from 1630 to 1635 cm^{-1} and 1520 to 1510 cm^{-1} in the SC, indicating conformational changes due to protein-starch interactions. Aggregation of the biopolymers into large particles was observed by SEM in the SC made with modified proteins. SC showed significant differences in the *in vitro* protein digestibility values, the lowest (85.1 ± 2.3%) occurring for P_{native}-S and the highest (92.7 ± 0.7%) for P_{US}-S. P_{pH11}-S presented the highest resistant starch content (92.6 ± 0.4%). It is concluded that the modification of P_{native} through heat, ultrasound and pH shifting allowed establishing different driving conditions for the formation of SC with S, and *in vitro* digestibility of protein and starch could also be modified.

Keywords: modified huauzontle protein, modified corn starch, soluble complexes, protein, and starch digestibility.

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4.1 Introduction

The increasing world population, coupled to several factors such as climate change, water scarcity and changing dietary patterns, among others, are imposing growing food security challenges [1]. In the years to come, meeting the growth in the demand of food will require of new strategies for increasing the production and access of foods to households [2]. To achieve this, the availability of food sources or ingredients, with appropriate nutritional and functional properties, must be ensured [3]. Proteins and polysaccharides are two of the main components found together in foods, and are responsible for providing their structure, texture, and stability [4].

Proteins from animal origin are considered to have superior nutritional quality and technological functionality than proteins from vegetable sources [5]. However, animal protein production is expensive and has a negative environmental impact [6]. This has led researchers to seek renewable and sustainable alternatives that may supply the nutritional components necessary for human health [7]. Plant proteins have been found to be capable of providing three-dimensional assemblies that allow food technologists the flexibility to develop new food products with appropriate nutritional quality, and which are more readily available and have lower costs than animal proteins [8]. Thus, an ongoing research topic is to explore the obtention of high-quality proteins from non-conventional sources such as pseudocereals [9]. Such is the case of huauzontle (*Chenopodium nuttalliae* Saff), a native plant species from Mexico that has a high protein content (20%), with an essential amino acid content that exceeds the standard recommendation and does not contain gluten [10]. However, there is much research to be done with plant proteins, as they have limited applications in the food industry in their native state, due their poor functional properties. An interesting option is to modify plant protein structural characteristics through diverse treatments, including heat treatment, pH change, ultrasonication and complexation with other molecules, allowing to improve their functional properties and define their possible final application in food [11, 12].

Starch is available and inexpensive but is little used in its native state in the industry due to limited functional properties [13]. Physical, chemical, and enzymatic modifications of starch have allowed to improve some of its functional characteristics (water solubility, hydrophobicity, resistance to digestion, etc.) [14]. Thus, it has been reported that octenyl-succinic anhydride (OSA)-modified starch displays enhanced amphiphilic characteristics, imparting more desirable properties to, e.g., bakery products [13].

The interactions between proteins and polysaccharides have been increasingly explored in the recent last years as the means for developing novel food structures with improved functionality [15]. The interactions between proteins and polysaccharides give rise to different types of colloidal systems including foams, nano emulsions, hydrogels, films, and microcapsules among others [16, 17], with unique structural features and properties [18]. These protein-polysaccharide complexes tend to exhibit better functional properties than that of the proteins and polysaccharides alone [19]. However, there are scarce studies regarding the digestibility of these complexes *per se.*

Based on the above, the objective of this work was threefold: (i) to establish the most suitable conditions (pH, relative charge density and weight ratio of biopolymers) driving the formation of soluble complexes between native and modified (thermal, ultrasonication, and pH shifting) huauzontle protein and a commercial octenyl succinic anhydride modified corn starch; (ii) to evaluate the structural features and morphology of the soluble complexes, and to (iii) assess the *in vitro* protein and starch digestibility of the soluble complexes.

This work is expected to shed light on the effect of protein structure modification on the formation conditions and *in vitro* digestibility of proteins and polysaccharides of soluble complexes composed of huauzontle protein-octenyl succinic anhydride modified corn starch, hoping that these results may contribute to foment the application of soluble protein-polysaccharide complexes as functional food ingredients.

Keywords: modified huauzontle protein, modified corn starch, soluble complexes, protein, and starch digestibility.

4.2 Materials and methods

4.2.1 Materials

Huauzontle (*Chenopodiun nuttalliae* Saff) seeds were obtained from a local producer from Tepoztlan (18°59´ 7´ N, 99°5´59´´ W, elevation above sea level of 1706 m), State of Morelos, Mexico. An octenyl succinic anhydride (OSA)-modified waxy corn starch (S; Purity Gum ® Ultra 32236110; low viscosity starch, soluble in cold water) was donated by Ingredion Mexico S.A. de C.V. Hydrochloric acid (HCl) and sodium hydroxide (NaOH) of analytical grade were purchased from J.T. Baker (Xalostoc, State of Mexico, Mexico). The enzymes amyl glucosidase from *Aspergillus niger* (A7095, 260 units mL^{-1}), porcine pancreatic α-amylase (A3176, type VI-B, ≥5 units mg^{-1}), glucose oxidase/peroxidase reagent (GOPOD kit; G3660), and 3,5-dinitrosalicylic acid (DNS; 128848) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and used for the protein digestibility assay. In all the experiments distilled and deionized water (DDW) was used. The graphs were made with the statistical package SigmaPlot version 12.0.

4.2.2 Huauzontle protein isolation

Huauzontle protein was isolated according to the method proposed by López-Monterrubio et al. (2020). The seeds were ground in a food processor (Nutribullet™, NB-101B, Hong Kong Ltd., Guandong, China) and subsequently passed through a No. 80 mesh sieve. The huauzontle seed flour was defatted with hexane in Soxhlet equipment for 3 h and left to stand for one hour at room temperature (25 ± 1°C) for evaporating the residual solvent completely. The defatted flour was dispersed in DDW (1:10 w/v) and pH was adjusted to 9 with 1 M NaOH (pH-Meter H1221, Hanna Instruments, Mexico City, Mexico). The resulting suspension was stirred for one hour at room temperature and centrifuged at 9200 ×g at 10 °C for 10 min (Centrifuge 5810 R, Eppendorf, AG,

Hamburg, Germany). The supernatant was recovered, and the pH was adjusted to 4.3 (isoelectric point of huauzontle protein) with 3 M HCl, kept in agitation for one hour and centrifuged at 9200 ×g at 4 °C for 10 min. The precipitated protein was washed thrice with DDW in a 1:10 ratio to remove the excess of NaOH. Finally, protein was dried to constant weight in a hot air oven HCF-62 (Riossa Digital, Mexico City, Mexico) at 35 °C for 24 h. The protein was manually ground and stored under refrigeration for subsequent analysis and coded as P_{native}.

4.2.3 Stock solutions

P_{native} was dispersed in DDW (1% w/w) at room temperature, pH was adjusted to 7 by adding 0.1 M NaOH or 0.1 M HCl, magnetically stirred for 2 h, and finally stored at 4 °C for 12 h to ensure complete protein hydration. The protein content in the stock solution was determined by the Lowry method as modified by [20], and it was 0.95 ± 0.01g per 1.0g. An S solution (1% w/w) was prepared by gentle stirring for 1 h just before the time of use to avoid syneresis and pH was adjusted to 7 by adding 0.1 M NaOH or 0.1 M HCl [21].

4.2.4 Protein modification process

P_{native} was subjected to three different treatments to modify its structure: (a) ultrasonication (P_{US}) using an ultrasound device (Sonics and Materials, Inc, Newtown, CT, USA), equipped with an 8 mm diameter stainless steel probe. A P_{native} stock solution was processed at 500 W for 10 min, (50% amplitude and frequency of 20 kHz; pulse duration 5 min on, 5 min off), using an ice bath to prevent heating of the protein. It has previously been reported that ultrasound treatments at a frequency of 20 kHz and a power of 500 W generate an ultrasonic intensity of 43 W/cm^2 [22]; (b) thermal processing (P_{heat}) by subjecting a P_{native} stock solution to 70 °C for 20 min; and (c) pH shifting (P_{pH11}) where the pH value of the a P_{native} stock solution was adjusted at pH 11 with 0.5 M NaOH. [10] reported that the maximum solubility of the huauzontle protein is achieved at pH 11. The alkaline solution was stirred for 1 h at room temperature (25 ± 1 °C) and pH was adjusted to 7.0 with 1 M HCl. Each treated huauzontle protein solution was

centrifuged at 1238 ×g for 30 min to eliminate the insoluble protein present and dried in an oven at 35 °C for 24 h.

4.2.5 ζ-potential and particle size of the biopolymers

The ζ-potential and particle size (d_h: hydrodynamic diameter) measurements of the P_{native} and modified proteins and S was performed with a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, Worcestershire, UK) [23]. Stock solutions were diluted with DDW to a concentration of 0.05% w/w and ζ-potential measured in a pH range of 3-7 at 25 °C. The pH of the solutions was adjusted with 0.1 M NaOH or 0.1 M HCl. The ζ-potential was determined by measuring the direction and velocity of biopolymer scattering as it moved along the applied electric field. The computer software converted the electrophoretic mobility measurements into ζ-potential values using Smoluchowsky's mathematical model. Stock solutions were kept refrigerated (4 °C) for 24 h before any measurement.

4.2.6 Huauzontle protein-S soluble complexes formation

In order to obtain a general picture for the complexation phenomena between huauzontle protein and S, the impact of the protein:S weight ratio at a given pH value was evaluated using absorbance measurements: (1) stock solutions of huauzontle protein and S were mixed in protein-S weight ratios of 1:0.25, 1:0.50, 1:1, 1:2 and 1:4, while always keeping the total biopolymer concentration of 1% w/w, by gently stirring of the mixtures for 1 h at room temperature; (2) at a given protein:S weight ratio, the pH of the mixtures was adjusted to different pH values 2-7 with 0.5 units increments followed by 24 h of equilibration at 4 °C; (3) the mixtures were centrifuged at 251 ×g at 10 °C for 30 min (Centrifuge 5810 R, Eppendorf, AG, Hamburg, Germany), in order to separate the insoluble huauzontle protein-S complex coacervate (CC) from the soluble complexes (SC) in the supernatant; (4) the absorbance of the supernatant was measured at 600 nm using a spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA) due to at this wavelength the huauzontle protein-S soluble complexes showed

significant higher absorbance than the solutions of either individual biopolymer. The optimum weight ratio and pH (pH_c) at which soluble complexes formation occurred between the huauzontle proteins and S was the point where the supernatant exhibited maximum turbidity (absorbance) due to the presence of soluble complexes. Samples of the supernatants were dried at 35 °C in a convection oven until constant weight was achieved.

4.2.7 ζ-potential and particle size of the SC

Fresh SC were prepared at the weight ratios and pH values determined as optimum (sub-section 2.6.). Samples were diluted at 0.05% w/w and ζ-potential and particle size were measured as indicated in sub-section 2.5.

4.2.8 Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra of the native and modified huauzontle protein, S and SC were obtained with a Frontier FT-IR/FIR spectrophotometer (Perkin Elmer, Waltham, MA, USA) equipped with a universal diamond crystal ATR sampling attachment [23]. Approximately 0.2 g of sample was placed and pressed with the ATR head. Measurements were obtained over a range of 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}. Forty scans per sample were taken and integrated to obtain the mean values of the spectra. To assess the huauzontle protein secondary structure Fourier self-deconvolution and second derivative analysis was performed, and the peaks were fitted in the amide-I region band (1700–1600 cm^{-1}) with the equipment software. Based on their center the Gaussian peaks could be assigned to their corresponding structure and the integral of each was divided by the sum of all determined peaks to identify the proportion (%) of each structure [24].

4.2.9 Morphology of the soluble complexes

Morphology of the SC was observed by Optical Microscopy and Scanning Electron Microscopy (SEM), as follows. SC samples were observed at a magnification of 100× with an optical microscope (Olympus BX53, Olympus Optical Co., Tokyo, Japan) coupled to an AxioCam ERc 5 s camera (Carl Zeiss

Microscopy GmbH, Oberkochen, Germany) and an image analyzer system (Motic Images Plus 2.0 software, Motic, Corp., Ltd., Chengdu, China). To confirm S and huauzontle protein presence in the SC, the samples were stained with potassium iodide and rhodamine (0.2% w/v) [25].

Samples of each SC were placed on the surface of aluminum supports with carbon adhesive tape and coated with gold for 5 min at 1.5 kV, 56 mA (JFC 1100 vacuum sputter coater, JEOL, Tokyo, Japan) and observed at a magnification of 5000× with a scanning electron microscope (JSM6360, JEOL, Tokyo, Japan) at 13 kV (Cuevas-Bernardino [26].

4.2.10 *In vitro* starch digestibility

In vitro digestibility of S alone and in the huauzontle protein-S complexes was determined following [27] procedure with modifications. Briefly, pancreatic α-amylase (0.0225 g per 10 U.mg^{-1}) was dispersed in 7.5 mL of acetate buffer (0.02 M) at pH 5.5, shaken for 30 min and centrifuged at 1500 $\times$g for 5 min. The supernatant was mixed with 0.75 mL of amyloglucosidase (300 U. mg^{-1}). The enzyme solution was prepared at the time of use. 10 mL of sodium acetate buffer (0.02 M, pH 5.5) was added to S and SC samples (200 mg). The samples were equilibrated at 37 °C for 30 min, then the enzyme solution (0.75 mL) was added; aliquots of 0.5 mL were taken after 20 and 120 min of hydrolysis and centrifuged at 5000 $\times$g for 1 min. Glucose content in the supernatant was measured by the dinitrosalicylic acid (DNS) method as follows: 200 µL were taken from the supernatant, 0.8 mL of distilled water and 1 mL of DNS were added and brought to a boil for 10 min. To stop the reaction, the tubes were placed in an ice bath. Finally, the absorbance was read at 540 nm in the spectrophotometer. The percentage of hydrolyzed starch in S and the SC was calculated by multiplying by a factor of 0.9 to change the values to glucose. The digested starch fractions were classified as: rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS).

Each fraction was calculated using the following equations.

$$RDS\ (\%) = \frac{(G_{20} - FG) \times 0.9}{TS} \times 100 \quad (5)$$

$$SDS\ (\%) = \frac{(G_{120} - G_{20}) \times 0.9}{TS} \times 100 \quad (6)$$

$$RS\ (\%) = 100 - RDS - SDS \quad (7)$$

Where FG is the free glucose content, TS the total starch content and G20 and G120 content of glucose released after 20 and 120 min of hydrolysis.

4.2.11 *In vitro* protein digestibility

Digestibility of the native (P_{native}) and modified (P_{heat}, P_{us} and P_{pH11}) huauzontle proteins, as well as of the proteins in the SC, was determined by the multienzymatic method of [28]. Porcine pancreatic trypsin (type IX, 15,310 units·mg^{-1} protein), pancreatic coil b chymotrypsin (type II, 48 units·g^{-1} of solid), porcine intestinal peptidase (P-7500, 115 units mg^{-1} of solid) and bacterial protease (type XIV, 4.4 units·mg^{-1} of solid) (Sigma-Aldrich Co., St. Louis, MO, USA) were used for the enzymatic digestion. Samples (63.8 mg) were added with 10 mL of DDW and pH adjusted to 8.0 with 1 M NaOH. One mL of the aqueous enzyme solution (1.58 mg of trypsin, 3.65 mg of chymotrypsin and 0.45 mg of peptidase) was added to the sample and digestion was allowed to proceed for 10 min at 37 °C. Afterwards, 1 mL of bacterial protease solution (1.48 mg) was added, and the digestion was continued for 9 min at 55 °C. The pH value was recorded after an additional 1 min and used to estimate *in vitro* protein digestibility according to the following equation:

$$Y = 234.84 - 22.56X \quad (8)$$

Where, Y is the *in vitro* digestibility of the protein (%), and X is the pH of the suspension after 20 min of digestion.

4.2.12 Statistical analysis

All analyses were conducted in triplicate from three independent experiments performed using a randomized experimental design and values were expressed as mean values ± SD. Data were subjected to simple classification analysis of

variance and in pertinent cases to Tukey's mean comparison analysis. Significance was established at $p \leq 0.05$. Data analysis was performed with the statistical package SAS version 9.4 (SAS Institute Inc., NC, USA).

4.3 Results and discussion

4.3.1 ζ-potential and particle size of individual biopolymers

The native and modified huauzontle proteins (Fig. 10) exhibited different isoelectric point (pI) values (**ζ** = 0 mV), which ranged from a pH value of about 4.3 for P_{native} to a pH of about 4.9 for P_{heat} and P_{US}. All the protein treatments exhibited a positive **ζ** value below the pI, which increased as the pH tended towards a value of 3. Likewise, for pH values above the pI of the proteins, a negative **ζ** value was displayed which became more pronounced as the pH tended to a value of 7. The changes in the **ζ** values as a function of pH are due to the presence of the functional groups of the protein. When the pH is lower than the pI the amino groups ($-NH_2$) are protonated giving rise to ammonium groups ($-NH_3^+$). On the other hand, negative values occur when the pH is higher than the pI, since the carboxylic acid groups (-COOH) are ionized giving rise to carboxylate groups ($-COO^-$). Above the isoelectric point the modified proteins presented significantly higher positive **ζ** values than those exhibited by the native protein. The greater number of positive charges in the modified proteins could be attributed to the unfolding of the polypeptide chains and exposure of ionizable groups caused by the treatments applied [29], [30]. In contrast, S presented negative **ζ** values whose magnitude increased as pH increased, due to the ionization of the carboxylic groups (-COOH) to form carboxylate groups ($-COO^-$) [31].

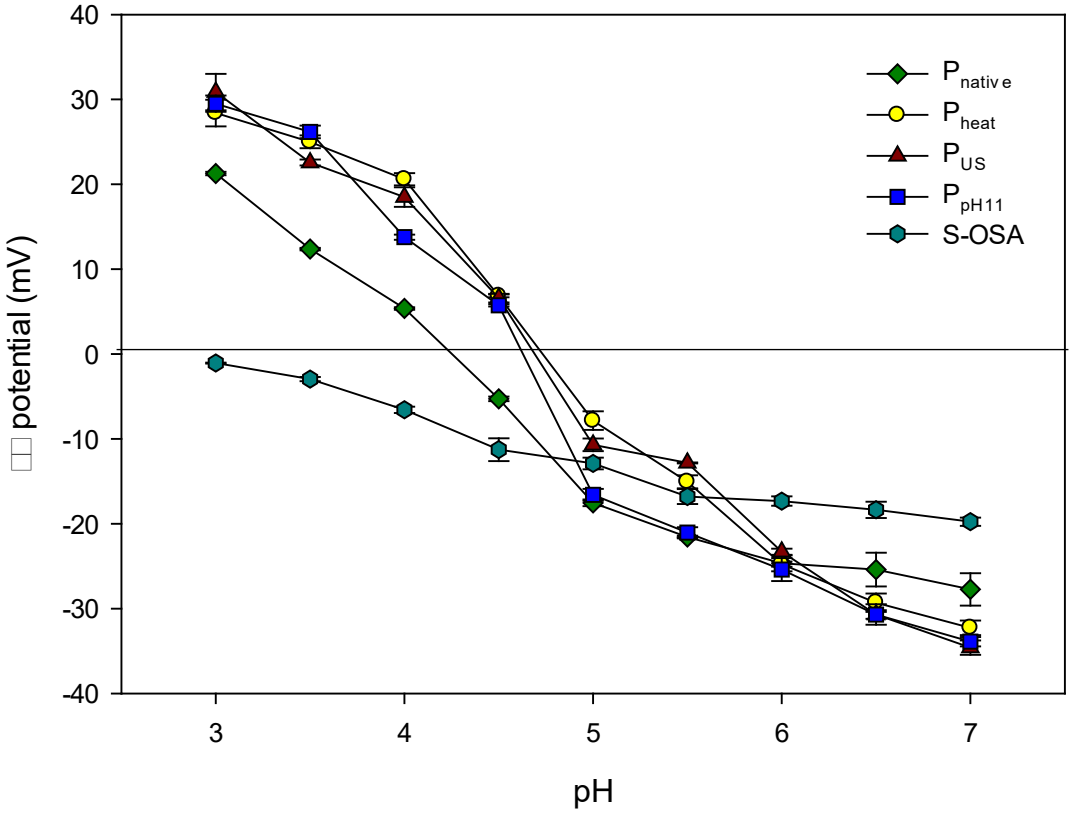

Fig. 10. Shows the ζ-potential of the native and modified huauzontle proteins and S.

Heat, ultrasound, and alkaline pH shifting treatments yielded huauzontle modified proteins with significantly smaller hydrodynamic diameter (nm), than that of the native protein, varying as follows: P_{native} (198.0 ± 13.7) > P_{pH11} (128.9 ± 4.5) > P_{heat} (94.0 ± 2.5) = P_{us} (86.5 ± 1.5) (Fig. 11). Reduction in the hydrodynamic diameter of P_{pH11} could be explained by a combined effect of an increase in the ionic strength and accelerated hydrophobic interactions [32]. Smaller particle size resultant of alkaline pH shifting treatment also was observed for ginkgo protein by [30], attributing it to refolding of the protein structure stabilized by intermolecular disulfide bonds. P_{heat} and P_{us} had the smallest hydrodynamic diameters. It has been informed that protein heating at temperatures above of 70 °C generates a more disordered and unfolded structure, resulting in changes in their secondary, tertiary, and quaternary structure [33]. Increases in solubility and decreases in

particle size for heating treated proteins have been associated to decreases in inter- and intramolecular hydrophobic interactions, hydrogen bonding, and disulfide bonds [34, 35]. The smaller ($p \leq 0.05$) hydrodynamic diameter of P_{us} respect to P_{native} and P_{pH11} could be due to the breaking of molecular bonds and the disruption of associative hydrophobic and electrostatic protein-protein interactions caused by the shear waves, shock waves and microflow produced during cavitation phenomenon [36, 37]. Similar results were reported by [38] for sonicated flaxseed protein, where turbulence and high shear forces occurring during cavitation caused the breakdown of protein molecules into smaller molecules.

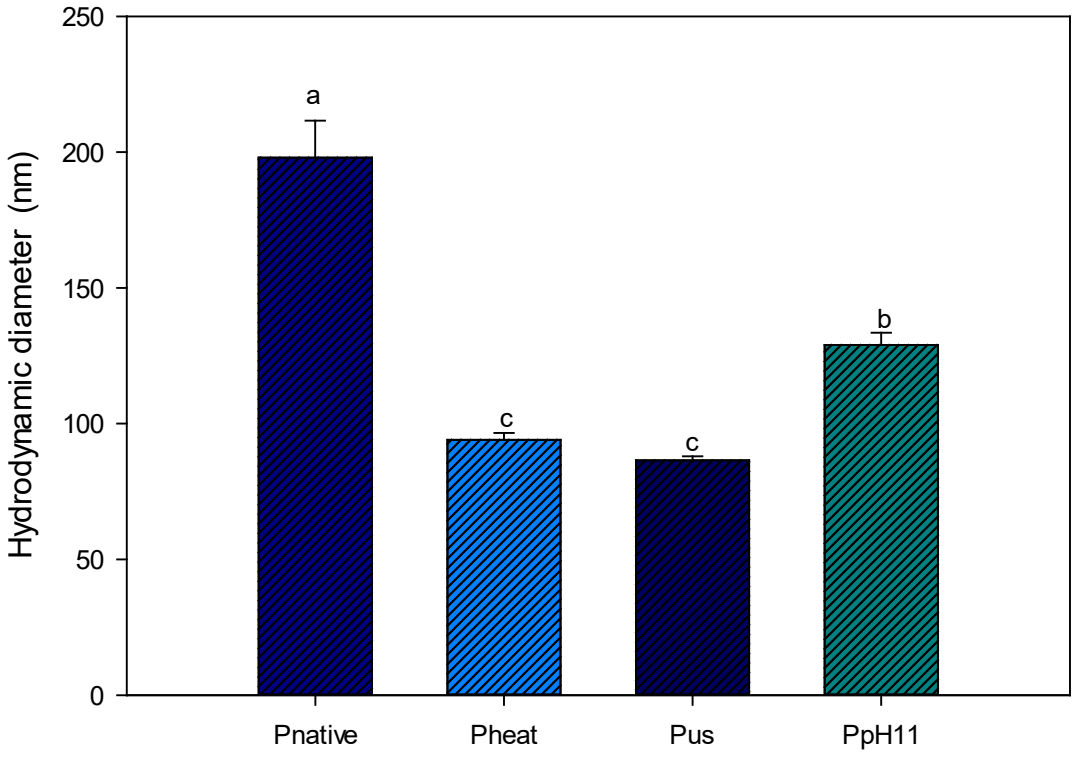

Fig. 11. Hydrodynamic diameter of huauzontle proteins: P_{native}, P_{heat}, P_{US} and P_{pH11}.

4.3.2 Fourier transform infrared spectroscopy of individual biopolymers

Fig. 12 shows the absorption spectra of P_{native}, P_{heat}, P_{pH11}, P_{US}, and S. Five main peaks were observed in the huauzontle protein spectra: Amide A, representative of NH stretching in the band of 3300-3100 cm^{-1}; Amide B, representative of symmetric and asymmetric vibrations of the groups $–CH_2$ and $–CH_3$ in the 2930-2860 cm^{-1} band; Amide I, representative of C = O stretching and NH bending in the 1635 cm^{-1} band; Amide II, representative of N-H bending and C-N stretching in the 1510 cm^{-1} band; and Amide III (1385 cm^{-1}) due to combination of N–H bending vibration and C–N stretching vibration with minor contributions from CO in plane bending and CC stretching vibration [10]. In addition, the band at 1452 cm^{-1} has been related to the bending of the O-H bond of the O-H carboxyl groups or the absorbance of acidic amino acids such as glutamic acid [39].

A change in the intensity of the peaks was observed in the modified proteins with respect to the P_{native}, in amide I, II, and III. [40] observed a change in the absorption peak of amide I in sonicated quinoa protein, which was attributed to the partial alteration of the carbonyl and hydroxyl group of the protein during modification. [41] mentioned that this behavior indicates a change in the secondary structure of the protein.

The region of 1500-400 cm^{-1} is considered the fingerprint of starches, and the changes registered in this region provide information about alterations in its polymeric structure and conformation [42]. The characteristic peaks for starches have been previously described: 1405 cm^{-1} assigned to $–CH_2$ bending and –COO stretch; 1040 cm^{-1} and 1015 cm^{-1} assigned to the crystalline and amorphous regions of starch, respectively; 1148 cm^{-1} due to vibrations of the glycosidic C-O-C bond; 930 cm^{-1} assigned to vibrations of skeletal glycoside bonds (α 1 → 4), and 759 cm^{-1} assigned to C-C stretch [42]. The peak found at 1570 cm^{-1} corresponds to the asymmetric vibration of the carboxylate group due to the OSA group of the modified starch [30].

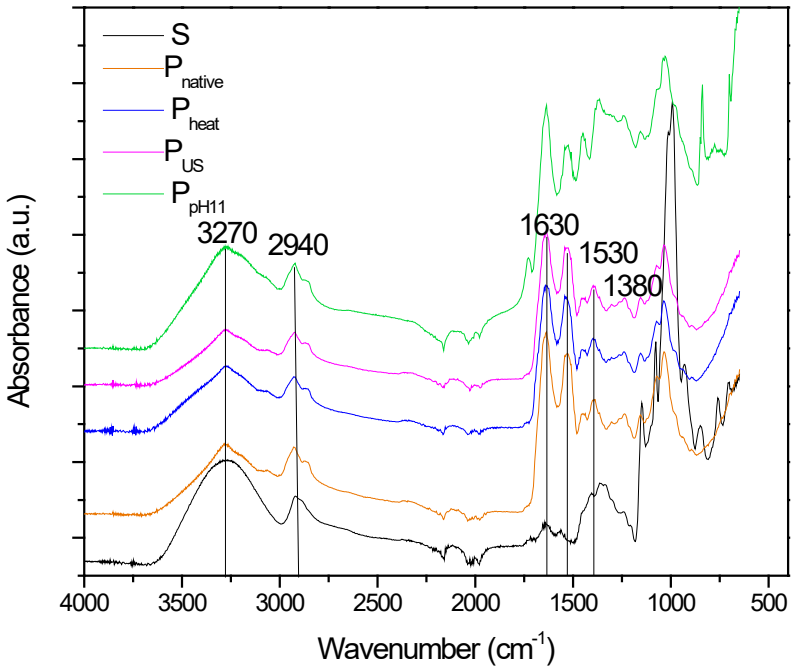

Fig. 12. FT-IR spectra of octenyl succinic anhydride-modified waxy corn starch (S) and huauzontle proteins (P_{native}, P_{heat}, P_{US} and P_{pH11}).

4.3.3 Secondary structure of the huauzontle protein

Deconvolution of the secondary structure in the region 1700 to 1600 cm^{-1} of amide I was performed and Gaussian peaks of the huauzontle proteins could be assigned to their corresponding structure based on their center: β-sheet (1640-1610 cm^{-1}), α-helix (1660-1650 cm^{-1}), and β-turn (1690-1660 cm^{-1}) [43]. Fig. 13a shows the percentages of each of the structures identified in the huauzontle proteins. Fig. 13b illustrates the deconvolution of the Amide I band by means of Gaussian functions. Three functions sufficed to describe accurately the experimental data. Significant changes on the secondary structures of the P_{native} (β-sheet, 33.56%; α-helix, 37.33%, and β-turn, 29.09%) were produced when heat, ultrasound and pH shifting were applied. The content of β-sheet and α helix structures decreased significantly in the P_{heat} and P_{US} treatments with respect to their native counterpart while the β-turn structure had a significant increase in both

treatments. [44] and [45] reported that β-sheet decreased in soy protein isolate upon heat application due to denaturation of 7S globulin. [46] suggested that sonication disrupts hydrogen bonds causing α-helix structures to become β-sheet, β-turn or random coil structures. [47] referred that ultrasound can cause unfolding of proteins, affecting their conformation by breaking hydrogen bonds and intermolecular hydrophobic interactions of α-helix structures thus improving the flexibility of the protein molecule. [48] suggested that the decrease in α-helix content is due to denaturation of protein molecules after heating, and that the increase in β-sheet content indicates rearrangement and reassociation of the denatured molecules to more stable structures.

On the other hand, treatment at alkaline pH showed a reduction of approximately 72% in the content of β-turn structure with respect to P_{native} and a high content of β-sheet structures. [49] determined that proteins undergo conformational changes when subjected to sonication and pH treatments, causing α-helical structures to shift towards β-sheet conformations. Alternatively, [50] determined that this type of behavior is due to the strong electrostatic repulsion between the charged amino acid side chains causing the hydrogen bonds to break and the structure to split. According to the results obtained in the present investigation, it can be inferred that the methods used to modify the secondary structure of the protein had significant effects.

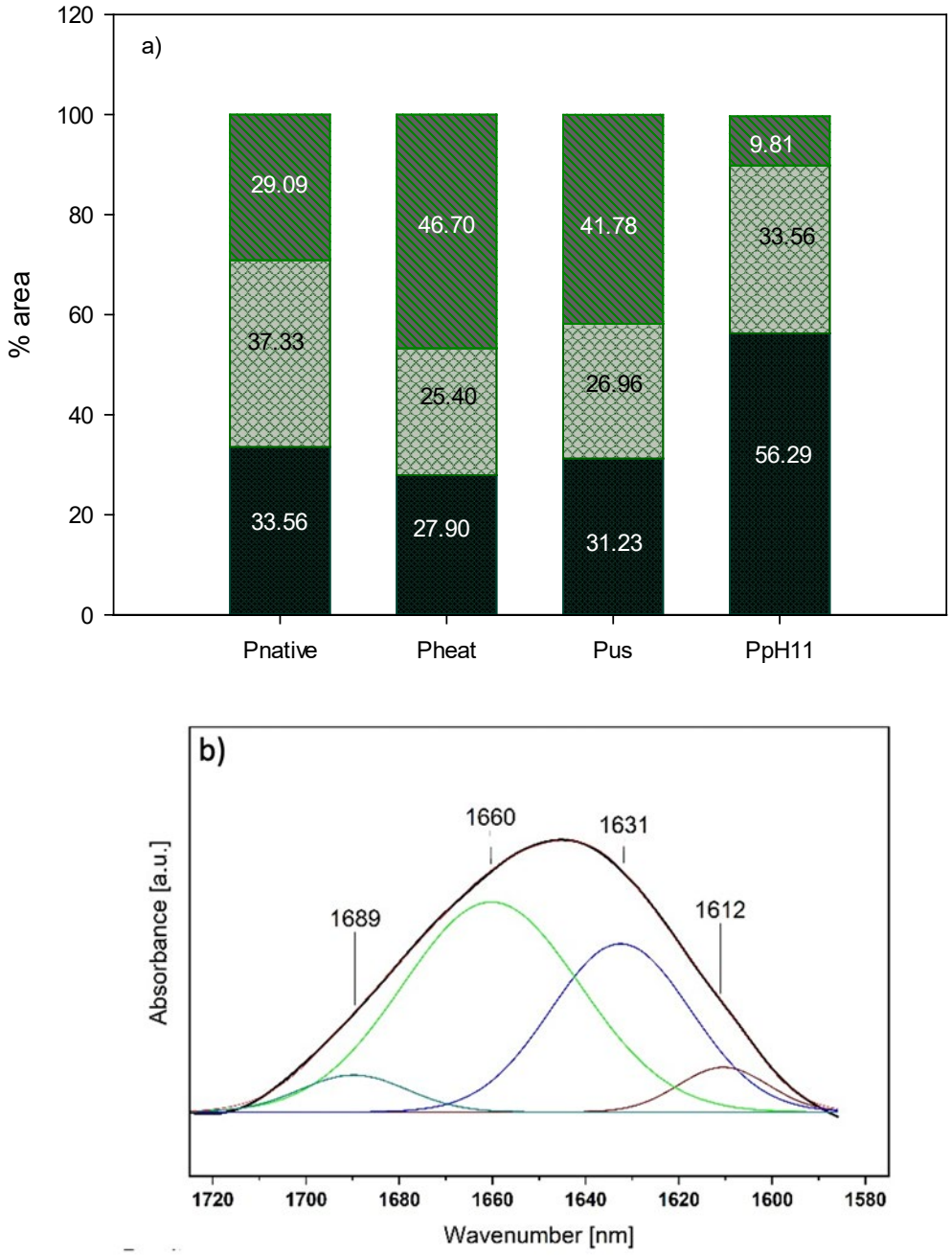

Fig. 13. (a) Secondary structure of huauzontle proteins: P_{native}, P_{heat}, P_{US} and P_{pH11}. (b) Illustration of the Gaussian deconvolution of the FTIR signal in the Amide I region.

4.3.4 Morphology of the huauzontle proteins

The native and modified huauzontle protein (Fig. 14) presented a structure in the form of thin, irregular, disordered sheets with rough edges similar to that reported for amaranth and quinoa protein by [51]. However, the modified proteins presented smaller structures than P_{native}, with P_{heat} and P_{US} that agree with the results shown in sub-section 3.1. The smaller sizes produced can be attributed to the action of cavitation, turbulent forces and the microflow caused by ultrasound, which has a mechanical effect on the surface of the biopolymers, causing the protein to break, reducing its size, and producing flatter surfaces [40]. The formation of smaller, irregular, and granular structures in the heat-modified protein could be due to depolymerization of the protein due to the breaking of chemical bonds caused by heat treatment, resulting in the formation of smaller molecular weight proteins [52]. The modification under alkaline conditions favors the unfolding of the protein molecule by increasing the exposure of free hydrophobic and sulfhydryl groups, the increase in intermolecular force on the protein surface induces tighter conformations which leads to smoother structures [32, 53]. It should be noted that small spherical bumps were observed on the surface of the native and modified proteins, which has previously been associated with the presence of globulins [54].

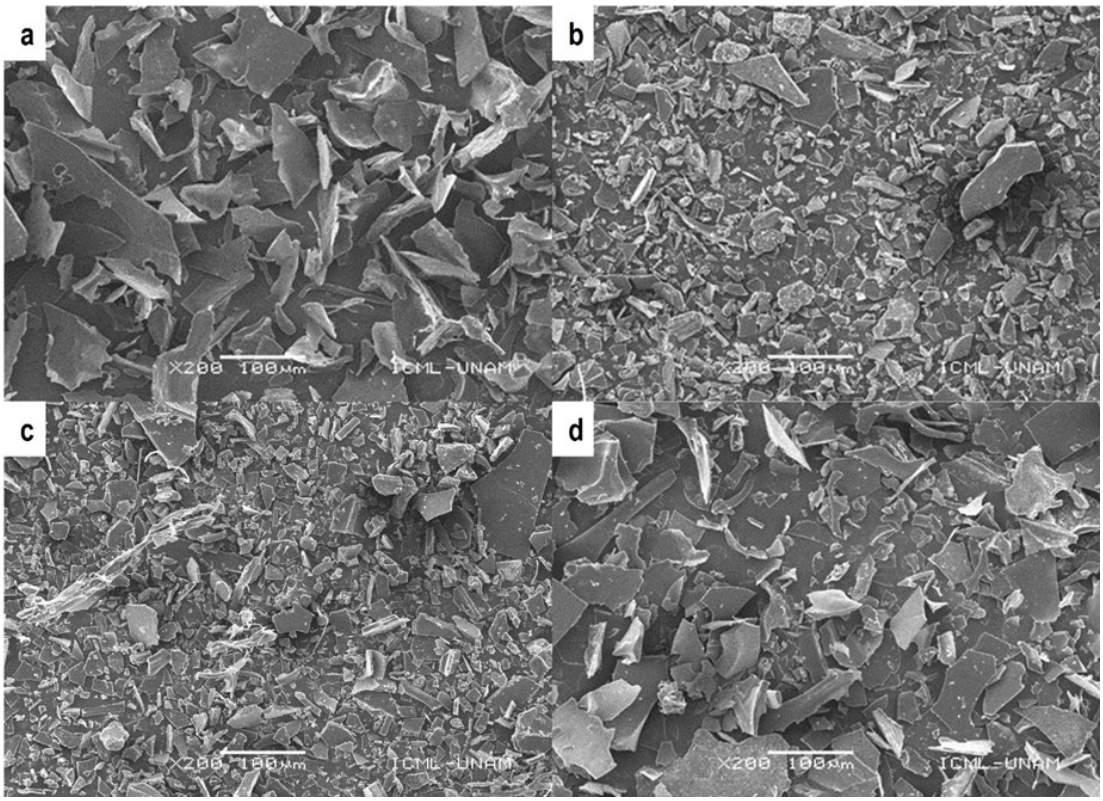

Fig. 14. Huauzontle protein scanning electron micrographs (magnification 200x): (a) P_{native}, (b) P_{heat}, (c) P_{US}, and (d) P_{pH11}.

4.3.5 Formation of SC between huauzontle protein-S

Two of the most important parameters that determine the biopolymer complexes formation are the pH and the weight ratio between the biopolymers, which influence the balance of the electrical charges and electrostatic attractive interactions between molecules and, therefore impact the formation and stabilization of the resulting complexes [55, 56]. Thus, with the aim of establishing the most favorable pH value and biopolymers weight ratio for inducing the formation of protein-S soluble complexes, the absorbance of the supernatant of the protein-S (with weight ratios from 1:0.25 to 1:4) solutions were measured at pH values ranging from 2 to 7. The reading throwing the highest absorbance lectures were taken as indicative of the most favorable conditions for the formation of the SC [57]. All the absorbance vs pH profiles of the huauzontle protein-S solutions (Figure 6) showed similar trends and were characterized by three

regions from high to low pH values as follows: (A) a region comprised in the pH range of 7.0 to 6.5, characterized by relatively constant low absorbance values, where individual huauzontle protein and S molecules co-exist in solution; (B) a region comprised in the pH interval ~ 6.5 to 3.5, where huauzontle protein-S complexes begin to form at a critical pH (pH_{cf}, ~ 6.5 to 6.0) by the binding of huauzontle protein molecules to the S chains, and remaining disperse in the solution due to an incomplete neutralization of the negatively charged S moieties and the positively charged huauzontle protein moieties [58]. SC formation continues as the pH decreases towards a critical pH value (pH_{sc}, ~ 5.0) where maximum soluble complexes formation occurs evidenced by the highest absorbance value achieved. At lower pH values, SC begin to aggregate and eventually precipitate as insoluble complexes (coacervates), with absorbance values decreasing. When a critical pH is achieved (pH_{cc}), coacervates precipitate and a maximum phase separation is achieved. As pH values continue to decrease in region C, protonation of carboxylate groups takes place, huauzontle protein-S complexes begin to revert into non-interacting individual biopolymer molecules may proceed [59].

In Fig. 15 it can be appreciated that the most favorable protein:S weight ratio and pH_{sc} for obtain P_{native}-S soluble complexes were 1:1 and 5.1, respectively. Whereas the most favorable protein:S weight ratio for all the modified huauzontle proteins was of 1:2, but the pH_{sc} value for driving the maximum absorbance was of 5.4 for P_{heat}-S, 5.5 for P_{pH11}-S, and 5.7 for P_{US}-S.

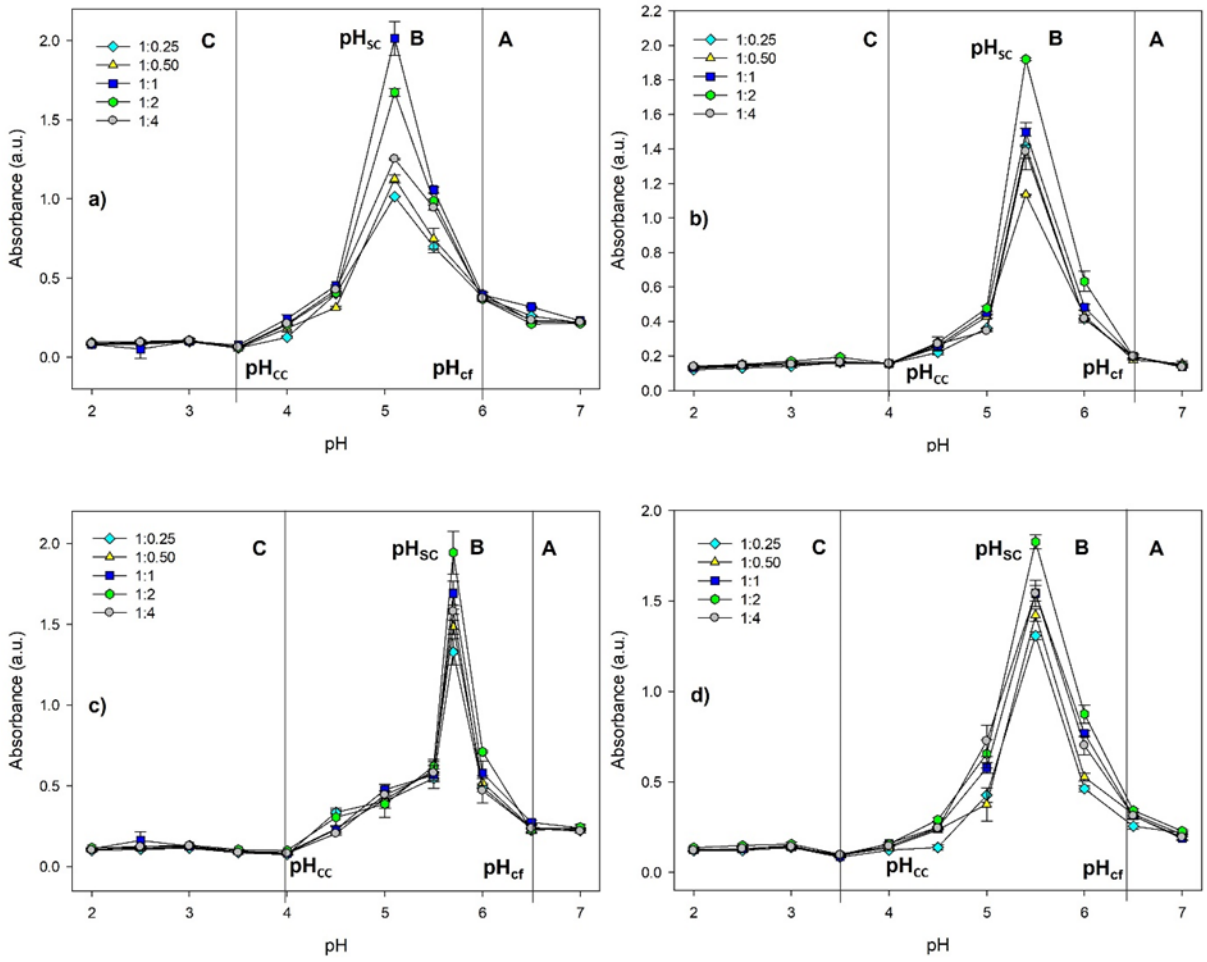

Fig. 15. Absorbance of supernatant: (a) P_{native} -S; (b) P_{heat}-S; (c) P_{US}-S, and P_{pH11}-S. Where: pH_{cf} = critical pH at which SC begin to form; pH_{cs} = critical pH at which maximum formation of SC occurs, and pH_{cc} = maximum phase separation pH at which complex coacervates are formed.

These results indicate that treatments altered the available active sites on the huauzontle protein chains, thus requiring different conditions for the interaction with S for yielding SC [56]. According to [60], the complexation of proteins and polysaccharides at pH higher than pI is governed by the binding of the anionic reactive sites of the polysaccharide and the small cathodic reactive sites of protein, resulting in anionic protein-polysaccharide aggregates, and therefore, the polysaccharide concentration being fundamental for the stability in solution of the complex. From the pH_{sc} values found for the SC obtention, huauzontle protein-S soluble complexes could be functional ingredients for a wide

spectrum of foods, such as protein drinks with pH values ranging between 5 to 6 [61, 62].

4.3.6 Characterization of the soluble complexes (SC)

4.3.6.1 ζ-potential and particle size of the SC

All the SC exhibited net negative charge, with *ζ-potential values* varying as follows: $P_{heat\text{-}S}$ (-29.63 ± 0.64 mV) = $P_{US\text{-}S}$ (-28.60 ± 0.95 mV) > $P_{pH11\text{-}S}$ (-27.77 ± 0.51 mV) > $P_{native\text{-}S}$ (-26.90 ± 0.82 mV). The ζ-potential is a fundamental parameter for the stabilization of dispersed systems such as emulsions, where higher values of ζ-potential have been related to greater stability. Since the stability of an emulsion depends on the combined effect of the thickness of the surface layer of the adsorbed biopolymer (steric stabilization) and the surface charge (electrostatic repulsion stabilization), a SC with higher ζ-potential values would indicate greater stability [63]. The pH_{sc} values (5.1 to 5.7) at which the SC were formed, led to the formation of SC with a negative surface charge, as the protein positive charges were unable to completely neutralize the negative charge of the S chains [16]. Huauzontle protein provided a low number of positive charges, so the negative charge of the S chains was not completely neutralized, leading to the formation of SC with a negative surface charge. This incomplete neutralization of the surface S charges, plays an important role on the stabilization of the SC, as the interactions between protein-S are sufficiently strong to maintain the biopolymers complexes integrity, but at the same time the electrostatic repulsion between the negatively charged moieties of S, deter the formation of a stronger, more integrated biopolymer complexes taking place which will eventually guide to their insolubility and precipitation. The above by solubilizing the SC through ion-dipole interactions with water molecules and avoiding precipitation by aggregation of the SC through electrostatic repulsion between charges [64]. The negative values of the ζ-potential favored the stability of the SC due to steric hindrance [26].

An increase in the hydrodynamic diameter of the complexes was observed, which varied as follows: P_{heat}-S (864.10 ± 13.89 nm) > P_{US}-S (659.90 ± 7.87 nm) > P_{pH11}-S (522.93 ± 39.10 nm) > P_{native}-S (294.03 ± 7.82 nm). The significantly higher d_h value exhibited by P_{heat}-S compared to the rest of the SC, could be imputed to the relative high concentration of polysaccharide which forms membrane around the denatured protein particles, which coupled with the greater viscosity of the protein due to the thermal treatment, lead to an increase in the hydrodynamic diameter of the complex [64]. Similar results were reported by [65], who observed an increase in particle size in protein-polysaccharide complexes when the protein was subjected to heat treatment. It should be noted that the particle size distribution presented bimodal curves for all the complexes formed (data not shown). [66], characterized the formation of complexes according to the type of particle size distribution, where the presence of monomodal peaks was characteristic of insoluble complexes, the presence of two separate peaks reflects that there was no interaction between the biopolymers, and bimodal peak was due to the formation of SC due to the interaction of positive patches contributed by the protein and negative charges due to the carboxylic groups of the polysaccharide.

3.6.2 FTIR of the SC

FTIR spectroscopy can be used to determine the structure of proteins and polysaccharides, as well as interactions when mixing biopolymers. The interaction of functional groups of proteins and polysaccharides favors the appearance of new bands or changes in the location of the absorption bands of the FTIR spectra [64]. In addition to this, it has been reported that protein-polysaccharide complexation can increase, maintain, or decrease the intensity of the characteristic bands of amide I, and this will depend on the type of polysaccharide used, and will derive from the molecular changes due to complexation [67]. Fig. 16 shows the FTIR spectra of the SC. The peaks corresponding to amide I of huauzontle protein shifted from 1630 to 1635 cm^{-1} and the peaks related to amide II were shifted from 1520 to 1510 cm^{-1}. According to [39], the shifts in the

characteristic bands of amide I and amide II can be attributed to conformational changes in the protein structure during complex formation, where random structures (e.g., α-helix) form a more organized configuration (e.g., β-sheet).

In addition to this, a reduction in the size of the bands corresponding to amide I and amide II and the disappearance of the characteristic peak of amide III (1380 cm^{-1}), in all the complexes was observed, which is indicative of conformational changes due to protein-S interactions [67]. [23] reported that the characteristic peaks of whey protein hydrolysate and cocoa pectin disappeared when the soluble complex was formed due to the electrostatic interaction between the ammonium groups of the protein (NH_3^+) and the carboxylate groups of the pectin (COO^-). In addition to this, the disappearance of characteristic peaks in the region of the carboxylate group of polysaccharides (1587-1643 cm^{-1}) is indicative of protein-polysaccharide interactions, highlighting the lower capacity of polysaccharide molecules to form intermolecular hydrogen bonds due to the presence of the protein [67]. On the other hand, the peaks observed in the protein spectra in the region of 1500 to 1200 cm^{-1} merged into a broad peak after the formation of the complexes. The fusion of characteristic peaks suggests the complexation between biopolymers. [25] observed the fusion of the absorption bands in the region from 1650 to 1500 cm^{-1}, attributing this behavior to the formation of starch-chitosan complexes.

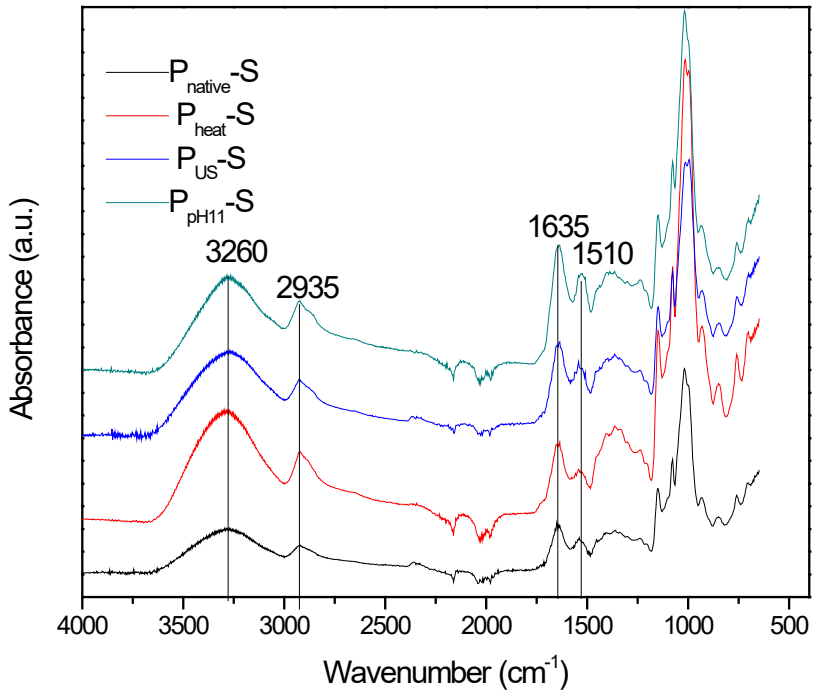

Fig. 16. FT-IR spectra of the soluble complexes composed of huauzontle protein-octenyl succinic anhydride-modified waxy corn starch (S): P_{native} -S, P_{heat}-S, P_{US}-S, and P_{pH11}-S.

4.3.7 Morphological properties of SC

In order to gain insights of the structure of the SC, their morphology was observed by optical and scanning electron microscopy (Fig. 17). The complexes adopted different conformations with respect to individual biopolymers and average size of SC ranged between approximately 300 and 800 nm. P_{native}-S complexes presented a cylindrical, irregular, and compact structure. Complexes formed by modified huauzontle proteins tended to aggregate forming larger particles. The formation of aggregates in the P_{heat}-S treatment agrees with the largest particle size and zeta potential reported (see sub-section 3.6.1). In addition to this, the denaturation of proteins by heating can lead to the formation of aggregates linked by disulfide bridges [68], hydrogen bonds and/or hydrophobic

interactions [69]. The P_{pH11}-S and P_{US}-S complexes showed an elongated shape, with a rough surface and cavities in their structure. Rough surfaces in protein-polysaccharide mixtures have been related to the nature of the protein and not to the nature of the polysaccharides [64]. [70] observed that the surface of propylene glycol alginate became rough when interacting with the pea protein, through electrostatic interactions between the positive ammonium groups of the protein and the negative carboxylate groups of the alginate, which was indicative of the adsorption of the protein on the surface of the polysaccharide. This type of morphology has also been associated with the protein-S ratio at pH above pI, where the attractive forces between biopolymers are not strong enough due to the lack of a larger amount of protein molecules available to interact with the polysaccharide [71].

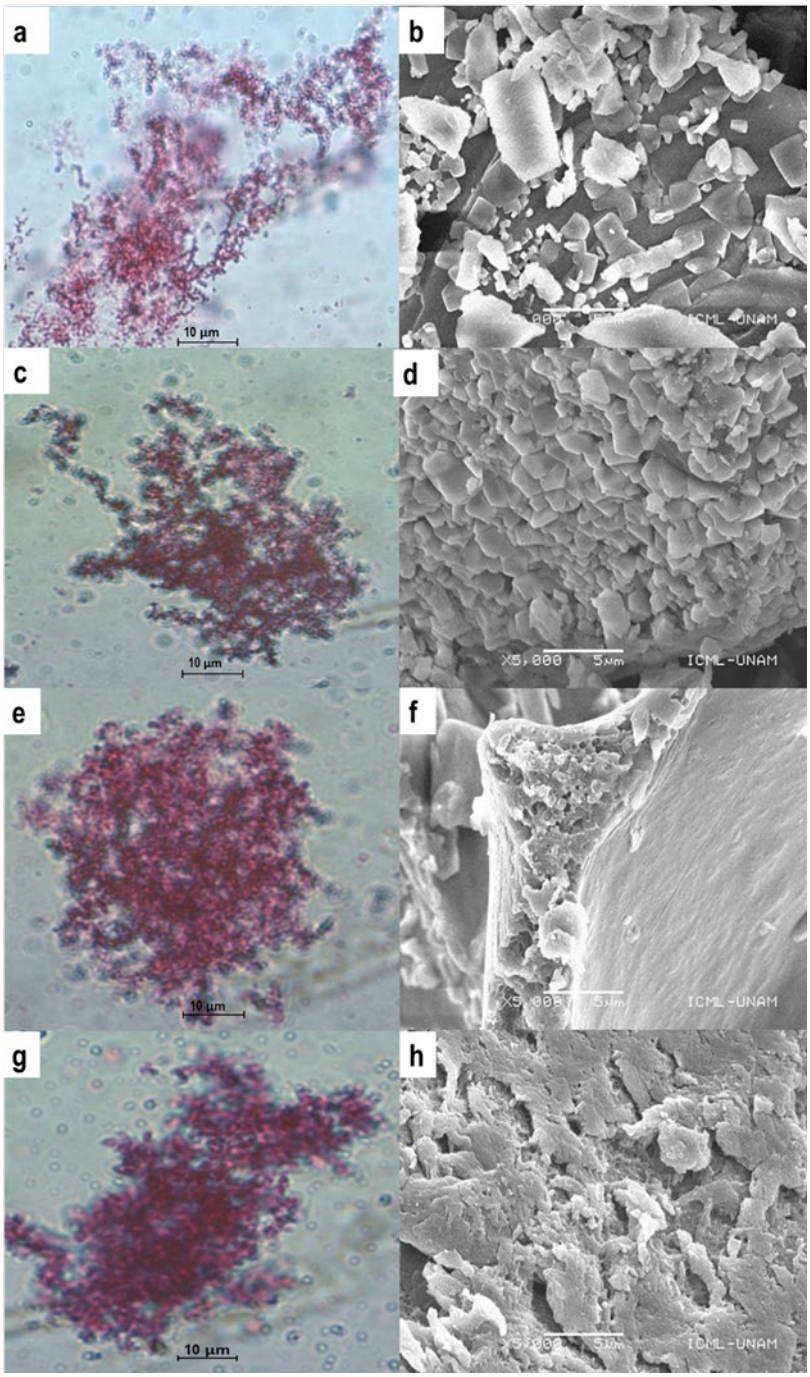

Fig. 17. Optical (right hand panel) and SEM (left hand panel) micrographs of the protein-S soluble complexes: (a, b) P_{native}-S; (c, d) P_{heat}-S; (e, f) P_{US}-S, and (g, h) P_{pH11}-S.

4.3.8 *In vitro* starch digestibility

The *in vitro* digestibility of S on its own and in the soluble complexes with huauzontle protein was characterized by its content in RDS, SDS and RS (Table 3). The fraction of RS in the SC varied as follows: $P_{pH11\text{-}S} > P_{US\text{-}S} > P_{heat\text{-}S} > P_{native\text{-}S}$. On the other hand, the fraction of RS in S (87.2 ± 0.4%) was in general significantly lower than that in the SC with modified protein, starch. It should be noted that P_{pH11}-S and P_{US}-S presented the highest RS values (92.6 ± 0.4 and 90.7 ± 0.4%, respectively). [72] attribute this behavior to the addition or embedding of the protein on the surface of the starch granules through hydrogen bonds and hydrophobic interactions, thus acting as a physical barrier and limiting the accessibility of the enzymes to starch. [73] mentioned that the protein acts as a physical barrier for digestion by binding to α-amylase, thus inhibiting its activity. Given the elevated levels of RS determined in all SC, these could be incorporated into baking and pastry products, since it was observed that the incorporation of RS improves the nutritional, textural and sensory properties, and in turn decreasing the glycemic index of the product final [74].

Most previous studies reported that the addition of proteins could decrease the digestibility of starches, however, in this study, P_{native}-S showed a significant increase in the *in vitro* starch digestibility (RS = 85.8 ± 0.2%). In this sense, [75] observed a similar behavior when the addition of protein-polysaccharide complexes decreased the RS content of wet sweet potato vermicelli. This behavior was attributed to the formation of a looser matrix around the starch molecules, which could favor amylolysis. This could have happened in this study, where a more compact matrix could be observed (Fig. 17) for the SC formed with modified proteins, while the SC formed with native protein presented a more open network. [76] attributed the decrease in the RS fraction to the protein:starch ratio, where a lower ratio presented lower RS values.

Increases in RS fraction in SC composed by modified huauzontle proteins were accompanied by decreases in the RDS fraction and relative increases in SDS fraction. [77] mentioned that starch digestibility is strongly influenced by the

presence of proteins, having mainly three mechanisms of digestibility influenced by protein-starch interaction: (i) protein can adsorb or encapsulate forming a physical barrier preventing enzymatic recognition, (ii) protein-starch interaction is governed by non-covalent interactions thus reducing starch digestibility, (iii) proteins exhibit inhibition of amylase activity thus decreasing starch digestibility.

Table 3. *In vitro* digestibility of starch on its own and in the soluble complexes with huauzontle protein.

Starch	RDS	SDS	RS
S	12.4 ± 0.2^{a}	0.5 ± 0.2^{b}	87.2 ± 0.4^{d}
P_{native}-S	12.9 ± 0.3^{a}	1.3 ± 0.3$_{a}$	85.8 ± 0.2^{e}
P_{heat}-S	11.0 ± 0.2^{b}	1.1 ± 0.2^{a}	88.7 ± 0.3^{c}
P_{US}-S	8.0 ± 0.4^{c}	1.3 ± 0.3^{a}	90.7 ± 0.4^{b}
P_{pH11}-S	6.4 ± 0.4^{d}	1.0 ± 0.1^{a}	92.6 ± 0.4^{a}

Different letters within the same column indicate that the means differ significantly (P≤0.05). S = octenyl succinic acid modified corn starch; P_{native} = native huauzontle protein, and P_{heat}, P_{US}, and P_{pH11} = modified huauzontle proteins through heat, ultrasound and pH shifting, respectively. RDS = readily digestible starch, SDS = slowly digestible starch, and RS = resistant starch.

4.3.9 *In vitro* protein digestibility

The nutritional quality of protein is determined by its digestibility, which is an indicator of protein bioavailability, therefore, it affects the bioavailability of amino acids [78]. Currently, the consumption of proteins of plant origin increases due to environmental and health factors, however, their digestibility is low compared to proteins of animal origin, this is due to the large size of storage proteins, lower solubility, and the presence of antinutritional factors [79]. In this sense, physical and chemical methods have been used to improve its nutritional properties. The *in vitro* digestibility of the native and modified protein and the SC was evaluated (Table 4). The lowest value was for the native huauzontle protein (82.0 ± 1.5%). These results agree with what was reported by [10], who reported digestibility values of 83% in huauzontle protein. On the other hand, P_{pH11} presented a high digestibility (87.2 ± 0.3%), indicating that the modification had significant effects on the structural conformations of the protein. [80] demonstrated that alkaline pH-

induced changes in protein conformation can modulate the accessibility of enzymes and influence their digestibility. The greater digestibility of P_{pH11} could be related to the decrease in the content of β-turn structures, which have a compact structure and have been related to lower protein digestibility [81]. All the modified proteins presented a higher *in vitro* digestibility than P_{native}. [82] mentioned that the application of high-intensity ultrasonic waves (20 kHz) altered the secondary structure of proteins, in addition to disintegrating any protein aggregate, which caused an increase in protein digestibility. Other researchers have shown that sonication can improve the digestibility of protein by disrupting intramolecular disulfide bonds, which promotes partial unfolding of structure [81]. Likewise, heat treatment causes protein denaturation as well as protein-protein aggregation due to hydrophobic interactions [83]. [37] determined that a decrease in the content of β-sheet structures favors the digestibility of protein. Table 4 shows that the P_{heat} treatment maintained the *in vitro* digestibility of the protein with respect to its native counterpart, probably due to a balance existing between the factors that favor the digestibility, i.e., a decrease in the content of β-sheet structures (see sub-section 3.3), and the increase in protein-protein interactions due to the heat treatment, which could inhibit enzymatic recognition.

Table 4. *In vitro* digestibility of native and modified huauzontle protein on their own and in the soluble complexes with octenyl succinic acid modified corn starch.

Indvidual protein	*In vitro* digestibility	Protein complexed	*In vitro* digestibility
P_{native}	82.0 ± 1.5^{cA}	P_{native}-S	85.1 ± 2.3^{bA}
P_{heat}	84.1 ± 1.2^{bB}	P_{heat}-S	89.5 ± 2.5^{bA}
P_{US}	86.0 ± 0.3^{abB}	P_{US}-S	92.7 ± 0.7^{aA}
P_{pH11}	87.2 ± 0.34^{aA}	P_{pH11}-S	87.6 ± 1.2^{bA}

^{ABCD}Different letters within the same row indicate that the means differ significantly (P≤0.05). ^{abcd}Different letters within the same column indicate that the means differ significantly (P≤0.05). S = octenyl succinic acid modified corn starch; P_{native} = native huauzontle protein, and P_{heat}, P_{US}, and P_{pH11} = modified huauzontle proteins through heat, ultrasound and pH shifting, resp *In vitro* digestibility of native and modified huauzontle protein on their own and in the soluble complexes with octenyl succinic acid modified corn starch ectively.

4.4 Conclusions

The conditions for the formation of soluble complexes (CS) between the native (P_{native}) and modified huauzontle protein (P_{heat}, P_{US}, and P_{pH11}) with octenyl succinic anhydride corn starch(S), strongly depended on the protein modification treatment (heat, ultrasound or alkaline pH shifting), due to different alterations in the secondary structures of the protein. Thus, the critical pH at which maximum formation of soluble complexes occurred varied from 5.2 (P_{native}-S) to 5.8 (P_{us}-S), being the required protein: polysaccharide weight ratio of 1:1 for P_{native}-S complexes and of 1:2 for P_{heat}-S, P_{US}-S and P_{pH11}P-S complexes. Treatments used to modify the huauzontle protein structure affected in turn the physicochemical properties (particle size, ζ-potential, morphology, structural features) of the SC and the *in vitro* digestibility of protein and starch that composed these SC. The highest *in vitro* protein digestibility was displayed by the P_{US}-S complex, while the highest resistant starch fraction was found in the P_{pH11}-S complex. These results indicate that the physicochemical properties and the protein and starch digestibility can be modulated by an adequate pre-treatment of the protein to be used in the formation of the SC with S, making these SC extremely attractive for their use in the food industry to achieve specific desired functional properties.

4.5 References

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5 Conclusiones generales

De acuerdo con los resultados de la investigación, las propiedades tecno-funcionales y fisicoquímicas como la formación de espuma, solubilidad y formación de geles fueron clave para identificar la diferencia entre proteína modificada y nativa. se comprobó que es posible formar complejos solubles entre la proteína de huauzontle y el almidón OSA, como lo confirma la absorbancia después de ser centrifugada y mantenerse estable, se corrobora que los complejos solubles se forman a pH superior al punto isoeléctrico de la proteína, el pH de formación dependió del tratamiento previo que se le dio a la proteína, las micrografías ópticas y de barrido permitieron visualizar la diferencia en la morfología de los biopolímeros individuales y acomplejados. Otra de las variables más importantes que permitieron corroborar las aseveraciones fueron los cambios que se observaron en la estructura secundaria de la proteína mediante la deconvolución.

De acuerdo con los resultados obtenidos el uso de proteína de huauzontle puede cumplir con condiciones que demanda la industria alimentaria una vez modificada su estructura, cabe resaltar que será necesario la aplicación de esta y su caracterización en un sistema alimentario, así como, identificar que compuestos antinutricionales como compuestos fenólicos, saponinas están presentes en el huauzontle que pudieran afectar su aplicación. Por último, se recomienda la modificación de la proteína a diferentes temperaturas para identificar el mayor número de grupos hidrófobos y así mejorar aún más las propiedades reológicas de la emulsión.