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Separation and Characterization of Water Soluble Protein Fraction from *Moringa oleifera* Seeds

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Abstract

A simple and relatively inexpensive method was developed in order to separate and purify the water soluble fraction of proteins present in dry dehulled Moringa seeds directly collected from local trees. Procedure includes: (a) extraction of the oil fraction from *M. oleifera* seeds using petroleum ether at room temperature; (b) aqueous extraction of defatted seeds; (c) fractionation of the resulting aqueous phase by cooling at 4 ^{o}C and separating the precipitated solid by centrifugation; and (d) freezing and vacuum drying of the wet solid. Protein fraction obtained consists of a mixture of molecular species of different sizes, with those around 26.5, 21.0 and < 14.2 KDa present in greater proportion, which shows interesting acid/base properties. Moreover, two important aspects have emerged from analyses performed to this protein sample: (a) a slightly acidic character was observed by potentiometric and conductimetric titrations which could be justified by the presence of tyrosine phenolic groups in the protein chains forming the mixture, as it has been inferred by FTIR studies, and (b) bathochromic displacements observed during UV-visible studies for the signal associated to $\pi \rightarrow \pi^*$ transitions of the peptide bonds can be attributed to conformational changes which modify natural α-helix structure of the protein, especially those caused by rupture of hydrogen bonds probably involving phenolic groups of tyrosine.

Keywords: Moringa seeds; Protein conformational changes; Conductimetric/Potentiometric titrations; FTIR characterization; Electrophoretic profiles.

1 Introduction

Moringa oleifera can be considered as a truly striking tree because of the larger number of interesting applications that have been either proposed or attained for its different parts, especially those related to the health and food sectors **[1]**. These applications are in sustained growth by the extensive research developing in different fields with these biomaterials. A brief list of some potential applications perceived from a quick literature review, and discriminated according to the employed parts of the plant, is presented in the table 1.

Table 1. Selected potential applications of different parts of *Moringa oleifera* tree.

Part	Field	Potential applications
Roots	- Medicine	- Extracts are promising candidates for treatment of ovarian cancer **[2]**.
	- Veterinary	- Aqueous extracts can be an alternative to synthetic antibiotics in combating poultry diseases **[3]**.
Stems	- Energy	- Enhanced cellulose preparation for production of bioethanol **[4]**.
	- Medicine	- Obtainment of anti-diabetic agents from the alcoholic and hydro-alcoholic extracts of Moringa stems **[5]**.
Leaves	- Food	- Dried leaves of Moringa can be considered as a potential source of natural antioxidants in human diet **[6]**.
	- Medicine	- Pain treatment using polar and no polar leaves extracts **[7]**.
Flowers	- Public health	- Larvicide for mosquito control **[8]**.
	- Food	- Food fortifier (i.e., to improve the nutritional value of food maize or millet gruel **[9]**).
Pods	- Water purification	- Preparation of adsorbents for aqueous phase removal of organic pollutants **[10]**.
	- Medicine	- Obtainment of hypotensive agents from alcoholic extracts **[11]**.
Seeds	- Food	- Production of oil **[12]**.
	- Water purification	- Coagulant for reduction of water turbidity **[13]**.

Probably, seeds are the most studied part of the so-called miraculous plant, especially because this is the raw material for obtain the reputed "Ben oil", which has been recognized since ancient times as one of the best base oils for perfumery. However, interest in Moringa's seeds has been more recently moving towards other fields, particularly trying to promote cheap water treatment systems to be applied in poor regions, including reduction of parasite eggs counts in agricultural irrigation systems **[14]**, bioremediation of water contaminated with usual [**15, 16**] or emerging **[17]** pollutants, flocculation of microalgae **[18]**, etc.

Regarding its use as a coagulating agent, it is widely accepted that activity of the aqueous extracts of *M. oleifera* seeds is caused by the presence of oligopeptides and proteins **[19-23]**. However, because of the varying procedures employed to obtain these proteins, including the use of dry seeds dehulled or not, defatted using different approach and solvents or not, water or aqueous saline extraction and the subsequent fractionation or not of the aqueous-soluble fractions, among other possible variations, it has been rather than difficult to compare the different results reported for both its characterization and their activities in some examined applications.

In order to achieve an easy and cheap method to obtain these proteins as well as to gain insight into the nature of active compounds from *M. oleifera*, a sample of proteins separated by precipitation at 4 ^{o}C of the water-soluble fraction from dehulled and defatted seeds has been chemically characterized. Results obtained are discussed and compared with those reported in recent studies.

2 Experimental

2.1 Materials. *M. oleifera* seeds were collected directly from the local trees on February 2017 at Cooperativa El Esfuerzo, Obispo Ramos de Lora municipality, Mérida state, Venezuela.

2.2 Reagents and Solvents. All reagents were employed as received from suppliers. IQE: NaOH, methanol; Riedel de Haen: HCl (37 %), NaCl (99.8 %) and petroleum ether (40-60 ^{o}C), 2-mercaptoehanol (99.0 %); Sigma-Aldrich: sodium dodecyl sulfate (99.0 %), glycine (99.0 %), acrylamide (99.0 %), sodium azide (99.0 %), sodium sulfate anhydrous (98.0 %); Merck: ammonium persulfate (98.0 %), sodium phosphate (99.0 %), sodium acid phosphate (98.0 %); Fischer Scientific: acetic acid (99.7 %); Bioreagents: N,N-methylene-bis-acrylamide (99.5 %).

2.3 Protein Extraction. Protein extraction was carried out according to preliminary results obtained in the laboratory, after modifying some previously reported procedures **[22, 24]**. The procedure used can be summarized as follows:

2.3.1 Separation and Defatting of M. Oleifera Seed. The pods were harvested directly from the trees in a state of marked dryness. Seeds were extracted from the inside and placed in a sunny place for one hour to eliminate as much moisture as possible; subsequently they were dehulled and ground manually using a mortar to obtain an oily mass. Approximately 10 g of this unctuous mass were placed in 40 mL of petroleum ether and left under magnetic stirring for 3 hours at room temperature (~ 23 ^{o}C), the solid subsequently separated by filtration using a filter paper equivalent to Whatman # 1. The defatted powder thus obtained was allowed to dry in an oven at 40 °C to remove the petroleum ether that might still be present.

2.3.2 Separation and Purification of the Protein. Defatted and dry powder was placed in 80 mL of distilled water for 3 hours, under magnetic stirring, at room temperature, after which it was filtered by gravity. Filtrate was refrigerated for about 8 hours at 4 °C emerging a whitish precipitate that was separated by centrifugation (4400 rpm, 4 °C, 15 min.). The solid was placed in 15 mL test tubes, washed with distilled water and centrifuged again, repeating this washing/centrifugation process for 3 times. Finally, the wet solid was placed in a round bottom distillation flask and left to freeze for approximately 12 hours in a refrigerator (-11 ^{o}C) to be subsequently subjected to a freeze drying process at room temperature using a rotary pump. After drying, whitish powder thus obtained was placed in a hermetically sealed bottle and stored until its use in a refrigerator at 4 °C.

2.4 Seeds and Protein Physical-Chemical Characterization

2.4.1Humidity. 100 mg of unctuous mass of M. *oleifera* seeds were weighed in a preweighed oven at 105 °C for 5 hours, with time starting when the oven temperature reached the set value. Subsequently, the crucible was transferred to the desiccator until it reached room temperature and weighed (m_3). This procedure was performed in triplicate. The percentage of humidity is calculated according to the equation 1:

$$\% \text{ Humidity} = 100.(m_2-m_3)/(m_2-m_1) \quad (1)$$

Dry protein was subject to this same process by triplicate, using 50 mg of sample.

2.4.2Ash. 100 mg of unctuous mass of *M. oleifera seeds* were weighed in a preweighed crucible (m_1). The crucible with the sample (m_2) was placed in the oven and kept at a temperature of 550 °C for 5 hours, with time starting when the oven temperature reached the set value. After the required time, the crucible was transferred to the desiccator until it reached room temperature and weighed (m_3). This procedure was performed in triplicate. The percentage of ashes is calculated according to the equation 2:

$$\% \text{ Ash} = 100.(m_3-m_1)/(m_2-m_1) * 100 \quad (2)$$

2.5 Protein Characterization

2.5.1 Acid/Base Potentiometric (Conductimetric) Titrations. 0.0260 g of protein were weighed and placed in a 25 mL graduated cylinder and then 10 mL of the freshly prepared NaOH solution was added under magnetic agitation. Protein solution was titrated with a newly standardized HCl solution (in triplicate), recording the pH (conductivity) values after the addition of each aliquot of HCl and stirring for 1 (2) min. Similarly, a blank was also titrated (10 mL of the freshly prepared NaOH solution).

2.5.2 FTIR Spectroscopy. Spectra were obtained in solid (KBr disc) and liquid phase (NaCl plate) using an FTIR Perkin-Elmer, Frontier model, Spectrophotometer between 4000 and 380 cm^{-1}.

2.5.3 UV-Visible Spectroscopy. 0.0138 g of protein were weighed and suspended in water into a 500 mL volumetric flask; subsequently, the solution is subjected to ultrasound for 30 minutes and filtered by gravity. This solution will be the starting solution for the preparation of protein solutions at different pH values by varying final NaOH concentrations (see table 2 for details). Briefly, the procedure used is as follows: 20 mL of starting protein solution were placed in a 25-mL volumetric flask, the appropriate volume of NaOH solution was added and completed with distilled water. The resulting solution was immediately stirred during 10 min and UV-visible spectrum of the resulting solution measured employing a Shimadzu Model Mino 1240 Spectrophotometer.

2.5.4 Size Exclusion Chromatography. 20 µL of a protein solution (1 mg/mL in phosphate buffer, pH = 6.8) were injected and left to run in the chromatograph Dione LC30, column Waters 112525, under the following conditions: pressure pump = 1900 psi, flow = 0.8 mL/min, temperature detector = 22 °C and phosphate buffer (pH = 6.8) as mobile phase.

2.5.5 Electrophoretic Studies. SDS-PAGE electrophoretic profiles were performed as described by Laemmli **[25]** in 15 % polyacrylamide gels. Samples were prepared in an Eppendorf tube by suspending 0.0100 g of protein in 100 µL of phosphate buffer solution (pH = 6.80; sodium acid phosphate (0.5 M)/sodium phosphate (0.5 M)/sodium chloride (0.5 M) and sodium azide as preservative). Samples were placed during 5 min in a water bath at 100 °C prior to loading in the gels.

Table 2. Preparation of protein aqueous solutions at different pH values for UV-visible analysis.

Experiment	N_{NaOH} (eq/L)	$V_{Protein}$ (mL)	V_{NaOH} (mL)	V_{H2O} (mL)	pH_{final}	λ_{max} (nm)	Abs.
1	---	20	---	5	6.64	194.27	0.3633
2	0.005	20	0.25	4.75	8.25	191.98	0.2128
3	0.005	20	1	4	10.18	196.0	0.1515
4	0.005	20	2	3	10.75	194.0	0.1519
5	0.005	20	3	2	10.94	197.0	0.1357
6	0.005	20	4	1	11.07	206.0	0.0741
7	0.005	20	5	0	11.20	208.0	0.0707
8	0.050	20	1	4	11.62	213.0	0.0674
9	0.050	20	2	3	11.90	214.0	0.0714
10	0.050	20	3	2	12.12	207.0	0.0963
11	0.050	20	4	1	12.25	219.0	0.0649
12	0.050	20	5	---	12.30	224.73	0.0772
13	1.000	20	5	---	13.23	216.00	0.2249

3 Results and Discussion

In order to harm proteins present in Moringa seeds of less as possible a simple process was conducted to obtain them from dry dehulled seeds, which includes (a) extraction of the oil fraction from *M. oleifera* seeds using petroleum ether (40-60 ^{o}C) during 3 h at room temperature (~23 ^{o}C); (b) aqueous extraction of defatted seeds; (c) fractionation of the resulting aqueous phase by cooling at 4 ^{o}C and then separating the precipitated solid by centrifugation; and (d) freezing and vacuum drying of the wet solid (named original protein hereinafter). Data from 6 experiments conducted and oil yield respect to initial dehulled seeds weight can be appreciated in table 3.

Oil extraction process yielded an average value about 16 % with respect to the initial weight of the peeled seeds. In general, it can be considered low compared to some previously reported values, i.e., 31.8 % and 33.5 % obtained by Mani *et al*. **[26]** from peeled seeds through a Soxhlet system, using petroleum ether and hexane, respectively, as solvents during 7 h, and 26.5 % recovered by Marrero *et al*. **[27]** from seeds with shell using hexane as solvent in an ultrasonic bath during 2 h; however, these differences can be justified because of the less exhaustive treatment that was applied in the present study to preserve as much as possible integrity of the proteins. Confirmation of the main characteristic groups present in the extracted oil was carried out, after solvent evaporation at 40 ^{o}C, by FTIR spectroscopy (Figure 1).

Table 3. Results of the oil extraction process from *M. oleífera* seeds using ligroin (40-60 °C) as solvent.

Experiment	N_{ss}	w_{ss} (± 0,0001) (g)	w_{ps} (± 0,0001) (g)	w_{ds} (± 0,0001) (g)	w_{oil} (± 0,0001) (g)	% Oil
1	55	16,3075	10,0266	7,9585	1,5522	15,4808
2	55	15,9678	10,0250	8,0891	1,6766	16,7424
3	56	16,9136	10,0103	7,5338	1,8665	18,6458
4	55	16,2316	10,0287	8,3383	1,7190	17,1408
5	55	16,2069	10,0386	8,1862	1,6008	15,9464
6	55	16,7027	10,0437	7,9320	1,4636	14,5723
Average ± SD		16,4 ± 0,4	10,03 ± 0,01	8,0 ± 0,3	1,6 ± 0,1	16,4 ± 1,4

N_{ss} = seeds with shell number; w_{ss} = seeds with shell weight; w_{ps} = peeled seeds weight; w_{ds} = defatted seeds weight; w_{oil} =oil weight; % Oil = w_{oil}.100/w_{ps}

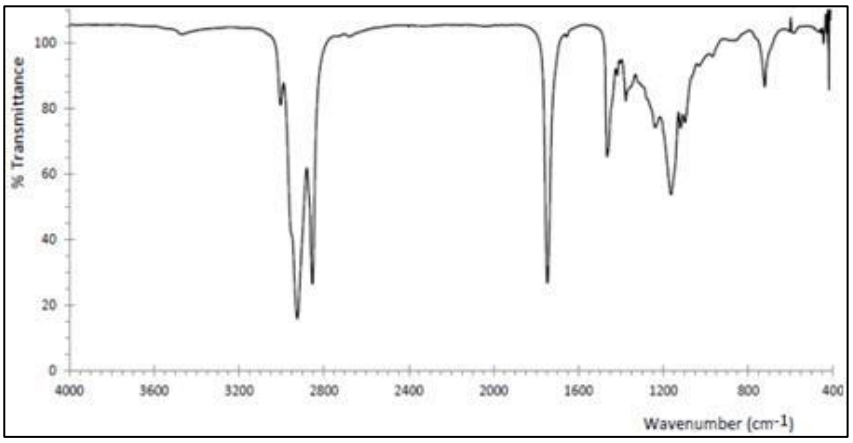

Fig. 1. FTIR spectrum of extracted oil from *M. oleifera* peeled seeds using petroleum ether as solvent during 3h. Liquid sample spread on a NaCl plate.

Table 4 shows humidity and ash percentage for dry dehulled seeds and humidity percentage for original protein. Found average values of % humidity (3,41%) and % ash (2,8%) for dry dehulled seeds are in good concordance with those previously reported by Santos *et al.* **[22]**. In turn, after purification by three washing with distillated water, centrifugation, freezing and vacuum drying, obtained protein exhibited an humidity percentage of 16,9 %, a value considerably higher but according with the usual hydrophilic character of the proteins.

Table 4: Obtained values of humidity and ash percentage for dry dehulled seeds and humidity percentage for original protein.

Sample	Dry dehulled seeds		Original protein
	% Humitidy	% Ash	% Humidity
1	3,483	2,880	17,357
2	3,387	2,679	16,732
3	3,360	2,781	16,568
Average	3,41 ± 0,06	2,8 ± 0,1	16,9 ± 0,4

On the other hand, FTIR spectrum of original protein (Figure 2) shows relevant signals associated to the expected amide groups such as two strong bands at 1656 and 1546 cm^{-1}, characteristics of C=O strength (amide I) and N-H bending (amide II), respectively. Additionally, signal at 3068 cm^{-1} could be associated to presence of aromatic amino acids (=C-H strength) especially because the interesting signal at 1173 cm^{-1}, which is in the region characteristic of the C-O vibration for phenolic groups **[28]**, as well as other spectroscopic evidence shown below, point to the presence of the amino acid tyrosine. This amino acid has been previously reported in protein samples obtained from *M. oleifera* **[29]**.

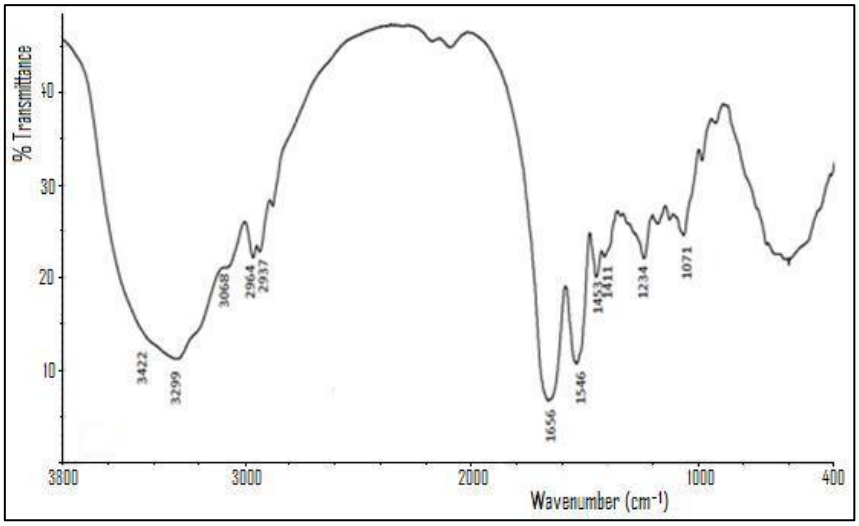

Fig. 2. FTIR spectrum of the protein obtained from dehulled Moringa seeds. KBr disc.

Furthermore, samples of aqueous original protein in excess of NaOH were potentiometric and conductimetrically titrated (in triplicate), using HCl as titrant; the pH and conductivity readings are plotted against the volume of titrant as shown in figure 3 and 4, respectively, where the titration curves of blank solutions (NaOH solution employed to prepare the protein solution) can be also observed.

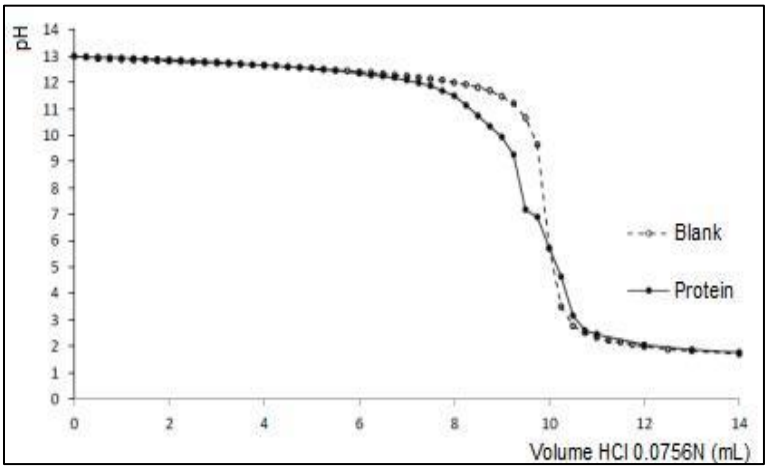

Fig. 3. Potentiometric curves of titration with aqueous HCl 0.0756 N of (a) protein sample and (b) blank.

Titration curve of the protein in Figure 3 shows two clearly defined inflection points (which could be better determined by its first derivative), occurring the first point (P1) at pH = 8.00 (neutralization of free OH^- groups) and the second one (P2) at pH = 3.17 (neutralization of the total OH^- groups). Difference between titrant volumes corresponding to these two points come into the volume of HCl 0.0576 N required to neutralize the OH^- groups bound to the protein, which makes it possible to determine the equivalent of acid groups present in the weight of titrated protein. Calculations are made according to the following relationships:

$$\text{eq. acid groups/g protein} = N_{NaOH}.(V_{P1}-V_{P2})/(1000.w_{prot}) \qquad (3)$$

where V_{P1} and V_{P2} are the volumes (mL) of titrant spent at the first and second inflection point, respectively; N_{NaOH} = normality of the NaOH solution used to dissolve the protein, and w_{prot} = weight of titrated protein (grams). Using the average values of V_{P1} and V_{P2} obtained during titrations

$$\text{eq. acid groups/g protein} = 0.0776 \text{ eq/L}.(10.5 \text{ mL} - 9.5 \text{ mL})/(1000 \text{ mL/L}.0.0261 \text{ g})$$

eq. acid groups/g protein = 0.00294 eq. acid groups/g protein

The inverse of the previous value provides the protein acid equivalent weight, i.e., 336.3 g protein/eq. acid group.

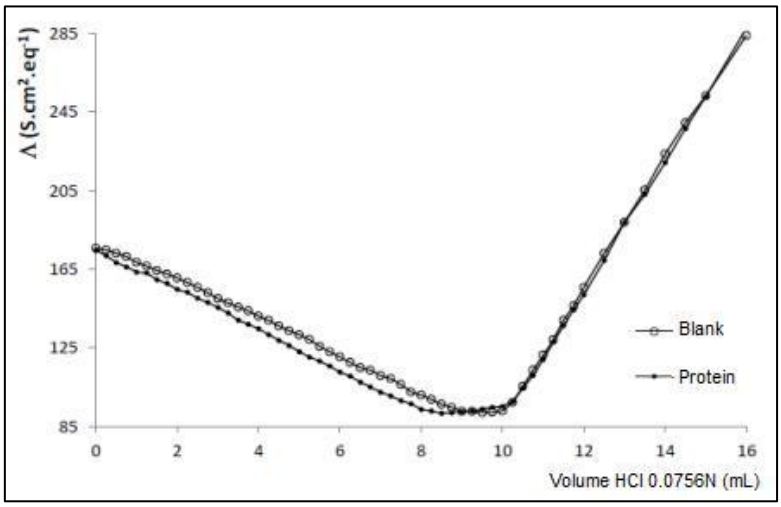

Fig. 4. Conductimetric curves of titration with aqueous HCl 0.0756 N of (a) protein sample and (b) blank.

Regarding to the conductimetric titration curve, three well defined regions of behavior can be seen in the Figure 4: (a) an initial zone, at low volume values of added titrant, where a decrease of the equivalent conductivity (Capital LAMBDA) is observed due to neutralization of OH^- ions (initially in excess) by the addition of HCl to form water (simultaneous generation of chloride ions does not influence far due to its lower ionic mobility); (b) an intermediate zone, where capital lambda increases slightly because now OH^- groups bound to the protein, which have a lower ionic mobility, are being neutralized; (c) a final zone, in which a pronounced increase of increase of capital lambda is observed as HCl is added because fundamentally to hydronium ions that are added in excess after neutralization of all the OH- ions present.

HCl volumes at the two points where slope changes occurring correspond to the volume of HCl required to neutralize the free OH^- groups (V_{P1}) and the total OH^- groups present in the system (V_{P2}); difference between them provides volume of HCl that neutralizes OH^- groups associated to protein. Analogously to potentiometric titration, this volume permit to compute the equivalents of acid groups per gram of sample using the equation 3, where now V_{P1} and V_{P2} are the volumes (mL) of titrant spent at the first and second slope change points, respectively; N_{NaOH} = normality of the NaOH solution used to dissolve the protein, and w_{prot} = weight of titrated protein (grams). However, because it was difficult to obtain precise values of V_{P1} and V_{P2} from the conductimetric titration curve, these values were graphically obtained from the first derivative, both in the conductimetric titration curve of the blank as of the protein (Figure 5).

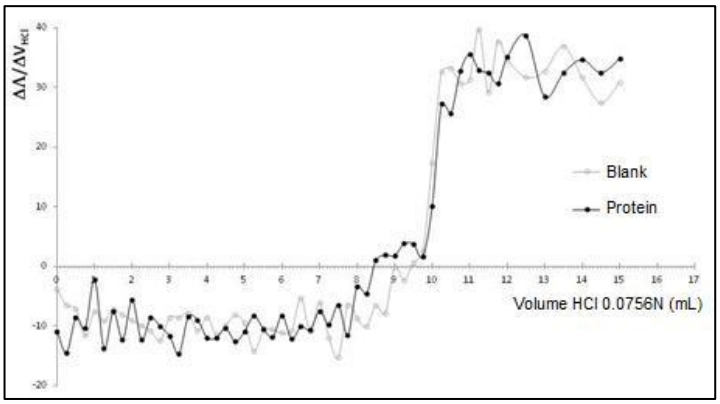

Fig. 5. First derivative curve for the graph of capital lambda vs HCl volume for protein and blank samples conductimetric titration.

Using average values of V_{P1} and V_{P2} obtained during conductimetric titrations permit also to compute the equivalent of acid groups/g protein:

eq. acid groups/g protein = 0.0756 eq/L.(9.45 mL – 8.45 mL)/(1000 mL/Lx0.0262 g)

eq. acid groups/g protein = 0.00289 eq. acid groups/g protein

Similarly, the inverse of the previous value provides an acid equivalent weight of the protein = 346.6 g protein/eq. acid group, a value very close to that obtained by potentiometric titration.

Protein samples precipitated during the potentiometric and conductimetric titrations were subjected to three treatments of washing with distilled water, centrifuged and then frozen and dried under vacuum to be later analyzed by FTIR and PAGE-SDS electrophoresis, in order to establish if these undergo some modification after being submitted to changes of pH during titrations. The results obtained are discussed later.

The purified protein sample, denominated as original (O), was subjected to electrophoretic analysis in order to determine the molecular weight of the protein obtained as well as its purity, i.e., if the extracted protein sample is a mixture of various proteins. During these analyzes, a sample of protein precipitated during titrations, denominated as precipitated (P), was also studied. As it can be observed in figure 6, wells 2 and 4, original protein sample consists of a mixture of different molecular sizes species, exhibiting a greater proportion those that appear at 26.0, 21.0 and under 14,2 kDa; other species that appear with less intensity can be observed at 41, 36, 17.5 and 16.5 kDa. The high intensity signal at 26.0 KDa could correspond to the protein reported by Santos *et al*. **[22]**, who identified it as a lectin of molecular weight = 26.5 KDa having hemaglutinating and coagulating activity. The changes that this protein undergoes after the precipitation in HCl (wells 3 and 5) include the decrease of its intensity in the electrophoretic profile and the appearance of new signals, of very low intensity, below and above it.

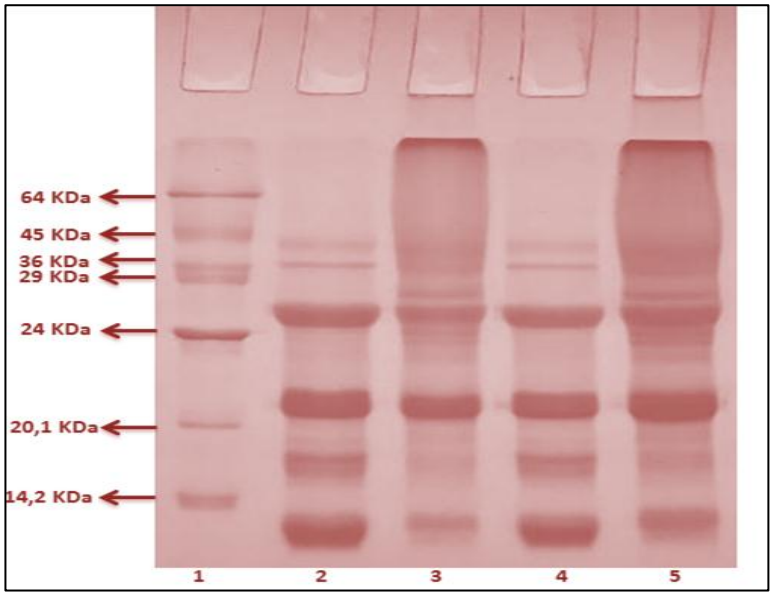

Fig. 6. SDS-PAGE electrophoretic profiles for: 1) Solution with protein molecular weight markers (2 µL); 2) Original protein solution (O), prepared by suspending 10 mg of purified protein in 100 µL of buffer (2 µL); 3) Precipitated protein sample (P), prepared by suspending 10 mg of precipitated protein (obtained during titrations) in 100 µL of buffer (2 µL); Original protein solution (3 µL of solution O); 5) Precipitated protein solution (3 µL of solution P).

A signal of great intensity observed at 21.0 kDa, which does not disappear in the sample precipitated during titration with HCl, does not seem have been previously reported, contrary to the intense signal below 14.2 kDa, which could be attributed to protein of 13 KDa reported by Ndabigengesere *et al*. **[30]**, who characterize it as a dimeric protein with coagulant properties and potentiality to purify turbid waters, although this cannot be conclusively stated due to markers with molecular weight of less than 14.2 kDa were not available. The intensity of this signal is strongly diminished in the profile of the sample precipitated in HCl, inferring that this protein is soluble in acid medium. Similarly, the faint signals at 16.5 and 17.5 practically disappear in the sample precipitated during titration with HCl.

Due to the interesting electrophoretic results obtained for the precipitated protein sample, it was characterized by FTIR hoping to observe some differences with respect to the original sample spectrum. Figure 7 shows an enlargement of the two regions where appreciable changes occur in the FTIR spectra of these protein samples. Thus, signal around 3428 cm^{-1} in the original protein undergoes an increase in its intensity in the spectrum of the precipitated one. This increase could be associated to –OH phenolic groups of tyrosine proposed above, which could suffer a greater displacement from the hypothetical phenoxide/phenol equilibrium towards the phenolic form because during the protein precipitation (dissolved in alkaline medium, pH ~ 12) with HCl the aqueous medium becomes markedly acidic (pH ~ 1.7). The rest of the signals remain without significant changes, except for a slight increase in the signal around 1173 cm^{-1} for the precipitated protein which, interestingly, also could be associated to phenol groups (C-O stretching) **[28]**.

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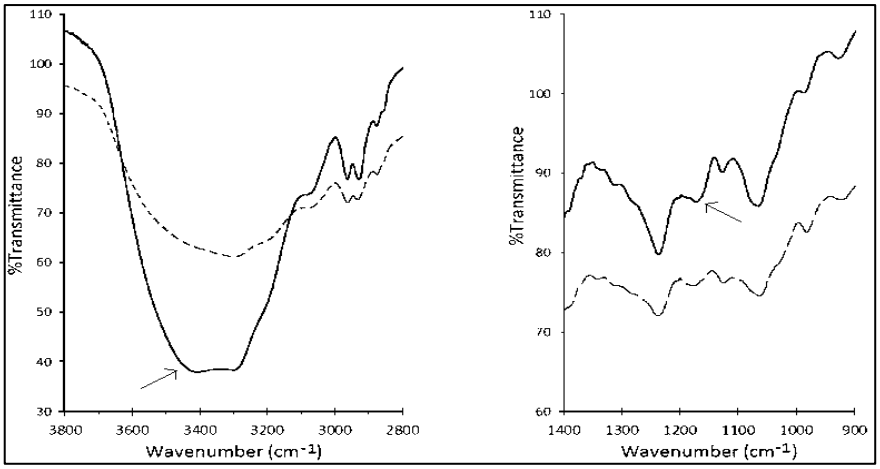

Fig. 7: Enlargement of regions where appreciable changes occur in the FTIR spectra of the original (---) and precipitated (—) protein samples. KBr disc.

In order to complement the protein characterization and trying to detect the occurrence of changes in its aqueous solutions as a function of pH, the original sample was studied by UV-visible spectroscopy (Figure 8a). As it can be seen, the protein in pure water, at its natural pH = 6.64, presented an absorption band at 194.27 nm as well as a less intense shoulder around 222 nm. The band at 194.27 nm appears in the characteristic absorption zone reported for the peptide bond **[31]** and would correspond to the electronic transition $\pi \rightarrow \pi^*$; on the other hand, the shoulder around 220 nm is attributed to the electronic transition $n \rightarrow \pi^*$ **[31]**.

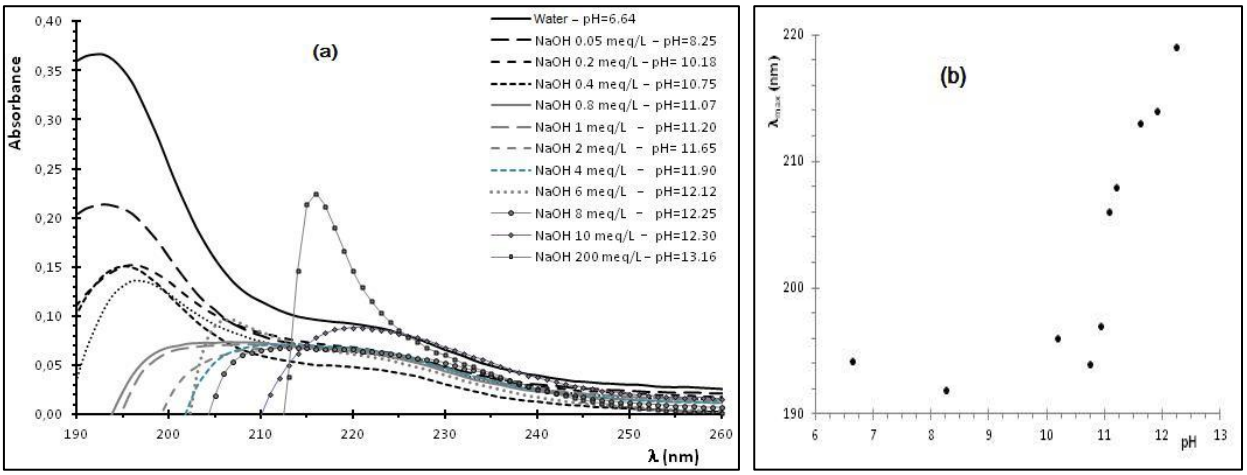

Fig. 8. (a) UV-visible spectra from aqueous solutions of original sample to different pH values (b) Offset of λ_{max} for the signal associated with the transition $\pi \rightarrow \pi^*$ in the UV-visible spectra of the original protein as a function of the pH of their aqueous solutions.

These signals will serve as a reference to compare the changes occurred in the samples with different pH values. Remainder spectra clearly show that absorbance at 194.27 nm decrease as well as that signal bathochromically shifts when pH is increased. Figure 8b allows to comparing more clearly this shift which starting dramatically at pH =10 and have usually been attributed to occurrence of conformational transitions that carry the proteins from a secondary α-helix structure to disordered structures, passing through laminar structures.

These transitions can be caused by several factors, i.e., changes of temperature, solvent, ionic strength and, as seems to occur in this case, by pH changes in the medium. In this sense, Kwaambwa and Maikokera **[32]** extracted a protein from seeds of *M. oleifera* for which proposed a secondary structure dominated by the α-helix form and whose FTIR spectrum was very similar to the obtained for protein studied in the present work. Their proposition is based on the observed FTIR signals as well as in a follow-up to the structural changes showed by the protein as a function of pH of the medium (between 4 and 12) by using circular dichroism. Similarly to the present study, protein obtained by Kwaambwa and Maikokera does not show significant changes until a pH of 10, while for higher values (11 and 12) a change in the secondary structure has been inferred due to the variations of the wavelength maxima shown by spectra of protein solutions in these last conditions.

According to above information it could be assumed that proteins obtained in the present study have a dominant secondary structure type α-helix (due to the similarity of its FTIR spectrum with that one reported by Kwaambwa and Maikokera), and

that bathochromic displacements observed in the signals associated to $\pi \rightarrow \pi^*$ transitions in the UV-visible spectra can be attributed to the occurrence of conformational transitions that modify the α-helix structure of the protein. It is also very important to note that signal associated to $n \rightarrow \pi^*$ transition does not seem to be affected by pH changes of the aqueous protein solutions.

Finally, although it was not possible to perform a calibration curve with molecular weight markers, Figure 9 shows the obtained SEC chromatogram for the original protein sample. It can be qualitatively appreciated that sample consists of a mixture of different sizes molecular species, which appear at long elution times (practically at the end), indicating that their molecular weight should not be very high, in perfect agreement with results obtained by electrophoresis, where three major protein fractions appear around 26.5, 21.0 and <14.2 KDa.

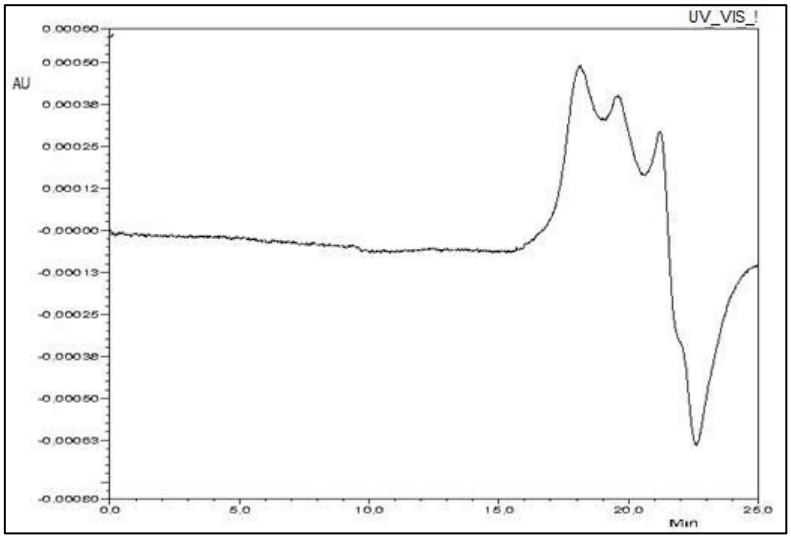

Fig. 9. SEC trace for the original protein solution (25 mg/mL) in sodium acid phosphate (0.5 M)/sodium phosphate (0.5 M), sodium chloride (0.5 M)) buffer.

4 Conclusions

A simple and relatively inexpensive method to separate the water soluble protein fraction from *Moringa oleifera* seeds has been developed. Protein fraction obtained consists of a mixture of molecular species of different sizes, with molecular species around 26.5, 21.0 and < 14.2 KDa present in greater proportion, which shows interesting acid/base properties, especially because the mixture can be purified to a greater extent by reprecipitation in aqueous acidic medium where protein with molecular weight < 14.2 KDa appears to be more soluble.

The two most outstanding aspects emerging from the characterization of the protein studied in this work are: (a) a slightly acidic character was observed by potentiometric and conductimetric titrations which could be justified by the presence of tyrosine phenolic groups in the protein chains forming the mixture, as it has been inferred by FTIR studies, and (b) bathochromic displacements observed for the signal associated to $\pi \rightarrow \pi^*$ transitions of the peptide bond in the UV-visible spectra can be attributed to occurrence of conformational transitions that modify the natural α-helix structure of the protein, especially those caused by the rupture of hydrogen bonds, probably involving phenolic groups of tyrosine.

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