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Isolation of a 66 KDa Protein with Coagulation Activity from Seeds of *Moringa oleifera*

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Abstract: The seeds of *Moringa oleifera* contain natural coagulants which can be used for clarification of water. Earlier reports have suggested mainly the presence of cationic peptides, ranging in molecular mass from 6 to 16 kDa with a pI value of around 10, for flocculation and coagulation properties. There is no report of any high molecular mass protein component involved in coagulation. Here, we report the isolation of a large molecular mass protein fraction having coagulation activity from the seeds *Moringa oleifera*. The protein was extracted in phosphate buffer and purified by cation and anion exchange column chromatography. The molecular mass of the protein was determined to be approximately 66 kDa by SDS-PAGE method. The coagulation activity of the protein was tested on soil suspension. The activity was found to be comparable with that of cationic peptides and alum. The protein showed no antimicrobial activity.

Key words: *Moringa oleifera* · isolation · protein · soil suspension · coagulation activity · antimicrobial activity

INTRODUCTION

Coagulation-flocculation followed by sedimentation, filtration and disinfection is used worldwide in the water treatment industry. Aluminum salts are by far the most widely used coagulants in water and waste water treatment [1-3]. The seeds of the tropical tree *Moringa oleifera* have been traditionally used for the clarification of drinking water in rural areas of Sudan and Malawi [4-6].

Numerous studies have shown that *Moringa oleifera* seeds possess effective coagulation properties [5, 7, 8] and they are not toxic to humans and animals [9, 10]. The active components in *Moringa oleifera* seeds were found to be soluble cationic proteins and peptides with molecular weight ranging from 6 to 16 kDa and isoelectric pH values around 10 [7, 11, 12]. One of these peptides named MO2.1 was purified and sequenced and was shown to have flocculent activity on a glass powder suspension [12] and also towards bacteria and clay [13]. It was demonstrated that recombinant form of this peptide possesses a bactericidal activity capable of disinfecting heavily contaminated water [14]. Also, a non-protein component of 3 kDa from *Moringa oleifera* has also been shown to have flocculation activity when tested against kaolin suspension [15, 16]. In the present study, we report the isolation of a 66 kDa protein having coagulation activity comparable to that of cationic peptides and alum on soil suspension.

MATERIALS AND METHODS

DEAE Macroprep and CM Macroprep were purchased from Bio-Rad Australia. Centricon and Centriprep were purchased from Amicon (Beverly, MA). Bovine serum albumin was purchased from Sigma Aldrich Pvt. Ltd. Molecular weight standards were obtained from Bangalore Genie, India. All other chemicals were purchased from Qualigens fine chemicals.

The seeds of the *Moringa oleifera* were collected locally and powdered. 10 g of the fine seed powder was suspended in 100 ml of 25 mM sodium phosphate buffer, pH 7.5 and incubated for 10-12 h at 4°C. The extract was filtered through muslin cloth to remove any cell debris and then centrifuged at 8000 rpm for 40 min at 4°C. The supernatant obtained was loaded onto a CM Macroprep column (1.5×20 cm) equilibrated in 50 mM sodium phosphate buffer, pH 8.0. The column was washed extensively and bound proteins were eluted with a step gradient of 0 to 1.0 M NaCl in same buffer. The flow through of CM Macroprep column was loaded onto the DEAE Macroprep column (1.5×20 cm) equilibrated in 25 mM sodium phosphate buffer, pH 7.5. After washing the column extensively, the bound proteins were eluted with a step gradient of 0 to 1.0 M NaCl in same buffer. The desired fractions were concentrated and desalted using 30 kDa cutoff Centriprep and Centricon.

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Protein concentration in crude extract and fractionated protein samples were estimated by standard dye-binding method using bovine serum albumin as standard [17]. Absorbances at 280 nm were also used to determine the protein content of different eluted fractions.

Sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis (SDS-PAGE) under reducing condition was done as described by Laemmli [18]. Relative molecular weight was determined by performing SDS-PAGE of protein with molecular weight standards under reducing condition. The molecular weight standards used were myosin, rabbit muscle (205 kDa), Phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa) and Insulin (3 kDa). The proteins were detected by staining the gel with 0.1% Coomassie brilliant blue R-250.

Samples of turbid water were prepared by adding 10 g of soil in 1.0 liter of tap water. The suspension was stirred for 30 minutes using a magnetic stirrer and then was allowed to settle down for 24 h. The supernatant was carefully removed and stored in a plastic bottle.

The soil suspension was diluted using tap water to produce an initial turbidity of 30 NTU. It was obtained by diluting 400 ml of supernatant in 5 liter of tap water. This turbid water was continuously agitated to prevent sedimentation and change of initial turbidity.

Various concentrations of protein, cationic peptides and alum were used for the coagulation activity assay. For coagulation assay, glass beakers of 100 ml were agitated on magnetic stirrer for 5 minutes. During agitation samples of purified protein, cationic peptides and alum were added in different beakers to a final concentration of 25, 50, 75 μg ml^{-1} and 100 μg ml^{-1}. After sedimentation for 1 h, 5.0 ml of the sample was collected from the middle of the beaker and residual turbidity of different samples were measured using a turbidimeter (HACH 2100AN model).

Antibacterial activity of protein was assayed using Radial Diffusion Method against E. coli. Petri plates were first autoclaved and poured with 20 ml of LB growth medium. They were allowed to solidify and then inoculated with overnight culture of the bacterial strain by spread plate method. Then wells were made with the help of sterile puncher and different amount of protein (5, 10, 15, 20, 25 μl) was loaded in different wells. Phosphate buffer served as negative control and the wells with ampicillin served as positive control. Incubation was done at 37°C for 12 h. The assessment of antibacterial activity was based on the measurement of diameter of zone of inhibition formed around the wells.

RESULTS AND DISCUSSION

The protein was purified by two step procedure on ion-exchange column chromatography. In first step, supernatant was passed through a cation-exchange column (CM Macroprep) equilibrated in 50 mM phosphate buffer, pH 8.0. The protein did not bind to the column and came in flow through. The bound cationic peptides were eluted at 0.5 M NaCl and concentrated in a 3 kDa cutoff Centriprep and then Centricon to a final concentration of 10 mg ml^{-1}. This step helped in achieving some degree of purification by minimizing proteins in flow through and purifying cationic peptides which were used for comparing the coagulation activity with the protein sample. In second step, flow through was loaded on to an anion-exchange column (DEAE Macroprep) equilibrated in 25 mM phosphate buffer, pH 7.5. The protein was fractionated by NaCl step elution and the fractions eluted at 0.3 M NaCl showing coagulation activity were pooled (Fig. 1).

These samples were concentrated and desalted on a 30 kDa cutoff Centriprep and Centricon to a final concentration of 10 mg ml^{-1}. The concentration step with 30 kDa cutoff Centriprep and Centricon ensured that no low molecular mass component is left in the protein solution. The purity of the protein was analyzed by SDS-PAGE analysis under reducing conditions. The Molecular mass of the purified protein was determined to be approximately 66 kDa on a 15% SDS-PAGE (Fig. 2).

The coagulation activity of this protein was tested and compared to alum and cationic peptides on soil suspension, a natural turbid water. The concentrations of protein, alum and cationic peptides used were 2.5, 5.0, 7.5 and 10 mg in different beakers of 100 ml. The change in turbidity in every sample was measured by turbidimeter. The change in turbidity due to protein sample was

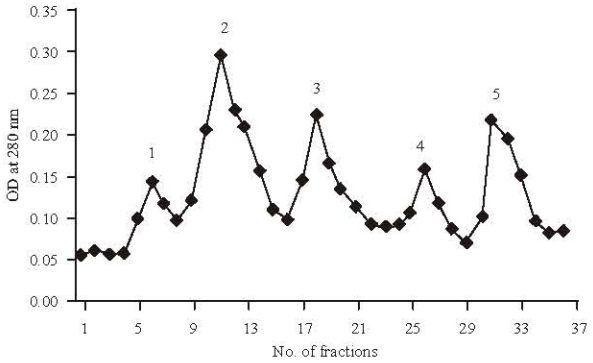

Fig. 1: Purification of protein on anion exchange column. Peak 3 with coagulation activity was eluted at 0.3 M NaCl in 25 mM Phosphate buffer, pH 7.5

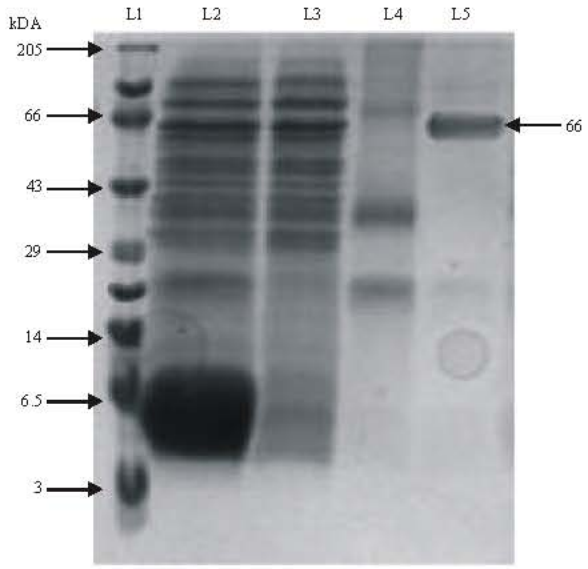

Fig. 2: SDS-PAGE analysis of the protein. L1, molecular weight markers; L2, total protein in buffer extract; L3, Flow through of cation exchange column; L4, Elution at 0.2 M NaCl on anion exchange column; L5, purified protein eluted at 0.3 M NaCl in 25 mM phosphate buffer, pH 7.5 on anion exchange column

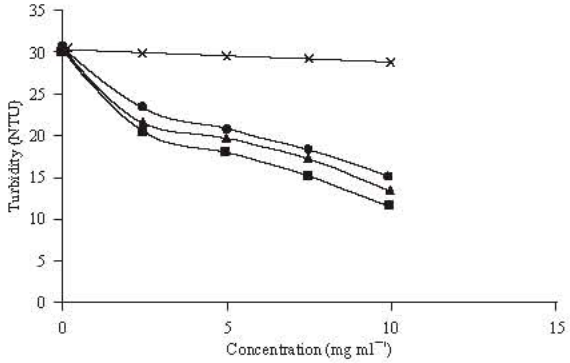

Fig. 3: Coagulation activity of purified protein (=) along with alum (<) and cationic peptides (5) on soil suspension. The Bovine serum albumin (r) was used as a control. All experiments were done three times and averaged

comparable to that of cationic peptides and alum. The turbidity decreased from 30 NTU to 14.8 NTU in beakers with protein sample at 100 μg ml^{-1} concentration. In case of alum, turbidity decreased from 30 to 11.6 NTU at equal concentration. Cationic peptides also showed similar pattern with turbidity being reduced from 30 NTU to 13.4 NTU. Coagulation assay done with BSA as a control showed no activity (Fig. 3). The turbidity, therefore, decreased by 51, 61 and 55% for purified protein, alum and cationic peptides respectively at a concentration of 100 μg ml^{-1}. Although the decrease in turbidity was better in case of alum and cationic peptides, the protein also showed significant and comparable coagulation activity.

The antimicrobial activity was tested on *E. coli* by radial diffusion assay. No zone of inhibition was detected in case of this high molecular mass protein. The results indicate that protein possesses only coagulation activity unlike cationic peptides which have both coagulation and antimicrobial activity.

CONCLUSIONS

In summary, the large molecular mass protein of approximately 66 kDa with coagulation activity on soil suspension has been purified using cation and anion exchange chromatography methods. The coagulation activity of the protein is comparable to synthetic coagulant alum and natural cationic peptides from the seeds of *Moringa oleifera* as tested on soil suspension, a natural turbid water. The purified protein does not have antimicrobial activity unlike cationic peptides isolated from same source which have both coagulation and antimicrobial activity. The flocculation-coagulation activity along with antimicrobial activity of seeds of this plant is a cumulative effect of different active components. The purified active components if used together for water treatment can be more effective in clarifying the water than the individual active components.

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