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Structural and Functional Characterization of Freeze-Dried Fibrillated Pinto Bean Protein Isolate

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ABSTRACT: Pinto bean protein isolate was heated at 85 °C pH of 2 for 5, 10 and 20 hours before forming fibrillar aggregates. Three different techniques-Congo red spectrum analysis, Fourier Transform Infrared spectroscopy (FT-IR) and Transmission Electron Microscopy (TEM)-were used to find these nanofibrils after freeze-drying. Congo red spectrum was right-shifted from about 500 nm in standard Congo red solution to about 534-536 nm in fibrillated pinto bean protein isolate. FT-IR spectra peak, moved from 1630 cm^{-1} in native pinto bean protein to 1619 and 1618, in protein solutions fibrillated for 10 and 20 hours, respectively. However, TEM scans revealed that the aggregates after 20 hours of heating were numerous than aggregates from 10 hours of heating. After fibrillation, the average percentage of protein degree of hydrolysis of pinto bean protein isolates heated for 5, 10, 20 hours, was increased initially and then decreased due to fibrillation. The main protein band with molecular weight between 40 and 55 KDa in native pinto bean protein electrophoregram was degraded to smaller molecular weight bands in fibrillated proteins. Emulsion Activity Index and Emulsion Stability Index, lowered upon fibrillation treatment; while Water Holding Capacity and Oil Holding Capacity were approximately 1.5 times greater than the native protein.

Keywords: Fibrillation, Freeze Drying, Functional Properties, Legume Seeds, Pinto Bean Protein, Protein Extraction.

Introduction

In terms of affordability, availability and sustainability, plant storage proteins have proven to be a strong substitute for animal proteins (Munialo *et al.*, 2014). Legume seeds offer a good balance of amino acids and a high protein content (Gueguen, 1985). Protein concentrations and protein isolates can be made from legume seeds (Pinuel *et al*, 2019). Proteins play a crucial role in the formulation and processing of food products because of the functional traits that affect consumer acceptability (Wani *et al.*, 2014).

The formation of amyloid (or amyloid-like) fibrils from various globular dietary

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proteins is gaining popularity (Tang *et al.*, 2010). Different factors govern food protein fibrillation, including time, temperature, moisture content, protein concentration, shear rate, existence of alcohol in the fibrillation medium, reducing agents, salt, enzymes and other food ingredients (Jansens *et al.*, 2019). When heated for a long time under conditions of above denaturation temperature, low ionic strength, and pH values far away from the isoelectric point, the proteins can form nanofibrils 1-10 µm in length, 1-10 nm in diameter, and multistranded twisted like structure (Hu and Li, 2021). According to a recent review of the research, food-derived amyloid fibrils are expected to have little impact on human health (Farrokhi *et al.*, 2019). Protein fibrils are attractive functional ingredient options. The functions of these fibrillar structures range from enhancing viscosity and gelling agent to foam and emulsion stabilizer (Kroes-Nijboer *et al.*, 2012). Many investigations on protein fibrillation from animal or plant origin have already been described. Mohammadian et al. (2018) generated fibrils from whey protein isolate at pH 2. Monge-Morera et al. (2021), investigated amyloid-like fibrils from hen egg white. Other researchers including Herneke et al. (2021) , Li et al. (2020) , Pang et al. (2020) and Wang et al. (2020) assessed proteins from plant source, for fibril formation.

Pinto bean protein isolate is a good source of aspartic acid and glutamic acid. Pinto bean protein contains higher levels of essential amino acids, higher thermal stability and emulsifying capacity compared to soy protein isolate. In addition, people are concerned about the safety of commercially available genetically modified soybeans and their long-term effects (Tan *et al.*, 2014).

The main goal of this study is to create pinto bean protein nanofibrils heated for a set amount of time in an acidic pH. To best of our knowledge, among legume proteins the fibrillation of pinto bean protein has not been published so far, and studies on this topic in other protein sources have focused on fibrillated protein suspensions, while we studied structural and functional properties of freeze-dried protein to evaluate it for use as an ingredient in food formulations.

Materials and Methods

A seed breeding institute sold improved pinto bean (*Phaseolus vulgaris*) seeds with the defined variety, Saleh, and a protein value of 20.8g/100g (dry weight basis). Prior to extraction, bean seeds were ground, sieved (using a 0.5 mm mesh), and kept at -18°C. All of the materials were of the analytical grade and were from the Merck Company.

- ***Pinto bean protein extraction and characterization***

In order to create a protein slurry, milled beans were combined with distilled water in a 1:10 (g/ml) ratio. The pH was raised to 8.0 using 1M NaOH, and it was then incubated for an hour at 20°C. In order to get rid of the insoluble materials, the slurry was centrifuged at 10,000g for 25 min at 20 °C. The supernatant was collected, and 1 M HCl was used to adjust the pH to 4.8. The supernatant was then centrifuged at 10000g (20 °C, 25min) after being incubated at 20 °C for 2 h with constant stirring. The albumin and sugar-containing supernatant was then discarded. The pellet was recovered and re-dispersed at a ratio of 1:10 (g/mL) in distilled water. To get removal of any remaining sugars and albumins, the redispersed suspension at a pH of 4.8 was centrifuged at 10000g (20 °C, 25 min) and the pellet was then resuspended in distilled water in a 1:3

(g/mL) ratio. The resuspended pellet's pH was adjusted to 8.0 using 2 M NaOH that had been dissolved, and the solution was then incubated at room temperature (20 ± 2 °C) for 1 hour to eliminate any remaining traces of insoluble materials. The pH of the supernatant was determined, and if necessary, it was corrected with 2 M NaOH to a final pH of 8.0. The bean protein that is obtained in this manner is the native protein solution (Munialo *et al.*, 2014). According to the Kjeldal method, protein content of native isolate (N) that was freeze-dried was 87% pure.

- ***Preparation of fibrillated pinto bean protein samples***

Sodium azide (0.02% w/v) was added to three sets of 50 ml protein suspension at a concentration of 25 mg/ml in deionized water in order to suppress microbiological development. The protein was thoroughly hydrated by stirring the mixture all night. 6M HCl was used to bring the pH down to 2. The biuret technique using the biuret kit for total protein, yielded a protein solution concentration of 21 mg/ml. The solutions were then heated at 85°C for 5, 10 and 20 h with gentle stirring for fibril generation, and after the requisite amount of time, they were quickly cooled in an ice bath to stop fibrillation (Munialo *et al.*, 2014). Since the majority of food ingredients are used in powder or dry form, the protein suspensions were used just for Congo red spectrum analysis while the fibril suspensions were freeze dried and preserved at -20 °C for analysis.

- ***Native protein isolate and its fibrillated form analysis***
- ***Congo red Spectral Analysis***

According to Tang et al. (2010), a spectrum analysis of Congo red was carried out. In order to determine the heating period at which the greatest absorbance peak occurs during the Congo red spectral analysis, samples were generated with three different heating times (5, 10 and 20 hours). Congo red dye was dissolved in 10 mM potassium phosphate buffer (pH 7.4) with 150 mM NaCl while being continuously stirred to create a red stock solution (10 mM). The stock solution was diluted 100 times to create a brand-new working solution. With a sample of 1 ml of phosphate buffer, an ultraviolet-visible spectrophotometer (Photonix AR 2017) was calibrated between 400 and 700 nm at room temperature (pH 7.4). Native protein isolate, which had the same concentration as the prepared fibrillated suspensions in aliquots of 100 µL at three different heating rates, was combined with 1 mL of the working solution and maintained at 25 °C for 15 min. On the spectrophotometer, the mixtures' visible spectra (in the 400–700 nm region) were captured in 1 cm path length cuvettes. The working solution for Congo red produced the blank spectrum.

- ***Fourier Transform Infrared (FT-IR) spectroscopy***

Using an Avatar FT-IR Spectrometer, the FTIR spectra of freeze-dried native or its fibrillated form were obtained by scanning a wave number range of 4000 to 500 cm^{-1}. For sample preparation, KBr is used (Wang *et al.*, 2020).

- ***Transmission Electron Microscopy (TEM)***

Transmission electron microscope was used to analyse the morphology of the freeze-dried bean protein powders (native or fibrillated) according to Gao et al. (2017) approach.

- ***Protein degree of hydrolysis (DH)***

80 mg of o-phthaldialdehyde 97% (OPA) was dissolved in 2 ml of ethanol

and was added to 3.81 g of disodium tetraborate decahydrate (Borax) and 100 mg of sodium dodecyl sulfate (SDS) which were previously dissolved in demineralized water. 0.25 ml Betamercaptoethanol was added to this solution, and demineralized water was used to increase the amount to 100 ml. This solution was freshly made.

50 mg of native or fibrillated freeze-dried protein samples with 3 ml of demineralized water were combined and then left at room temperature for 30 min. The solution was centrifuged at 12000 rpm for 20 min. 3 ml of OPA reagent and 0.4 ml of supernatant were combined for 5 s. This solution was kept for exactly 2 min then the combination was read at 340 nm in the spectrophotometer. A standard curve for serine was used to read absorptions.

- ***SDS-PAGE***

According to Tang et al.'s (2010) approach, SDS-PAGE was carried out using 12% separating gel and 4% stacking gel. Protein suspensions (freeze-dried native or fibrillated) mixed with 10 ml electrophoretic sample buffer containing Tris-HCl (1M), SDS (6ml), glycerol (6ml), bromophenol blue (6mg) and dithiotreitol. Fibrillated proteins were solubilized in acidic deionized water with a pH of 2. They were heated for 5 min. Onto the stacking gel, aliquots containing 20 µg of protein per well were added. After being stained with 0.1% Coomasie Brilliant Blue in 50% trichloroacetic acid, the gel was washed with a solution of methanol and water that contained 7% (v/v) acetic acid and 40% (v/v) methanol.

- ***Functional properties measurement***
- ***Emulsion Activity Index (EAI) and Emulsion Stability Index (ESI)***

Wu et al. (1998) method was used for EAI and ESI measurements. An emulsion was made using 0.1% protein powder (native or fibrillated) and sunflower oil with 3 to 1 ratio. 50 µl of the emulsion was mixed with 5 ml 0.1% SDS solution. The absorbance was read at the time emulsion prepared and 10 min later at 500 nm. Below is the calculation for EAI:

$$EAI(\frac{\mathrm{m2}}{\mathrm{g}}) = \frac{2T(A0 \times Dilution\ factor)}{C \times \varphi \times 10000}$$

Where:
T= 2.303
A0= absorbance 0 min after emulsion preparation
Dilution factor = 100
C= protein mass/volume (g/ml) of protein suspension
Φ = oil volume fraction of the emulsion

And ESI was measured from the formula below:

$$ESI = \frac{A0 \times \Delta t}{\Delta A}$$

Where:
Δt = 10 min and ΔA = A0 - A10

- ***Water and oil holding capacities (WHC and OHC)***

Ma et al. (2022) method was used to determine WHC and OHC of protein powders. 0.1g native or fibrillated pinto bean protein isolate was mixed with 1.5 ml distilled water (for WHC) and sunflower oil (for OHC) in a centrifuge pre-weighed tube. It was kept in room temperature for 30 min and then was centrifuged for 5000 g, 30 min. Supernatant discarded and the increase in sediment weight was reported as percentage.

Results and Discussion

- ***Congo red spectral analysis***

Congo red experiences changes in optical characteristics as a result of

preferentially binding to the repeating β-sheet structure of amyloid fibrils (Ye *et al.*, 2018).

The study of native proteins and protein solutions heated for 5, 10, and 20 hours at pH of 2 is shown in Figure 1. Compared to the fibrillated proteins, the original protein displayed a distinct absorbance peak (about 500 nm). The absorbance peak at 534-536 nm was higher with longer heating times. The sample that had been cooked for 20 h had the highest absorbance peak, while the protein suspension that had been heated for 10 h had the next-highest peak. The lowest amyloid-like fibril development was observed in the protein solution heated for 5 h, which exhibited the lowest absorption at 534-536 nm.

According to Tang et al. (2010) research, the Congo red spectrum in the presence of native (untreated) materials did not show any absorbance peak, however the spectra in the presence of heated samples showed an absorbance peak at about 534 nm, which is a feature of amyloid fibrils. With heated samples at 15–360 min, they didn't notice any significant changes in relative absorbance at 534 nm, presumably indicating that the fundamental structural components needed to make amyloid fibrils were similarly released; nevertheless, as the time passed, the intensity of the absorbance rose. Tang et al.'s (2010) results thus confirmed our findings regarding the wavelength of amyloid fibril absorption, which was reported to be 534 nm, or the increase in Congo red spectrum intensity with increasing heating time.

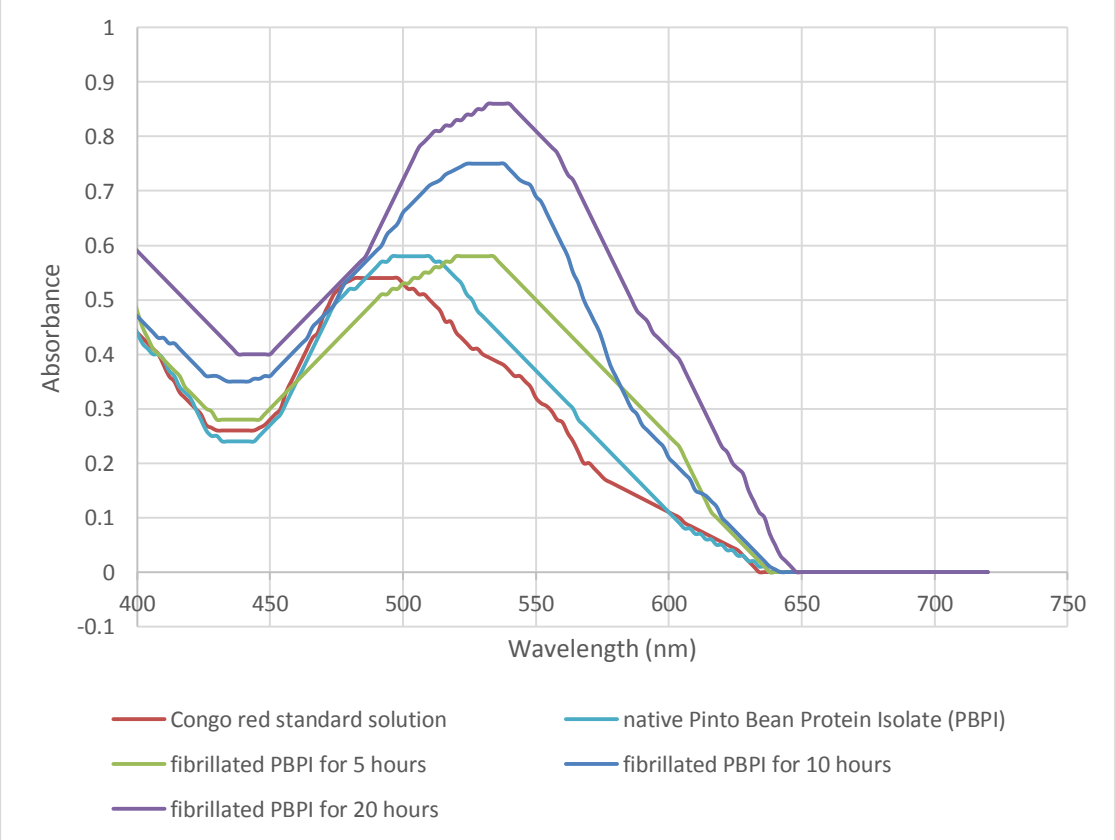

Fig. 1. Congo red spectral analysis of pinto bean protein isolate (native or fibrillated)

Incubation times longer than 6 hours led to the formation of pea protein β-sheet aggregates, according to Munialo et al.'s (2014) findings. The hydrolysates started to combine with heating periods up to 12 and 24 h, and at that point they tended to form threadiness aggregation, according to Wang et al. (2020) investigations on soy protein fibrillation [14].

- ***FT-IR***

Infrared spectroscopy is a vibrational spectroscopic technique that focuses on the vibrations of atoms in a molecule in the 4000-400 cm^{-1} range, particularly stretching and bending motions. In general, FTIR spectroscopy is used to determine the secondary structure information of proteins (Zhang *et al.*, 2021).

The FTIR spectra of the native and fibrillated pinto bean protein isolates after 10- and 20-hours heating times are shown in Figure 2. As these two freeze-dried bean protein heated samples had the highest peak in congored absorbance spectrum, they were used for FTIR spectroscopy. After 10- and 20-hour heating, the peak location of amide I in our native protein moved from 1630 in native protein to 1619 and 1618 in fibrillated samples 10- and 20-hours heated respectively, indicating the production of β-sheet structures. Changes in the peak location of the amide I, II, and III sections suggest protein structural transformation (Liu *et al.*, 2020). The wave number of β-sheet structures of amyloid fibrils is decreasing (Mong-Morera *et al.*, 2021).

Our findings are consistent with those of Ruysschaert and Raussens (2018), who discovered that amyloid fibrils often had absorbance between 1611 and 1630 cm^{-1}, whereas native β-sheet proteins have absorbance ranging from 1630 to 1643 cm^{-1}. These changes are linked to the creation of more extended β-sheets and/or longer β-strands, as well as more planar sheet formation in amyloid fibrils. There is a shift towards lower wave numbers when β-sheet structures are present in amyloid fibrils (ca. 1630 and ca. 1611 cm^{-1}).

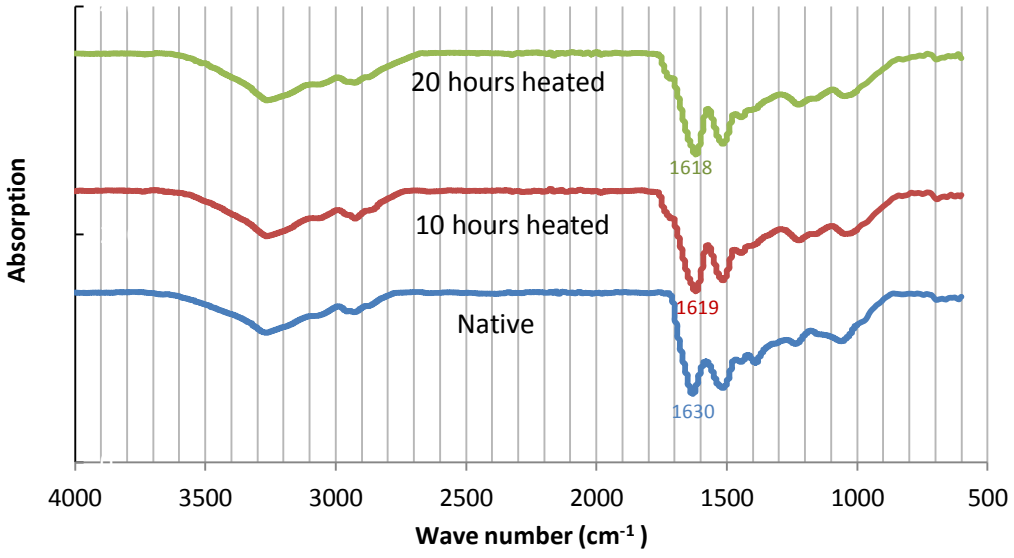

Fig. 2. Fourier-Transform Infra-Red (FT-IR) spectra of native pinto bean protein isolate and fibrillated pinto bean protein isolate heated for 10 and 20 hours.

- ***Transmission Electron Microscopy (TEM)***

TEM images of freeze-dried native protein and fibrils after 5, 10 and 20 h of native protein heating are displayed in Figure 3. Granular morphology was seen in a freeze-dried native pinto bean protein isolate. The granules bonded together and produced short filaments after 10 h of heating and low pH. However, after up to 20 h of heating, the hydrolysates exhibited curly aggregation and were longer in length.

Munialo et al. (2014) TEM images for pea protein fibrils appeared to curly structure which is the same as our study except that we used resuspended freeze-dried fibrils which were shorter in length. β-lactoglobulin fibrils in Gao et al. (2017) studies, were 100-400 nm in length because of the freeze-drying and resuspension exerted on fibril suspensions. In Wang et al. (2020) tests, the soy protein isolate hydrolysates, had granular shape after high temperature (85 °C) and low pH treatments on day 0. Due to a lack of catalysis, after 6 hours of heating time, no fibril structure could be formed. The granule sizes of the hydrolysates rose slightly as the heating duration was increased (incubation 0-day, heating time 6–24 h). When the heating period was increased, the polypeptides showed a fairly organized structure as well as a fibrous pattern. When heated for 6 to 10 h, the soy hydrolysates displayed isolated tiny granules (about 100 nm in diameter); however, when heated for 12 to 24 h, the hydrolysates began to aggregate and tended to form threadiness aggregation at 24 h. According to their findings, using moderate incubation times, in addition to heating time, considerably compacts fibril development (Wang *et al.*, 2020).

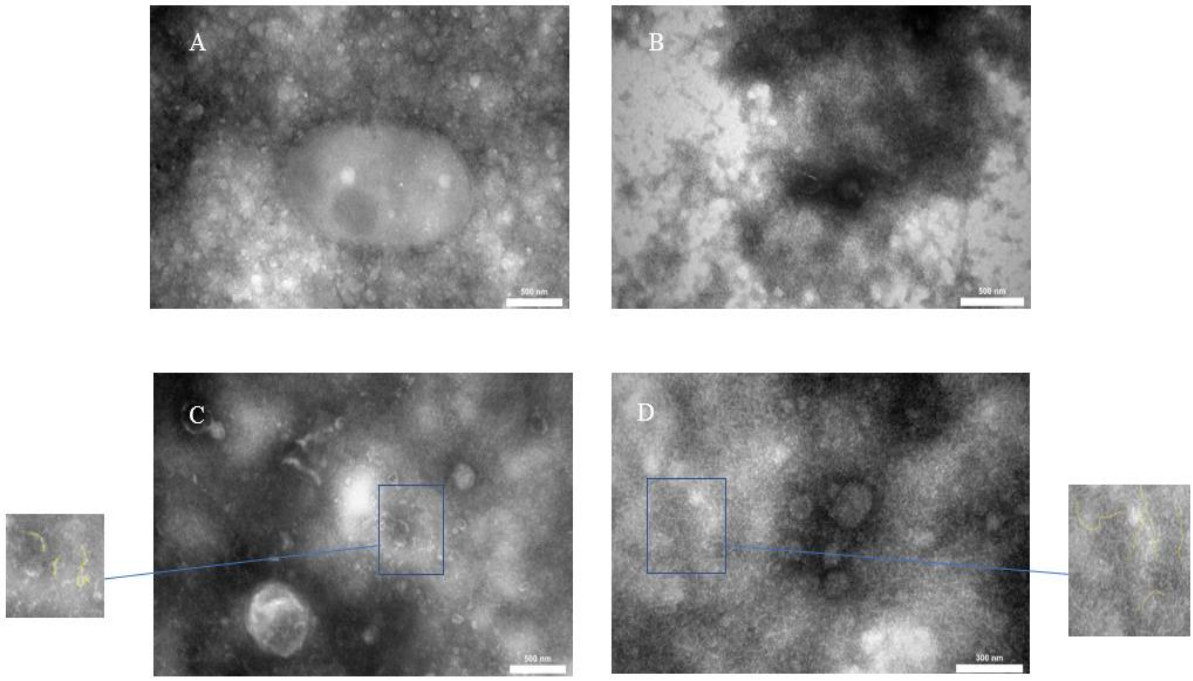

Fig. 3. Transmission Electron Microscopy (TEM) images of freeze-dried native (A) and fibrillated pinto bean protein isolate after 5 hours (B), 10 hours (C) and 20 hours (D) heating.

- ***Protein Degree of Hydrolysis (DH)***

Hydrolysis of the protein into smaller peptides is promoted at high temperatures. Many proteins have been demonstrated to have a relationship between hydrolysis and fibril production (Kroes-nijboer *et al.*, 2012). Thermal denaturation and acidic hydrolysis unfolded the globular protein structure, exposing functional groups that are normally buried in the core of the globular protein. Active peptide monomers capable of assembling and forming nucleus oligomers are released during this process. When a certain concentration is reached, protofibrils begin to develop and expand from oligomers, eventually assembling into longer mature fibrils (Wang *et al.*, 2020). Reduced electrophoresis analyses in Tang et al.'s (2010) experiments revealed that heating caused increasing polypeptide hydrolysis. Previous research has revealed that β-lactoglobulin undergoes peptide hydrolysis before assembling into nanofibrils, according to Ye et al (2018). Aspartate amino acid peptide bonds, in particular, are susceptible to hydrolysis. SDS-PAGE analysis of selected ThioflavinT studies samples indicates practically full breakdown of whey proteins into peptide fragments within the first 24 h of incubation (Ye *et al.*, 2018). Thermal denaturation and aggregation (to form amorphous aggregates), polypeptide hydrolysis of the aggregates, commencement of fibril assembly (or production of protofibrils), and fibril elongation (to generate highly ordered fibrils) or chain termination were demonstrated by Liu and Tang (2013). Table 1 shows the protein DH of native and fibrillated protein samples. The dissociation generated a considerable increase in DH after 5 hours of heating in acidic solution. However, after 10 and 20 h of heating, DH was reduced, most likely due to the incorporation of peptides generated by hydrolysis into fibrils.

- ***SDS-PAGE***

Figure 4 displays electrophoregrams of samples of native and fibrillated pinto bean protein. A prominent band between 40 and 55 KDa, which represents the vicilin fraction (Keivaninahr *et al.*, 2021), can be seen in the isolated native protein. This band eventually degraded into low molecular weight peptides with a range of 35–50 KDa after being heated for ten and twenty hours at an acidic pH. Heating fragmented the polypeptides of phaseolin, resulting in the creation of pieces of reduced molecular mass (e.g., <10 kDa after 360 min), according to gel electrophoresis examination in Tang et al. (2010) investigations. Heating the sample for more than 10 h, according to Munialo et al. (2014), causes the bands over 21 KDa to disappear.

Table 1. Protein degree of hydrolysis of native and fibrillated pinto bean protein isolate (PBPI)

	Degree of hydrolysis %
Freeze-dried native PBPI	$1.67^{d} \pm 0.52$
Freeze-dried fibrillated PBPI (5-hours heated)	$38.75^{a} \pm 1.22$
Freeze-dried fibrillated PBPI (10-hours heated)	$28.24^{b} \pm 1.26$
Freeze-dried fibrillated PBPI (20-hours heated)	$14.69^{c} \pm 0.68$

Means ± SD (n= 3) with different letters in each column indicate significant difference ($P < 0.05$)

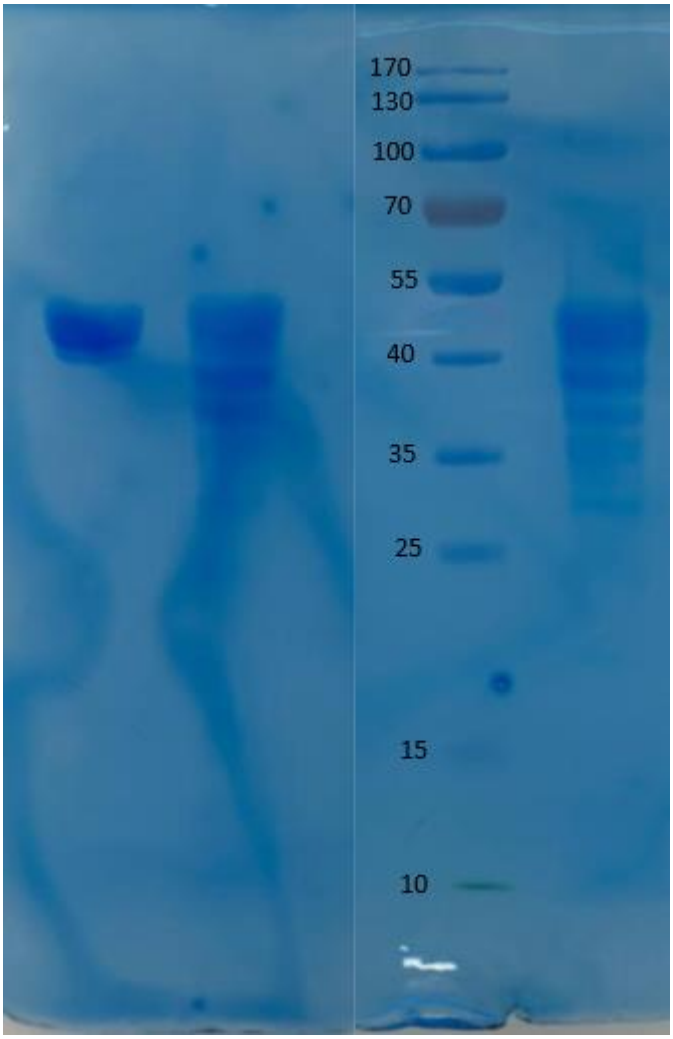

Fig. 4. SDS-PAGE pattern of Native pinto bean protein isolate, Fibrillated pinto bean protein isolate heated for 10 hours, Reference protein bands, Fibrillated pinto bean protein isolate heated for 20 hours, from left to right.

- ***Emulsion Activity Index (EAI) and Emulsion Stability Index (ESI)***

EAI and ESI of the isolated native protein and the fibrillated form, is shown in Figure 5. Both EAI and ESI was lower in fibrillated protein than the native. Increasing heating time, according to congored spectral analysis and TEM images, was along with higher β-sheet structures and worm-like aggregates indicative of fibrils. EAI and ESI were improved upon increasing heating time, but were not comparable to native protein. Mohammadian and madadlu (2018) reported higher EAI and ESI in fibril-stabled emulsions. Freeze-drying fibrillated pinto bean protein produced in our study, seems to shorten the fibril length and this may be the cause for the lower EAI and ESI.

- ***Water Holding Capacity(WHC) and Oil Holding Capacity (OHC)***

Both WHC and OHC of fibrillated pinto bean protein in all the three time treatments, were higher than the native protein significantly ($p<0.05$), shown in Figure 6. Maximum WHC was for the pinto bean protein isolate heated for 20 hours in acidic pH. Farrokhi et al. (2019) reported a higher WHC and OHC for nanofibrillated whey protein isolate.

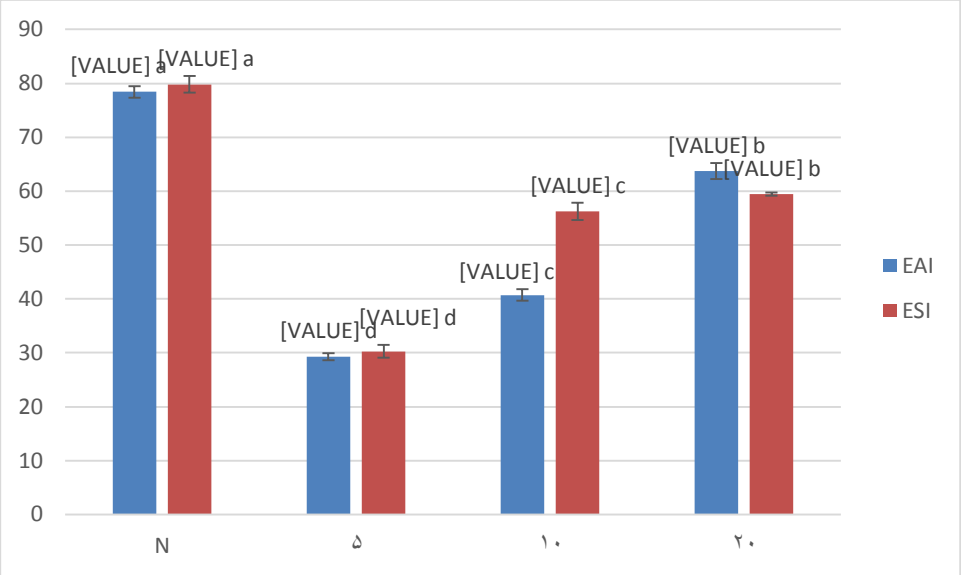

Fig. 5. Emulsion Activity Index (EAI) and Emulsion Stability Index (ESI) in: N(Native pinto bean protein), 5 (Pinto bean protein isolate heated for 5 hours in acidic pH), 10 (Pinto bean protein isolate heated for 10 hours in acidic pH) and 20 (Pinto bean protein isolate heated for 20 hours in acidic pH). Different small letters for the same color columns are significantly different $p<0.05$.

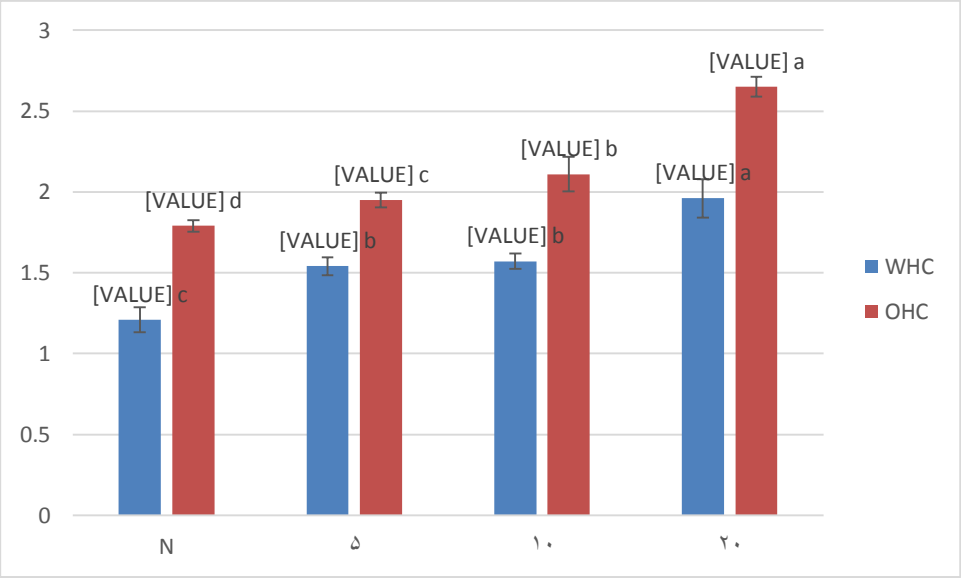

Fig. 6. Water Holding Capacity (WHC) and Oil Holding Capacity (OHC) in: N(Native pinto bean protein), 5 (Pinto bean protein isolate heated for 5 hours in acidic pH), 10 (Pinto bean protein isolate heated for 10 hours in acidic pH) and 20 (Pinto bean protein isolate heated for 20 hours in acidic pH). Different small letters for the same color columns are significantly different $p<0.05$.

Conclusion

Pinto bean protein isolate was successfully used to create amyloid-like protein fibrils. Heating duration was critical in the production of fibrils. Congo red spectral analysis is an effective method for identifying fibril formations. The self-assembly of polypeptides into observable fibrils was differentiated by an increase in the number of regularly secondary structures, as seen by rising intensity in Congo red spectral analyses and this

fibrillar structure was detectable through FTIR pattern and TEM images after freeze drying to be used as an ingredient in food technology. Increasing the heating time resulted in a decrease in protein hydrolysis, confirmation of fibril production from peptides produced during heating, and acidic hydrolysis. Water holding capacity and oil holding capacity of pinto bean protein isolate treated for fibrillation imporatntly ($p<0.05$) grew about 1.5 times, which made it suitable for absorbing water or oil in food formulations.

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