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Effect of Isoelectric Precipitation and Ultrafiltration Treatment on Extraction and Physicochemical Properties of Proteins from Date Palm Pollen

[1]Haifa Sebii, [1]Sirine Karra, [1]Ines Gafsi Makhlouf, [2]Sabine Danthine, [2]Christophe Blecker, [1]Hamadi Attia, [1]Souhail Besbes and [1]Brahim Bchir

[1]*University of Sfax, National Engineering School of Sfax, Laboratory of Analysis Valorization and Food Safety, Soukra Road, BP W-3038 Sfax, Tunisia*

[2]*University of Liege, Gembloux Agro-Bio Tech, Laboratory of Food Science and Formulation, Passage des Deportes 2 B, Gembloux B-5030, Belgium*

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Corresponding Author:
Haifa Sebii
Laboratory of Analysis Valorization and Food Safety, University of Sfax, National Engineering School of Sfax, Soukra Road, BP W-3038 Sfax, Tunisia

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Abstract: The present study investigates a comparison of two methods of extraction of date palm pollen protein. Two obtained pollen protein concentrates using the isoelectric precipitation and the ultrafiltration methods, (PPCIP and PPCUF) were obtained. From a physical point of view, PPCIP was a crystalline powder whereas PPCUF had an amorphous structure. The surface properties of the two protein concentrates varied according to pH and concentration. Indeed, it turned out that at pH 7, the two extracts were more capable of reducing the surface tension than at pH 4. Findings also proved that the surfactant proteins had a molecular mass >10 KDa. In addition, the solubility values were greater at pH 7 than at pH 4. These results were in line with the isoelectric points determined in the ZETA potential study which gave a pHi around 3 for the two extracts.The differential calorimetric analysis revealed that PPCIP had two denaturation peaks that could be attributed to the different constituents contained in the extract whereas PPCUF presented only one peak relative to the denaturation of DPP proteins. This difference was simultaneously noticed in the Thermogravimetric analysis.

INTRODUCTION

Date Palm Pollen (DPP) is the male reproductive cell of the date palm (*Phoenix dactylifera* L., Palmae). The pollen grains are the male gametophytes or the male fertilizing element of the phanerogams. These are natural micro-particles that plants use for the safe transport of their genetic material, often over large geographic distances[1]. It is formed at the stamens in the anthers and germs by producing a pollen tube allowing the transfer of its nuclei to the female gametophyte in the gynoecium.

Isolated plant proteins are widely used as food ingredients because of the high cost of animal proteins, their promising functional properties and their accessibility to the poor population. The functional properties of food proteins are the main criterion for the

formulation and processing of food products. These properties of protein isolates are influenced by the method of preparation and by the storage environment[2]. It should be noted that methods of extracting and separating proteins, such as alkaline extraction or neutral extraction followed by precipitation or ultrafiltration steps have been studied[3, 4].

Alkaline extraction, known as the isoelectric precipitation method is a simple procedure to obtain a relatively pure protein with a high yield. It is the most common way to prepare protein isolates in the food industry. This extraction is normally performed under mild alkaline conditions (pH 8.0-12.0) by adjusting the pH with NaOH to solubilize the protein[5].

Ultrafiltration is aninteresting separation technologywell spread in the food industry. The ultrafiltration process becomes an essential part of food technologyas a tool for concentration and separation[6, 7]. Ultrafiltration is filtration through a finer-pore membrane. The membranes used are either mineral or organic. An ultrafiltration membrane is characterized with adifferential selectivity and capable to retain proteins of high MW while allowing lower-molecular-weight proteins and other smallmolecules to pass through in the permeate. An ultrafiltration membrane has differential selectivity and can retain high MW proteins while allowing low MW proteins and small molecules to permeate[8]. The technique requires the implementation of high pressures. Membrane ultrafiltration improves the efficiency of conventional processes and introduces new ingredients or additives to the market. Ultrafiltration is a non-destructive method and it does not involve a change of state or reaction. Therefore, it is energetically economical and particularly suitable for the treatment of labile compounds such as biomolecules. The principle of this method is to fractionate by passing through a porous membrane under the action of a pressure gradient, the constituents of a liquid according to their size and/or load characteristics. From a given liquid, two fractions are obtained: the retentate retained in the feed/recycle circuit and the permeate or filtrate which is the liquid having passed through the membrane. Thus, after solubilization of the proteins, the application of ultrafiltration will allow them to be concentrated after passage through a membrane, having a well-defined cut-off point in a retentate. Factors that may affect effective protein separation by ultrafiltration include membrane type, molecular weight cut off and volume/concentration ratio.

Hassan[9] reported that DPP is a rich source of proteins (31%). Sebii *et al*.[10] claimed that a huge quantity of DPP could be unused in Tunisia, since, in the region of fax, around 50% of date palm trees are male and 100 female trees require 2-4 good pollinator trees as a pollen source[11]. Thus and to add value to this neglected natural product, a step of extraction of the proteins is carried out. To the best of our knowledge, the only study that deals with the extraction of DPP protein were to investigate the effect of sonication pretreatment on DPP protein concentrate quality based on its physico-chemical, surface and thermal properties[10]. Thus, this study aims to discover the best way to obtain the pollen protein concentrate by comparing the isoelectric precipitation and the ultrafiltration methods.

MATERIALS AND METHODS

Raw material: As described by Sebii *et al*.[10], DPP was manually collected from male date palm trees Sfax, Tunisia 2015 and spathes were gently shaken to separate pollen flour from flowers then sieved to remove residual particle and directly frozen in a watertight container at -20°C for further uses.

Preparation of protein concentrate

Isoelectric precipitation extraction: DPP was mixed with distilled water at a 1:10 (w/v) ratio. pH was adjusted to 12 using NaOH (1M). The mixture was magnetically stirred for 2 h at 30°C then centrifuged at 10000 rpm at 4°C for 30 min. The extraction procedure was repeated twice to enhance protein extraction. Supernatants were collected before further precipitation. Isoelectric precipitation was carried out by adjusting the pH of supernatant to 3 with HCl (1M) and keeping it at 4°C overnight. Finally, the protein concentrate was recovered by centrifugation 10000 rpm at 4°C for 30 min. Protein concentrate was neutralized, dialyzed 5 days against ultrapure water and freeze-dried using a freeze drier (Bioblock scientific Christ ALPHA 1-2, IllKrich-Cedex, France) (Fig.1).

Ultrafiltration: Instead of recovering protein using isoelectric precipitation, UltraFiltration (UF) was done to concentrate protein from supernatants obtained by the method previously described. A pilot-scaleUF unit (Rhodi a Orelis, France) was used. The UF pilot is composed of a pump and two pressure transducers (0-6 bars) located at the inlet (Pi) and outlet (Po) of the membrane module. Trans membrane pressure TMP was calculated as TMP = (Po+Pi)/2. The used membrane was a CARBOSEP membrane made of zirconium and titanium oxide and carbon support with a Molecular Weight Cut-Off (MWCO) of 10 kDa (M5). The inner and the outer diameters of the membrane were equal to 6 and 10 mm, respectively, its length was 400 mm and the filtration area was 0.0075 m^2. To remove the filter cake, the membrane was cleaned after each run with NaOH (10 g L^{-1}, T = 80-85°C) for 30

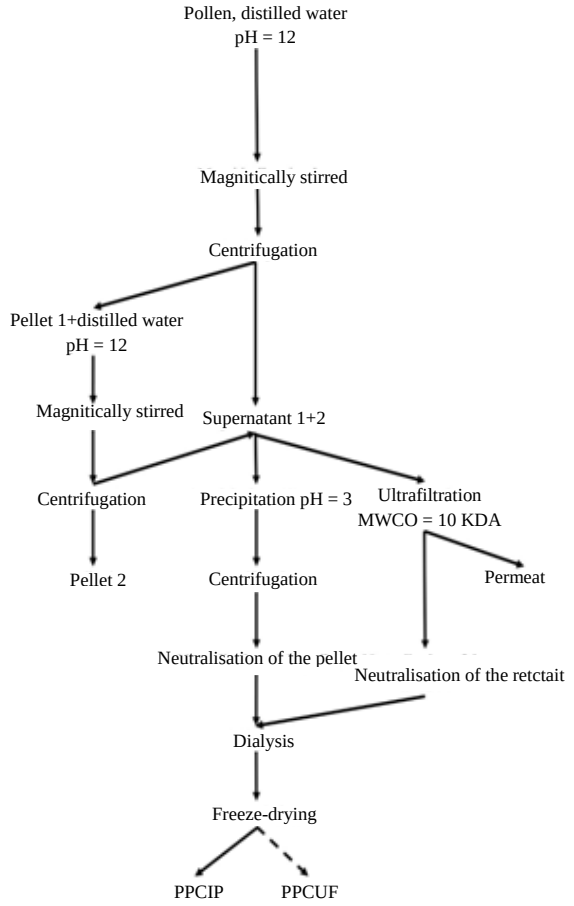

Fig. 1: Extraction procedure conventional isoelectric precipitation and ultrafiltration methods

min with a TMP about 2-3 bars and followed by an acidic treatment with HNO_3 (3-5 m^{-1} at 55-60°C) for 30 min at a TMP of 2–3 bars, until the original water flux was restored. The retentate was neutralized, dialyzed 5 days against ultrapure water and finally freeze-dried (Fig. 1).

The volume concentration factor VCF (the ratio of initial supernatant volume to final retentate volume) was fixed at 6-fold and the corresponding Protein Concentration Factor PCF (the ratio between the protein concentration in the retentate and feed) was calculated after protein quantification. All experiments were performed at 4°C.

Physico-chemical properties

Dry matter and mineral content: Dry matter, mineral content was done according to the Association of Official Analytical Chemists.

Total protein content: Dumas method (elementary analysis: rapid N exceed) was used to determine protein content[12].

Color: Cie Lab parameters (L*, a*, b*) were determined using a color flex EZ (Hunterlab, USA), calibrated with black and white tile. The L* reflects the lightness of the sample, it ranges from 0 (black) to 100 (white) while the a*and b* values represent the redness and the yellowness of a powder, ranging from -100 (green) to +100 (red) and from -100 (blue) to +100 (yellow), respectively.

Optical microscopy: According to Karra *et al*.[12], an optical microscope (Nikon Eclipse E400, Nikon Corp., Kanagawa, Japan) is used to investigatethe morphology of PPCIP and PPCUF. The used microscope is equipped with a digital camera (Digital Sight DS-U3, Nikon Corp.) and connected to the computer in which an image processing software (Lucia Version 4.21, Laboratory Imaging Ltd., Prague, Czech Republic) is installed. Powders were put between the microscope slide and the coverslip. Visual observations were done with a 10x objective. The polarized mode was used to determine crystalline substances.

X-ray diffraction: Bruker D8-Advance Diffractometer (Bruker, Germany) was used to study the (XRD) patterns of the protein concentrate powders. The data were collected in the 2 θ ranges 5-40° with a step size of 0.02°and a counting time of 0.5 s/step[10].

Nitrogen solubility index: The nitrogen solubility index was studied at two pH 4 and 7. A 1% suspension from both samples was prepared and pH was adjusted with NaOH (1M) or HCl (1 M). the slurries where centrifuged 10 min at 10000 g at 25°C and nitrogen content of the supernatant was determined with the dumas method. Nitrogen solubility index was calculated as the percentage of nitrogen in the supernatant referred to as the initial nitrogen concentration of the suspensions. NSI might be calculated using the following equation:

$$NSI = \left(\frac{\text{N in the supernatant}}{\text{N in the initial dispersion}} \right) \times 100$$

Zeta potential: Zeta potential allowed to study the surface charge of both protein extracts as depends on pH (2-12). The surface charge of 0.1% protein dispersions from PPCIP and PPCUF wasanalyzed withthe Delsa Nano C Instrument (Malvern Instruments, Westborough, MA)to determine the isoelectric point through the determination of the surface charge. The curve was obtained using the means of values of three replicates[10].

Surface tension measurement: A 0.5 and 1% protein dispersions from different extract were prepared at two pH 4 and 7. An automated drop volume Tensiometer TVT1 (Lauda, Germany) was employed for the measurement of the surface tension. The dynamic mode

with a syringe volume of 2.5 mL and a drop creation time from 0.07 to 0.8 s/µL was chosen. All measurements were in 25±0.5°C. The speed of drop formation changes the lifetime of the drop. Increasing the drop creation time leads to increasing the concentration of the surfactant molecules in the drop which decreases the surface tension.

Thermal properties

Differential scanning calorimetry: Heat flow was recorded during heating from -50 and 250°C at a scan rate of 5°C/min with TA Instrument Q1000 DSC. Hermetic pans were used. The reference was an empty pan having an equal mass within 0.1 mg. The cell was purged with Nitrogen 50 mL/min. The temperature was calibrated with two standards (Indium, Tonset: 156.6°C, DH: 28.7 J g^{-1}; Eicosane, Tonset: 36.8 °C, DH: 247.4 J g^{-1}).Specific heat Capacity (Cp) was calibrated using a sapphire.

Thermogravimetric analysis: The Thermogravimetric analyzer (Mettler Toledo DSC/TGA 1-star system) was used to measure weight change during heating the samples from 25 to 800°C with a step rate of 5 °C/min. 10 mg of each sample was placed in a ceramic pan. The Nitrogen gas flow rate was kept constant at 35 ml/min. The experiments were performed in triplicate to test the repeatability of the device. Data is collected automatically to get the weight loss rate curve.

Statistical analysis: All given values were the mean of three replications and were expressedas the mean±standard deviation (x±SD). Significant differences between the mean values ($p \leq 0.05$) were determined by using the student's t-test.

RESULTS AND DISCUSSION

Chemical composition: The proximate composition (dry matter, ash and protein) of pollen protein concentrates extracted with conventional isoelectric precipitation and ultrafiltrationmethods were given in Table 1. Protein content was relatively high (>60%) for both extracts which allow us to classify them as protein concentrates. A significant difference ($p<0.05$) was noticed between PPCIP and PPCUF having 80 and 73% protein, respectively. This can be attributed to the retention of high MW protein through the membrane during the ultrafiltration process. Then, the observed difference demonstrates that DPP proteins are constituted with proteins having mostly a MW superior to 10 Kda (MWCO of the ultrafiltration membrane) and PPCIP contains a mixture of proteins with a MW superior and inferior to 10 KDa. The dry matter content was similar in both protein extracts (85.8 and 85.5% for PPCIP and PPCUF, respectively). However, significant differences ($p<0.05$) have been reported between two samples in ash contents. PPCIP presents lower ash content (7.94%) than PPCUF (9.11%) which proves that ash is probably linked to proteins having a MW superior to 10 KDa which has led to its retention when passing through the ultrafiltration membrane.

Physical properties

Color: Color is considered an important quality parameter that affects the consumer's food product acceptability. Several conditions could influence the color of foodstuff including drying process (freeze-drying, spray-drying, oven drying). The colors characteristics given in Table 1 of the two pollen protein concentrate (PPCIP and PPCUF) were significantly different ($p<0.05$) in spite of the similar value of yellowness (28.1and 30.3 for PPCIP and PPCUF, respectively). PPCUFhad the highest value of L*(60.1) but the lowest value of a*(2.9).

Ghribi *et al*.[13] reported that freeze-drying is the most effective method that controls the Maillard reaction that causes the darkness of the product since freeze-dried chickpea protein concentrate presented a lightness near 65 which clearly explains the obtained lightness value of both freeze-dried extracts in this study. Based on color parameters values, we may also conclude that PPCUF displays the more attractive color due to its highest lightness and lowest redness values[14] and that both extracts could be added in food formulations as novel ingredients without having a great impact on the color parameters of the food systems.

X-ray diffraction: The X-Ray Diffraction (XRD) was conducted to provide detailed information about the crystallographic structure of PPCIP and PPCUF (Fig. 2a). Results proved that PPCUF flours were characterized by an amorphous structure contrary to PPCIP flours which were characterized by crystalline one. Probably, the crystallinestructure of PPCIP flours is due to the protein fraction whose molecular mass is <10 KDa. Based on several types of research that confirm that an amorphous structure provides better techno-functional properties when compared withcrystalized flours, we suggest that PPCUF flourscould givegreat functionality when used in functional food applications[10, 13].

Optical microscopy: The morphology of PPCIP and PPCUF was shown in Fig. 2 b. Under polarized mode, the micrograph of both concentrates confirmed what has been discussed in the X-ray diffraction part. The clear variation in areacolor and the presence of yellow and blue colors in the case PPCIP (Fig. 2 b) confirming the presence of crystalline regions which might limit the functionality of proteins such as solubility and surface properties[15].

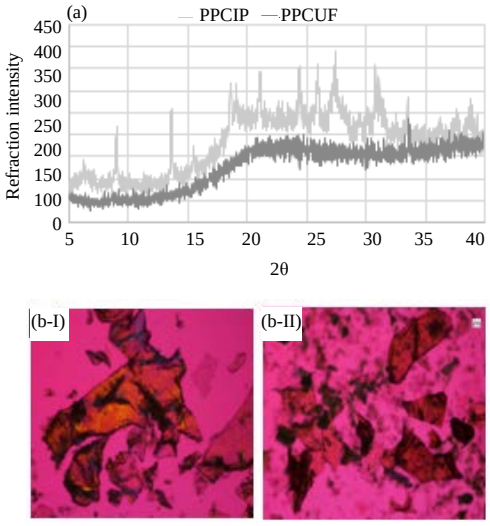

Fig. 2: Physical properties of data palm pollen concentrates obtained with isoelectric precipitation PPCIP and with ultrafiltration PPCUF (a) X-ray diffraction and (b) Optical microscopy (I) PPCIP and (II) PPCUF

Table 1: Physico-chemical properties of date palm pollen protein concentrates

Parameters	PPCIP	PPCUF
Dry matter (%)	85.80±0.28[a]	85.54±0.24[a]
Ash (%)	7.94±0.32[a]	9.11±0.15[b]
Protein (%)	80.94±0.34[b]	73.54±0.32[a]
Color parameters		
L*	46.98±1.05[a]	60.06±0.35b
a*	8.69±0.06[b]	2.90±0.06a
b*	28.70±0.68[b]	30.29±0.24[b]
NSI* (%)		
pH 4	48.13±0.15	45.36±1.34
pH 7	58.36±1.76	92.75±0.77

NSI: Nitrogen solubility index; DM: Dry Matter. All the data are expressed as mean±SD and are the mean of three replicates; Means with the different superscript letters within the same line are significantly different ($p<0.05$)

Nitrogen solubility index: Protein solubility is important in food systems since it affects other functional properties and serves as a useful indication of the performance of protein extracts.

NitrogenSolubility Index (Table 1), determined in two pH 4 and 7, showed that PPCUF was much moresoluble than PPCIP at pH 7 with 92.7 against 58.3%. Also, in pH 4, Nitrogen Solubility Index values decreased to become 45.3 and 48.1% for PPCUF and PPCIP, respectively. The difference in the solubility of PPCs may be attributed to different extraction methodsand the composition of each concentrate. Results suggest that high MW components existing in PPCUF are more soluble than those with different MW in PPCIP. Such findings could be explained by the fact that a high

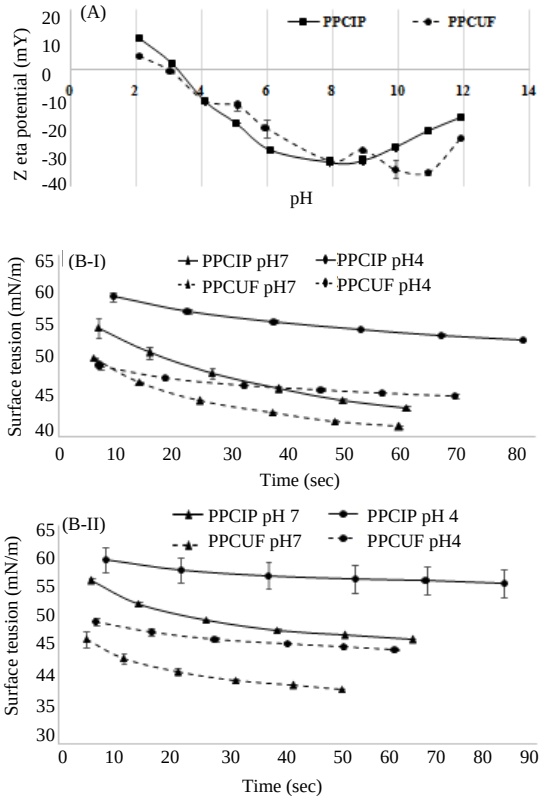

Fig. 3: Surface properties of date palm pollen concentrates obtained with isoelectric precipitation PPCIP and with ultrafiltration PPCUF (A) Surface charge and (B) Surface tension (I) Concentration 0.5 g/100 mL and (II) Concentration 1 g/100 mL

MW component contains more exposed hydrophobic regions enhancing the water-molecule interactions and increasing solubility. Conventional isoelectric precipitation method, contrary to ultrafiltration one, had probably affected the protein structure and caused its partialdenaturation and aggregation in both pHs which prevented its solubilization.

These observations confirm what has been suggested in the X-ray diffraction and the optical microscope sections showing that crystalline powder (PPCIP) is less techno-functional than amorphous one (PPCUF). DPP protein extract obtained by the ultrafiltration method showed a higher solubility index in pH 7 than those obtained for chickpea protein concentrate (48.33 %)[13].

Since, proteins with low solubility have limited functional properties and more limited uses[16], thus, PPCUF could be the originof novel ingredients or techno-functional additives effective for use in food, cosmetic and pharmaceutical fields (Fig. 3).

Table 2: Weight loss values obtained by Thermogravimetric analysis of date palm pollen protein concentrates

Step 1		Step 2		Step 3		Step 4	
Temperature °C	Weight loss (%)	Temperature °C	Weight loss (%)	Temperature °C	Weight loss (%)	Temperature °C	Weight loss (%)
PPCIP 0_120	-12.29±0.02[b]	120_185	-8.53±0.03[a]	185_245	-31.36±0.15[b]	245_310	-9.90±0.02
PPCUF 0_150	-9.64±0.04[a]	150_235	-30.14±0.06[b]	235_300	-16.01±0.04[a]	- -	

Means with the different superscript letters within the same line are significantly different ($p<0.05$)

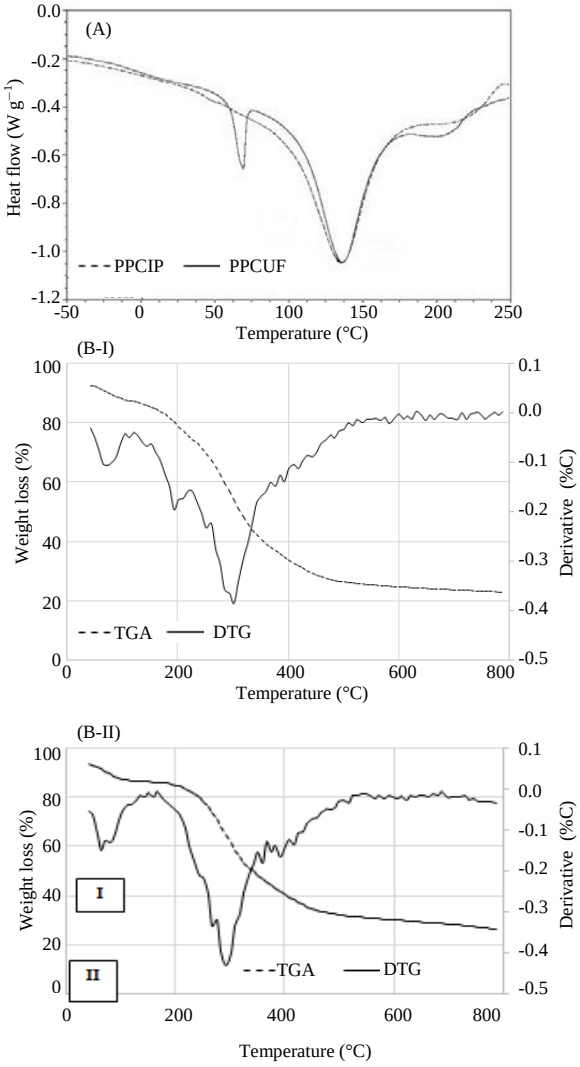

Fig. 4: Thermal properties of data palm pollen protein concentrates obtained with isoelectric precipitataion PPCIP and with ultrafiltration PPCUF (A) Differential scanning calorimetry and (B) Thermogravimetric analysis (I) and PPCIP (II) PPCUF

Surface properties

Zeta potential: Zeta potential provides a measure of the net surface charge and potential charge distribution at the interface. The zeta potential of PPCUFand PPCIP protein extracts were positive at pH 2 (+10.6 mV and+4.5 mV for PPCIP and PPCUF, respectively) became less positive with increasing pH until it reached zero (around pH 3), which is the main reason of the molecular attraction forces leading to the decrease in solubility values. It became increasingly negative as the pH was further increased, until it reached a value of -26.8 mV and -34.2 mV for PPCIP and PPCUF, respectively at pH 10 in which molecular repulsion forces are important and help increasing solubility values (Fig. 4a). From obtained values, it's clear that PPCUF is the most electro-negatively charged and this explains the higher solubility values discussed in previous section. Similar isoelectric points were obtained in our previous study for pollen protein concentrate extracted with isoelectric precipitation method and for pollen protein concentrate extracted with the sonication pretreatment[10].

Surface tension measurement: A protein is characterized as a good surfactant if it can rapidly adsorb at the interface, undergo rapid conformational change and rearrangement or reorientation at the interface and form a cohesive film at the interface.

The ability of protein concentrates prepared in this study (PPCUF and PPCIP) to lower the interfacial tension between the air-water interface was investigated (Fig. 4b). Both concentrates were able to decrease the interfacial tension. Nevertheless, several differences were observed whatever was the pH and the concentration. In fact, PPCUF and PPCIP reduced the surface tension at pH 7 better than at pH 4. This can be attributed to the solubility values as previously discussed. In fact, proteins were more surface-active in the pH of their solubility, thus, in their isoelectric point, they were less surface-active[10].

In addition, protein concentration in dispersions affected significantly the surface tension of pollen protein concentrates. At pH 7, PPCIP exhibited a lower surface tension at 0.5 g/100 mL protein dispersion than that of 1 g/100 mL protein dispersion reaching 43.9 and 46.6 mN/m, respectively. However, at the same pH value, PPCUF was more surface-active at 1 g/100 mL protein dispersion than that 0.5 g/100 mL protein dispersion attaining at the equilibrium point 38.7 and 41.3 mN/m, respectively. Obtained values could be explained in the for PPCIP, by the fact that the mixture of proteins having different MW could be only surface active at a low concentration which might reflect that proteins with MW inferior to 10 KDa are the responsible for the limited surfactant property. They could prevent proteins with MW

superior to 10 KDa from reaching the interface. For PPCUF, proteins with MW superior to 10 KDa are the main responsible for reducing the surface tension. According to these results, we confirm our founding in our previous study[10] that whatever the extraction method (isoelectric precipitation, isoelectric precipitation with sonication pretreatment or ultrafiltration) pollen protein concentrates constitutes natural surfactants which can be used to ameliorate the organoleptic properties of food systems. Reduced surface tension reflects improved functional properties including emulsifying and foaming properties which are important characteristics of protein products utilized in the ground and canned meat formulations, baked goods, doughnuts, pancakes, soups and whipped cakes and desserts[8].

Thermal properties

Differential scanning calorimetry: DSC is an effective tool to predict the behavior of protein powders in food systems. It determines the denaturation temperature and the glass transition temperature and the quantity of energy needed to denaturize proteins. Results, presented in Fig. 4a, showed that PPCIP exhibited two endothermic peaks at 70 and 135°C related to the denaturation of the different constituent existing in this concentrates. For PPCUF, one major denaturation peak around 135°C was observed. Ghribi *et al*.[13] reported a single endothermic peak ranging from 127-136°C for chickpea protein concentrates. Karra *et al*.[12] claimed that the endothermic peak around 130 °C is attributed to male date palm flowers proteins. From these values, we might suggest that the observed peak at 135°C is mainly related to the existing protein in both concentrates and since it persists in PPCUF, then, we could conclude that proteins having a MW superior to 10 KDa might denaturize at 135°C. For PPCIP, the second peak is probably due to the component that conferred the crystalline structure, discussed in the X-ray diffraction part and that might be soluble dietary fiber or lipid fraction[12, 17].

Thermogravimetric analysis: DPP protein concentrates were also submitted to TGA in order to discover the weight loss during heating. Results from Thermogravimetric analysis were in agreement with those obtained in DSC. In fact, the Thermogravimetric curve (Fig. 4b) could be divided into steps as shown in Table 2. Steps generally include water loss, carbohydrate decomposition, the oxidation of carbonaceous material, carbonization of the material and ash formation[18]. The weight loss values demonstrate that the weight loss of the PPCIP (60%) was relatively greater than the PPCUF (55%). Data showed that the weight loss was divided into three steps for PPCUF with an additional step in for PPCIP. For the latter concentrate, the first step could probably be attributed to the loss of the component that gave the first endothermic peak in DSC. Then, the second step which weight loss is similar to that of the first step of PPCUF, might be the loss of proteins having a MW >10 KDa that denaturized around 135 °C in DSC. The maximum weight loss (31 and 30% for PPCIP and PPCUF, respectively) was recorded until 245 and 235°C for PPCIP and PPCUF, respectively. This step might correspond to the carbohydrate decomposition and carbon aceous material's oxidation. The rest of the weight loss, in the remaining steps was due to the carbonization of the material and the ash formation.

The thermal properties of the DPP protein concentrates clearly demonstrates the high thermal stability of their components, especially, for PPCUF whose molecules remain at the native form until 135 °C. In food industries, several technologies, implying thermal treatment could be applied such as spray-drying, pasteurization, extrusion, etc. Based on these analyses (DSC and TGA) and taking into account the weight loss of both concentrates, we might conclude that PPCIP could be used in water-rich products such as meat products while PPCUF could be used in extruded products such as snacks to add crunchiness.

CONCLUSION

As a conclusion, proteins having a MW greater than 10 KDa, obtained by the ultrafiltration technique are characterized by) An amorphous structure that might be the source of better techno-functional properties in comparison with crystallized powders) better thermal stability up to temperatures around 135°C versus denaturation at lower temperatures for compounds having a MW below 10 KDa contained in protein concentrate obtained with isoelectric precipitation method.) Interesting surface properties thanks to their solubility compared to those obtained by isoelectric precipitation method. Thus, applying ultrafiltration to recover DPP proteins is a promising method that helps to provide a novel ingredient that might be used in food formulations.

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