MOLECULAR CHARACTERIZATION OF CRUDE SEED EXTRACTS FROM *Moringa oleifera*

María Martha Sandoval Arreola, José Ramón Laines Canepa and José Roberto Hernández Barajas

SUMMARY

Some natural coagulants have shown to be efficient in removing turbidity and color from water, including the extract of Moringa oleifera *seeds. In this study, the nature of the coagulant in* M. oleifera *was determined by establishing the protein content in extracted solutions and the corresponding molecular weight. The extraction of coagulant from* M. oleifera *was performed using three types of solvents: distilled water, seawater, and a NaCl 1N solution. The protein content of the extracts was determined by the Pierce BCA colorimetric assay, using a Genesis 10 S UV-VIS Spectrofotometer®. For the determination of protein molecular weight, the electrophoresis in polyacrylamide gel (SDS) technique in 12% gel was used, following Laemmli protocol. Each coagulant was prepared using the Okuda method, modifying the extraction process using liquid nitrogen. Protein content was 3160, 3400, and 3640mg BSA·l^{-1}, for distilled water, seawater, and NaCl solution, respectively. The molecular weight determined for the* Moringa *coagulant extracted with distilled water corresponds to a dimer with a weight of 16 and 14kDa for each band. The molecular weight for the salt solutions was the same, 32.35, 6.00 and 5.00kDa for each band. The results show that the modified method for the extraction of the coagulant liquid N_2 yielded homogeneous protein content in each of the solutions and a higher fragmentation with salt solutions. This fragmentation allowed saline solutions to achieve greater removal of turbidity and color. Therefore,* Moringa *seeds is a promising alternative for water treatment.*

Introduction

The coagulant effect of *Moringa oleifera* seeds has been studied seeking an alternative use in water treatment. Its removal efficiency of color and turbidity has been the subject of several investigations at laboratory scale and has been considered as a possible competitor to aluminum sulfate. However, it has been found that its application causes an increase of organic matter in treated water due to the addition of inactive components present in the crude extracts, which cause degradation of the treated water and makes it impossible to store (García *et al*. 2010). To promote widespread use of *Moringa* seed as a coagulant, the application of a suitable method of purification of the crude extracts is required, in order to eliminate the non-active components and to know the nature and characteristics of the active component in the solutions. In this sense, there have been several studies considering the solvent used. Several studies have described the protein as the major active component in the extracts with distilled water, although they vary in determining the molecular weight and isoelectric point. Gassenschmidt *et al*. (1994) identified a molecular weight of 6.5kDa and an isoelectric point >9. Ndabigengesere *et al.* (1995) have described it as a dimeric protein with molecular mass of 12-14kDa and an isoelectric point between 10 and 11. Okuda *et al*. (2001a, b) showed that the active component is in the protein fraction of molecular weights between 12 to 14kDa for the distilled water solution, whereas when the active *Moringa* ingredient is extracted in saline solution, it has a molecular weight of 3kDa, indicating that it is not the same active component. More recently, García *et al*. (2010) identified a protein of molecular weight of 17-26kDa, composed of three subunits of molecular weight of ~10kDa, suggesting that the active component of the crude extract may be formed by a trimer with subunit S-S bonds. The isoelectric point was determined to be between 10 and 11. Madrona *et al.* (2012). found values of molecular weight of ~30kDa for the extract in distilled water. Dörries (2005) determined molecular weights of 4-8kDa when useing a saline solvent. Finally, Bodlund (2013) reports a 6.5kDa protein in salt solutions. In the present work, the behavior of proteins using sea water shall be assessed as well. It seeks to determine the nature of the coagulating proteins as a basis for the development of new strategies for industrial application.

Material and Methods

Preparation of Moringa oleifera seed extract

Moringa Oleifera seeds were purchased from the Pio Renova Company, Mexico City. They were peeled and pulverized in a commercial food stainless steel grinder (Robot Coupe®). The powder obtained was subjected to Soxhlet extraction to remove

KEYWORDS / Coagulating Protein / Electrophoresis / *Moringa oleifera* / Seed Extract /

Received: 09/13/2015. Modified: 06/22/2016. Accepted: 06/28/2016.

María Martha Sandoval Arreola. Master of Environmental Sciences, Universidad Juárez Autónoma de Tabasco, Mexico. Professor, Instituto Tecnológico de Lázaro Cárdenas, Mexico.

José Ramón Laines Canepa. Doctor in Sciences and Tropical Systems Management, Universidad Juárez Autónoma de Tabasco (UJAT), Mexico. Professor, UJAT, Mexico. Address: División Académica de Ciencias Biológicas, UJAT. Highway Villahermosa-Cárdenas km.0.5 S/N, junction Bosques de Saloya, ZP 86150, Villahermosa, Tabasco, México. e-mail: Josra_2001@yahoo.com.mx

José Roberto Hernández Barajas. Doctor in Chemical Engineering Sciences, Instituto Tecnológico de Celaya, Mexico. Professor-Researcher, Universidad Juárez Autónoma de Tabasco, Mexico.

COMPORTAMIENTO Y CARACTERIZACIÓN DE EXTRACTOS CRUDOS DE SEMILLAS DE *Moringa oleifera*

María Martha Sandoval Arreola, José Ramón Laines Canepa y José Roberto Hernández Barajas

RESUMEN

Algunos coagulantes naturales han demostrado su eficiencia en la remoción de turbiedad y color en el agua, entre ellos las semillas de Moringa oleifera. *En este estudio se determinó la naturaleza del coagulante en* M. oleifera *mediante el establecimiento del contenido de proteína en soluciones extraídas y el peso molecular correspondiente. La extracción del coagulante se realizó utilizando tres tipos de solventes: agua destilada, agua de mar y solución 1N de NaCl. El contenido de proteína de los extractos se determinó por el ensayo colorimétrico Pierce BCA, utilizando un espectrofotómetro Genesys 10S UVVIS®. Para la determinación del peso molecular de proteínas se utilizó la técnica de electroforesis en gel de poliacrilamida (SDS) usando geles al 12% y siguiendo el protocolo de Laemmli. Cada coagulante se preparó utilizando el método de Okuda, modificando el proceso de extracción utilizando nitrógeno líquido. El contenido de proteínas fue de 3160, 3400 y 3640mg BSA·l^{-1} para la extracción con agua destilada, agua de mar y NaCl, respectivamente. El peso molecular determinado para el coagulante de moringa extraído con agua destilada corresponde a un dímero con peso de 16 y 14kDa para cada banda. El peso molecular para las soluciones salinas fue el mismo: 32,35; 6,00 y 5,00kDa para cada banda. Los resultados indican que el método modificado para la extracción del coagulante con N_2 líquido permitió obtener contenidos de proteínas homogéneos en cada una de las soluciones y una mayor fragmentación con las soluciones salinas. Esta fragmentación permitió que las soluciones salinas tuvieran una mayor remoción de turbiedad y color. Las semillas de moringa podrían ser una alternativa prometedora en el tratamiento de aguas.*

COMPORTAMENTO E CARACTERIZAÇÃO DE EXTRATOS CRUS DE SEMENTES DE *Moringa oleifera*

María Martha Sandoval Arreola, José Ramón Laines Canepa e José Roberto Hernández Barajas

RESUMO

Alguns coagulantes naturais têm demonstrado sua eficiência na remoção de turbiedade e cor da água, entre eles as sementes de Moringa oleifera. *Neste estudo se determinou a natureza do coagulante em* M. oleifera *mediante o estabelecimento do conteúdo de proteína em soluções extraídas e o peso molecular correspondente. A extração do coagulante foi realizada utilizando três tipos de solventes: água destilada, água do mar e solução 1N de NaCl. O conteúdo de proteína dos extratos foi determinado pelo ensaio colorimétrico Pierce BCA, utilizando um espectrofotómetro Genesys 10S UVVIS®. Para a determinação do peso molecular de proteínas se utilizou a técnica de eletroforese em gel de poliacrilamida (SDS) usando géis de 12% e seguindo o protocolo de Laemmli. Cada coagulante se preparou utilizando o método de Okuda, modificando o proceso de extração mediante utilização de nitrogênio líquido. O conteúdo de proteínas foi de 3160, 3400 e 3640mg BSA·l^{-1} para a extração com água destilada, água de mar e NaCl, respectivamente. O peso molecular determinado para o coagulante de moringa extraído com água destilada corresponde a um dímero com peso de 16 e 14kDa para cada banda. O peso molecular para as soluções salinas foi o mesmo: 32,35; 6,00 e 5,00kDa para cada banda. Os resultados indicam que o método modificado para a extração do coagulante com N_2 líquido permitiu obter conteúdos de proteínas homogêneos em cada uma das soluções e maior fragmentação com as soluções salinas. Esta fragmentação permitiu que as soluções salinas tivessem maior remoção de turbiedade e cor. As sementes de moringa poderiam ser uma alternativa prometedora no tratamento de águas.*

oil by using cyclohexane as a solvent. The solid phase extraction was dried in an oven at 104°C to evaporate the residual cyclohexane. The dried powder was ground in an agate mortar to give uniform grains and was stored at room temperature in tightly sealed glass jars for later use.

Coagulant extraction

An amount of 10g of dry powder was weighted, frozen with liquid N_2 and milled again in an agate mortar to obtain a fine powder. It was dissolved in 1lt of solvent (distilled water, 1N NaCl or seawater) and stirred for 20min at 60rpm. It was vacuum filtered using Whatman 40 paper filter, and later through Millex®-HV 0.45µm filters. The solution was frozen at -15 C to prevent aging, until use.

Total protein content

Total protein content of crude *Moringa* extracts was determined by the Pierce's bicinchoninic acid method (BCA) with colorimetric detection. The control curve was made with bovine serum albumin (BSA) at 0, 2, 4, 6, 10, 15, 20 and 25µl·ml^{-1}. Absorbance was measured at 562nm by means of a Genesis spectrophotometer 10SUV-VIS Spectrofotometer®.

Protein concentration

The protein precipitation technique with trichloroacetic acid (TCA) was used, using the following procedure: A mixture of 20% TCA was applied to the protein samples; the samples were kept on ice for 30min, after which time they were centrifuged for 20min at 14000rpm. The precipitated protein pellets were washed three times with ethanol/ethyl acetate (1:1) and centrifuged for 10min at 14000rpm after each wash.

Denaturation of the protein samples

Final protein pellets were dissolved in 2X Laemmli. They were incubated at 95°C for 10min, then cooled down on ice for 5min and finally centrifuged to begin its analysis by electrophoresis.

Molecular weight determination

Denatured samples were analyzed by polyacrylamide gel electrophoresis (SDS) using 12% gels and following the protocol of Laemmli (1970). A Coomassie G-250 blue solution was used to visualize the protein bands. In order to estimate the molecular weights a prestained SDS-PAGE Broad Range (Catalog # 161-0318) molecular weight standard was used, consisting of proteins of known molecular weights.

Results and Discussion

Total protein content of the crude extracts

The values obtained in the determination of the total

content of extracts prepared are presented in Table I. Okuda (1999) reported a value of 3166mg BSA·l^{-1} of the total content of protein for crude extracts of *Moringa* extracted with tap water. Similarly, Madrona *et al.* (2012) determined for saline solutions a value of 4499.19mg BSA·l^{-1} and for extract with water, a value of 1832.6mg BSA·l^{-1}.

The difference in the values obtained in these three studies indicates that the contents of extracted proteins depend on the method of extraction and purification used. This is a very important factor when considering that the proteins contained in *Moringa* seeds are coagulant agents. Therefore, a higher concentration would be reflected in improved efficiency in water clarification. The lower values in protein content are reported for extracts using distilled water.

TABLE I
TOTAL CONTENT OF PROTEINS IN *Moringa* CRUDE EXTRACTS

Crude extracts	Sample 1 (nm)	Sample 2 (nm)	Mean (nm)	Protein (mg BSA/l)
Moringa in distilled water	0.416	0.514	0.47 ± 0.07	3160
Moringa in 1N NaCl solution	0.610	0.459	0.53 ± 0.11	3640
Moringa in sea water	0.504	0.496	0.50 ± 0.01	3400

Determination of molecular weight of proteins

- With distilled water

Electrophoresis in polyacrylamide gel for the crude extract using distilled water yield a variety of results, supporting the results found in several studies. In Figures 1 and 2 the presence of dimers and trimers can be observed in the resulting gel when distilled water was used as the solvent, as has been found in other studies. For example, Garcia *et al.* (2010) found a trimer with units of a molecular weight of ~10 kDa; on the other hand, Santos *et al.* (2005) and Madrona *et al.* (2012) reported a molecular weight of 30kDa. These results confirm the presence of isoforms of the same protein. The calculation of the molecular weights by the Quantity One 4.6.5 program is shown in Table II. For the extract of *M. oleifera* with distilled water, two very close bands of 16 and 14kDa were observed, so this result suggests the existence of a dimer with two subunits of different of molecular weight, around 15kDa. This result coincides with that of Santos *et al.* (2005) and Madrona et al. (2012), who reported a molecular weight of 30kDa. These units are probably joined by S-S links, as indicated by García *et al.* (2010), who found a trimer with units of ~10kDa. Dörries (2005) suggested the presence of other bands, corresponding to isoforms of the same protein, indicating the presence of proteins of higher molecular weight, only present during the first two days. In Figure 1, this behavior is observed with the presence of a higher molecular weight protein (~41.485kDa).

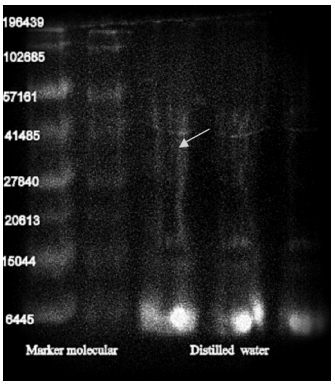

Figure 1. Dimer identification in *Moringa* extract crude using distilled water as solvent.

- With sodium chloride and sea water

The results for the extract with NaCl 1N solution and seawater are shown in Figure 3. Molecular weights estimated by the software Quantity One 4.6.5 were 6.0 and 5.0kDa, respectively, which form a heterodimer of 11.0kDa. Gassenschmidt *et al.* (1994) and Ghebremichael et al. (2005, 2006) found a cationic protein of 6.5kDa. Bodlund (2013) conducted a study of various seeds, including *Moringa*, finding a molecular weight of 6.5kDa for the *Moringa* proteins when it had

TABLE II
DETERMINATION OF THE MOLECULAR WEIGHTS USING QUANTITY ONE 4.6.5 SOFTWARE

Crude extract	Number of bands detected	Molecular weight (kDa)
Moringa in distilled water	2	16.00 14.00
Moringa in 1N NaCl solution	3	32.35 6.00 5.00
Moringa in sea water	3	32.35 6.00 5.00

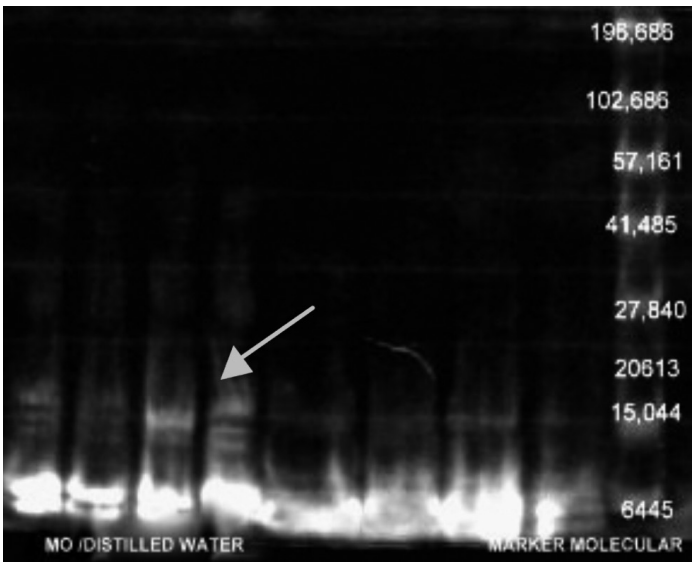

Figure 2. Trimer identification in *Moringa* extract crude using distilled water as solvent.

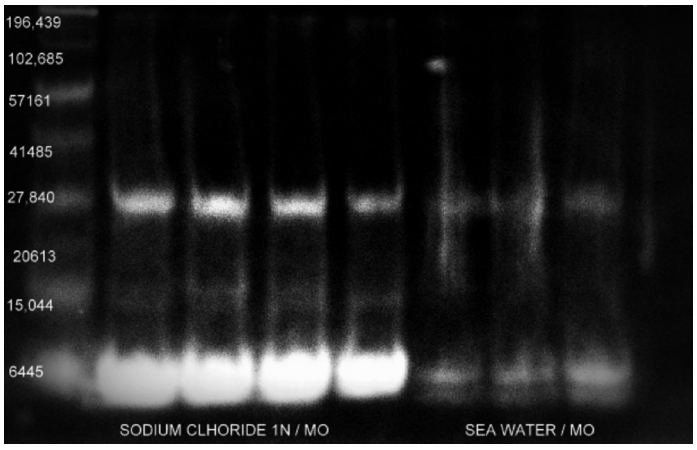

Figure 3. Electrophoresis on 12%polyacrylamide gel for *Moringa* in sea water.

been purified and the presence of other bands in the crude extract; interestingly, in this study a protein of ~30kDa is highlighted, which suggests that it was not possible to break the peptide bonds of protein by the effect of the salt and that it is necessary to improve the method by increasing the stirring time of the solution.

Because *Moringa* seed extracts consist of large amounts of polar amino acids with positive and negative charges, the rupture caused by the effect of salt allows the existence in the molecule with a larger number of radical groups, which in turn promotes the formation of chemical bridges and the adsorption between the coagulant and colloidal substances, allowing brine solutions to be more effective in the coagulation process.

In the case of sea water, no data was found for comparison. However, Figure 3 shows the same type of proteins than those obtained with crude extracts of *Moringa* in NaCl, indicating a probable similarity in the type of proteins extracted with the salt.

Gassenschmidt (1995) suggested a coagulating mechanism for small proteins as they have a positive charge and bind to the surface of negatively charged particles. This leads to the formation of particles with different charge zones and due to the collision between them, follicles formation is promoted.

Conclusion

Active agents found in *Moringa oleifera* aqueous solutions correspond to dimeric proteins with a molecular weight of 30kDa. Active agents of salt solutions of *M. oleifera* are proteins of molecular weights between 6 and 5kDa, forming a heterodimer with a molecular weight of 11-12kDa.

According to the results, the presence of isomorphic forms of the same protein appears to be an acceptable explanation to the behavior found in the same coagulant when distilled water is used as the solvent in the extraction of proteins. Likewise, they suggest a possible degradation due to the effect of glucosinolates and derivatives, which may affect the stability of the samples.

The selection of the extraction method used for improving coagulation efficiency in the water treatment is crucial because it determines the amount of proteins than could be extracted from *M. oleifera* seeds.

ACKNOWLEDGMENTS

This publication is financed with resources from PROFOCIE 2014.

REFERENCES

Bodlund I (2013) *Coagulant Protein from Plant Materials. Potential Water Treatment Agent*. Thesis. Alba Nova University. Stockholm, Sweden. 37 pp.

Dörries SC, Marison IW. (2005*). Coagulants of* Moringa Oleifera *Lam. Seeds: Purification and Characterization.* Thesis Nº 3251. École Polytechnique Fédérale de Lausanne. Switzerland. 133 pp.

García B, Arnal JM, Verdú GIR (2010) Purification of a natural coagulant extracted from *Moringa oleifera* seeds: isolation and characterization of the active compound. Food Innova. International Conference on Food Innovation. (19/25-29/2010). Universidad Politécnica de Valencia. Spain.

Gassenschmidt U, Jany KD, Tauscher B, Niebergall H (1995) Isolation and characterization of a flocculating protein from *Moringa oleifera* Lam. *Biochim Biophys Acta 1243*: 477-481.

Ghebremichael KA, Gunaratna KR, Henriksson H, Brumer H, Dalhammar G (2005) A simple purification and activity assay of the coagulant protein from *Moringa Oleifera* seed. *Water Res. 39*: 2338-2344.

Ghebremichael KA, Gunaratna KR, Dalhammar G (2006) Single-step ion exchange purification of the coagulant protein from *Moringa oleifera* seed. *Appl. Microbiol. Biotechnol. 70*: 526-532.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*: 680-685.

Madrona GS, Branco IG, Seolin VJ, Alves-Filho BA, Fagundes-Klen MR, Bergamasco R (2012) Evaluation of extracts of *Moringa oleifera* Lam seeds obtained with NaCl and their effects on water treatment. *Acta Sci. Technol. 34*: 289-293.

Ndabigengesere A, Narasiah KS, Talbot BG (1995) Active agents and mechanism of coagulation of turbid waters using *Moringa oleifera*. *Water Res. 29*: 703-710.

Okuda T, Baes A. U, Nishijima W, Okada M (1999) Improvement of extraction method of coagulation active components from *Moringa oleifera* seed. *Water Res. 33*: 3373-3378.

Okuda T, Baes AU, Nishijima W, Okada M (2001a) Isolation and characterization of coagulant extracted from *Moringa oleifera* seed by salt solution. *Water Res. 35*: 405-410.

Okuda T, Baes A. U, Nishijima W, Okada M (2001b) Coagulation mechanism of salt solution. Extracted active component in *Moringa oleifera* seeds. Water Res. *35*: 830-834.

Santos AFS, Argolo ACC, Coelho LCBB, Paiva PMG (2005). Detection of wáter soluble lectin and antioxidant component from *Moringa oleifera* seeds. *Water Res. 39*: 975-980.