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Moringa oleifera (drumstick tree) seed coagulant protein (MoCP) binds cadmium - preparation and characterization of nanoparticles

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Abstract

Moringa oleifera is grown globally. It is a multipurpose tree and the seeds are rich in phytochemicals with antimicrobial activities. The crude powder of seeds clarify the turbid and metal contaminated water. *M. oleifera* (drumstick tree) seed coagulant protein (MoCP) was isolated to homogeneity from the crude extracts by carboxymethyl cellulose chromatography (CMC) and gel filtration. The molecular weight of the protein on gel filtration was 13 kDa and in SDS-PAGE it migrated as a single band under reducing conditions with molecular mass of 6.5 kDa (dimeric). Immobilized MoCP selectively binds cadmium from aqueous solutions (pH 2.0-7.0) with maximum binding at pH 6.0 in 180 min when tested at 10-600 minutes. It also bound the metal in the concentration range of 30-70mgL^{-1}. The adsorption kinetics was better described by pseudo second order and the data better explained by freundlich isotherm model than Langmuir isotherm model as in Freundlich model the correlation coefficient (R^2) is high and the calculated q_{max} is very close to the experimental q_{max} rather than Langmuir isotherm model. Furthermore, the nanoparticles of MoCP were prepared and characterized using transmission electron microscopy (TEM). The authenticity of the isolated protein and the nanopraticles prepared was confirmed by specific reactivity with the MoCP antibody raised earlier in our laboratory.

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Introduction

Heavy metals removal from aqueous solutions through biosorption has been well documented in literature (1). Biosorbents in view of their abundance and low-cost are used for the effective removal and recovery of heavy metal ions from wastewater. *Moringa oleifera* phytomass preparations have been applied for dye and turbidity removal in waste waster including fluoride and toxic metals (**Table 1**) (2-30). Cadmium toxicity to biota has been well established in literature. Therefore, removal of toxic substances from water is very important with respect to environmental health, and low cost economical considerations (31). Many methods have been documented for the removal of heavy metals from the aqueous media which include flocculation, ion-exchange, chemical precipitation, and evaporation (32). However, these methods are costly and have low efficiency for the removal of heavy metal contaminants. These methods cause secondary wastes during treatment. Biosorption is the most effective method used for the treatment of waste water (33) . Many biological materials such as seed powder to bark of the plants have been used for industrial scale removal of contaminants (34, 35). However, the component responsible for the adsorption is not known for many biomaterials. In addition from unprocessed biological material it requires a lot of biomass for industrial scale purification. *M. oleifera* seeds showed cleared turbid water and due to the presence of coagulant protein (MoCP) (36). Active coagulant protein identified from the seeds is a dimeric protein and has isoelectric pH between 10 and 11 (37).

The biophysical properties of this protein have been revealed by some studies. Research work in the laboratory is focused on the large scale isolation and affinity purification of biologically important proteins/glycoproteins and to functionally characterize them. As part of this program we have recently purified MoCP and raised an antibody for the same (38). Purified MoCP migrates as a single protein band in SDS-PAGE under reducing conditions with an apparent molecular

Table 1. *Moringa oleifera* applications in biosorption

Brief research finding	Reference
Biosorption of Lead (II) ions from aqueous solution	2
Removal of reactive dyes from their aqueous solutions	3
Removal of organic pollutants from aqueous solutions by biosorption	4
Toxicity assessment and modelling in water purification by whole cell bioreporter	5
M. oleifera husks as biosorbent chromium	6
M. oleifera for phytoremediation of heavy metals polluted soil.	7
Carbon nanosheets derived from *M. oleifera* stems as electrode material for high-performance electric double-layer	8
Removal of Cd (II) and Pb (II) from aqueous environment	9
Fluoride remediation with *M. oleifera*.	10
Evaluation of aluminum sulfate and water-soluble *M. oleifera* seed lectin to reduce turbidity and toxicity of polluted stream water.	11
Bioremediation of heavy metals from water collected from various lakes in Bangalore, India using *Moringa* leaf and bark extract	12
Nutritional and medicinal applications of *M. oleifera*	13
Sorption of cadmium from aqueous system by shelled *M. oleifera* seed	14
M. oleifera leaf extract reduces intracellular cadmium accumulation and oxidative stress in *Saccharomyces cerevisiae*	15
Remediation of contaminated water using *M. stenopetala* and *M. oleifera* seed powder	16
Heavy metals removal from wastewater by *Moringa* pods	17
Development of a magnetic coagulant based on *M. oleifera* seed extract for water treatment.	18
Pharmacologically relevant phytochemcials in *M. oleifera* against photo-oxidative damages imposed by gamma radiation	19
Biosorption of Pb(II) from aqueous solutions using chemically modified *M. oleifera* leaves.	20
Optimization of Cd(II), Cu(II) and Ni(II) biosorption by chemically modified *M. oleifera* leaves powder.	21
Biosorption of Pb^{2+} from aqueous solutions by *M. oleifera* bark:	22
Biosorption activity of *M. oleifera* seeds powder.	23
Removal of lead, iron and cadmium ions by means of polyelectrolytes of *M. oleifera* whole seed kernel.	24
pH dependence of sorption of Cd^{2+}, Zn^{2+}, Cu^{2+} and Cr^{3+} on crude water and sodium chloride extracts of *M.stenopetala* and *M. oleifera*.	25
Coagulant properties of *M. oleifera* protein preparations: application to humic acids removal.	26
Removal of cadmium from aqueous system by shelled *M. oleifera* seed powder.	27
Agroforestry and nutritional potential of *M. oleifera*	28
M. oleifera seed presscake extract for removal of direct black 19 from synthetic wastewater	29
Removal of heavy metals from aqueous solutions by *M. oleifera* seeds	30

mass of 6.5 kDa. On gel filtration it showed a molecular mass of 13 kDa consistent with earlier findings (36). Agro-based lignocellulosics, seed power including seed extracts are considered for Waste water treatment and turbidity removal (34). In view of the recent developments in the improvement of extraction method of coagulation active components from seeds for drinking water treatment, coagulant protein / flocculating protein and applications in treating turbid waters the present work assumes considerable significance. Heavy metals removal using seed powder, apotransferrin loaded nanoparticles and application of nanocomposites is gaining momentum in this field (38,39). Several seed proteins have been shown to effectively bind heavy metals such as Cd and we identified seed proteins from *Strychnos potatorum* to be useful in removal of heavy metals from aqueous media (40).

Therefore, the present study was initiated with the following objectives. i) isolate and purify MoCP from the seeds and immobilize the purified protein and test its ability to bind cadmium from aqueous solutions, ii) prepare nanoparticles of MoCP and make a preliminary characterization in order to test their potentiality in the long run as efficient tools for removal of toxic metals from aqueous solutions.

Materials and Methods

Plant Material

The seeds of *M. oleifera* used in the present study were obtained from the local supplier. *M. oleifera* seeds were deshelled manually and were ground into fine powder with the help of a motor and pestle. The seed powder was defatted with acetone, dried and used for further studies. All other chemical reagents used in the study were of high quality and procured locally from firms.

Extraction and isolation of *M. oleifera* coagulant protein (MoCP)

To isolate the seed protein MoCP, the seeds were deshelled, ground and defatted. From 20g the total proteins were extracted from overnight using 20 mM phosphate buffer saline pH 7.4 containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The extracted suspension was clarified by centrifugation at 12000 rpm for 30 minutes in a refrigerated centrifuge and the supernatant was collected. This supernatant was termed as crude extract. The methodology followed to purify protein is as described with minor modifications (36) . The crude extract was precipitated with ammonium sulphate ((54.2 g/L, 80% final saturation) and the protein pellet was obtained by centrifugation as described above and was dissolved in 20 mM phosphate buffer saline pH 7.4 and further dialysed extensively against the same buffer using a 10k cut off dialysis membrane that retains the intact MoCP. The clear dialysed sample was briefly centrifuged as above and the clear supernatant was applied onto anion exchange CM-52 chromatography gel (25 ml) which was pre-equilibrated with 20 mM phosphate buffer pH 7.4. The gel was extensively washed with 20 mM phosphate buffer pH 7.4 and sequentially eluted with 0.3M NaCl and 0.7 M NaCl. Protein in the column fractions was monitored at 280 nm. The proteins eluted from 0.7M NaCl fractions that contained the MoCP were pooled and concentrated. It was then subjected to gel filtration (Sephadex G-100) chromatography to enrich the MoCP and to remove any minor contaminants. Protein containing fractions corresponding to MoCP (Mr 13,000 kDa) compared with standard proteins, were pooled and concentrated and used in further studies.

SDS PAGE and Western blotting

Aliquots of the protein eluted from the gel filtration column were subjected to SDS-PAGE analysis under reducing conditions (43). After the electrophoresis proteins were detected using coomassie brilliant blue dye

In another experiment, the purified MoCP was separated on a 12.5 % SDS-PAGE and the proteins transferred to a nitrocellulose membrane for western blotting. Both MoCP as well as the MoCP nanoparticles prepared were identified on the membrane using an antibody to the purified MoCP raised in a rabbit and available in the lab.

Preperation of affinity gel containing MoCP

In short, 2 ml of gel was washed sequentially on a sintered glass funnel with ice cold isopropanol, cold water and 0.1M HEPES buffer pH7.4. MoCP protein (mg) in 0.1M HEPES buffer pH 7.4 was added to gel and allowed to couple overnight in cold by rotation on a rotator.The purified MoCP obtained after gel filtration was coupled to affigel-10 (Bio-Rad labs) according to manufacturer's instructions.

Analysis of cadmium binding

The glassware used in the present study was rinsed with 10% (v/v) HNO_3 and washed extensively with double distilled water to remove any other interfering materials present. Stock solution of cadmium chloride was prepared by dissolving 1.6306 g in 200mL of Milli-Q water and final volume was made up to 1000 mL with Milli-Q water. This stock solution contains 1000 mgL-1 of Cd (II) and solutions containing different concentrations of Cd (II) were prepared by diluting the stock solution appropriately. The cadmium levels were measured by using a flame atomic absorption spectrometer (GBC 932 plus, Australia). A standard solution of cadmium (1000 mg L-1) was used for atomic spectrometer which was procured from Sisco Research Laboratories (India). Affigel-10 used for immobilization of protein was procured from Bio-Rad laboratories.

To evaluate if the immobilized MoCP can bind the metals the following experiments were done. The gel prepared above was packed into a column of 2 ml and equilibrated with 25 mM phosphate buffer pH 7.4. The gel prepared was completely used to enable optimal binding using a rotator over night in cold.

The column was washed with de-ionized Milli-Q (Millipore) water. To the gels, 4 ml of (30 mg/l) cadmium metal ion was

added and rotated for six hours at 4°C to ensure optimal binding. Under different conditions discussed below, the unbound solution was collected; the gel was washed with de-ionized Milli-Q water for removing the excess metal ions. The bound metal ions were eluted using 0.15 M HCl. The metal concentrations were measured using flame atomic absorption spectrometer (GBC 932 plus, Australia). The wavelength used for analysis of the metal in this study was 228.8nm. The effect of different pH solutions on the equilibrium adsorption of Cd(II) ions was investigated at a wide range of pH values (pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0). Further studies such as the binding efficiency at different time points, concentrations of metal ions were conducted at pH 6.0.

The adsorption isotherms of cadmium binding at different concentrations was analysed by two adsorption isotherms, Freundlich and Langmuir adsorption isotherms. The Freundlich isotherm represented by the equation $= \text{Log } q_e = \log K_f + (1/n) \log C_e$; where C_e (mg L−1) is the equilibrium concentration; q_e (mg g−1) is the amounts adsorbed per specific amount of adsorbent at equilibrium, K_f (mg g−1) and n are constants which are adsorption capacity and intensity of adsorption, respectively.

Nanoparticles preparation and characterization of MoCP

For the preparation of nanoparticles, the procedure described by (44) with minor modifications was adopted. Purified MoCP 10 mg was dissolved in 500µl of 1x PBS buffer of pH 7.4 and kept on ice for 5 min. 15 ml olive oil was added drop wise with continuous stirring on a cyclomixer.

This emulsion was sonicated 15times, for 30sec with 1min gap. The sample was frozen in liquid nitrogen immediately for 10 min. It was then kept at 4°C for 4 hours and the nanoparticles pellet was collected by centrifugation at 10000 rpm for 10min. The residual oil was removed by washing the pellet with chilled diethyl ether 3-4 times. The clear pellet was dissolved in 20 mM PBS buffer pH 7.4 and the nanoparticles sample was stored at 4°C.

The nanoparticles prepared were characterized by SEM (PHILIPS FEI-XL ESEM, USA). The sample was uniformly dispersed on a clean glass cover slip using a spin coater and dried in a dust free zone. It was studied under SEM following manufacturer's instructions. For SEM analysis of sample metal stubs were coated with double-sided adhesive tape, the cover slip with nanoparticles sample was kept on the sticky surface and sample was coated with gold in Sputter Coater. Samples were stored in dry, dust free environment during the analysis. The size and morphology of the samples were studied.

Results

Extraction, isolation and purification of MoCP

This was done as described under the methods. The protein fractions eluted with 0.7M NaCl from the CM-52 cellulose contained MoCP protein when analyzed by SDS-PAGE analysis (results not shown). The protein fractions containing the MoCP were pooled, concentrated and separated on a Sephadex G-100 gel to remove minor contaminating proteins. MoCP eluted as a single major peak from the gel (data not shown) corresponding to a molecular mass of 13 kDa. Aliquots of this fraction on SDS-PAGE under reducing conditions exhibited a molecular mass of 6.5 kDa (monomer of MoCP) (**Fig. 1**). Purified MoCP was immobilized to affigel at a concentration of 4-5 mg/ml gel.

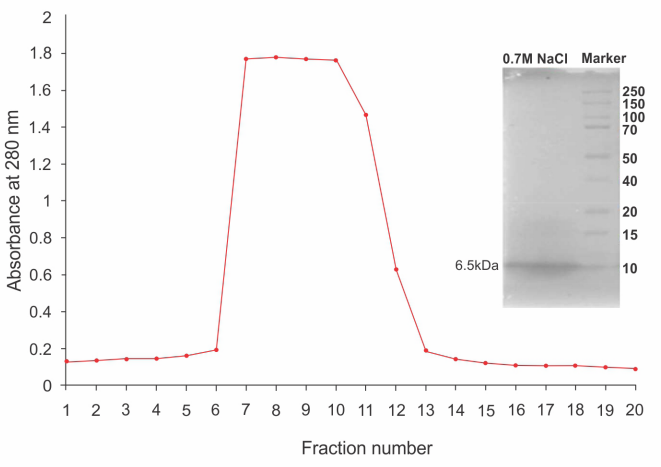

Figure 1. Elution profile of the proteins from the extracts of the seeds on CM-Cellulose gel using 0.7M NaCl, and inset : 10% SDS-PAGE analysis of the fraction 9 from 0.7 M NaCl eluates under reducing conditions showing the monomer of MoCP.

Effect of pH on binding of cadmium to MoCP

To characterize the cadmium binding to MoCP parameters like pH, time, and concentration of the cadmium were taken in our study as described under methods and the metal binding was estimated by flame atomic spectroscopy. The maximum binding was detected at pH 6 (**Fig. 2**). Since pH 6.0 was found to be optimal pH for binding, this was used in further separate experiments.

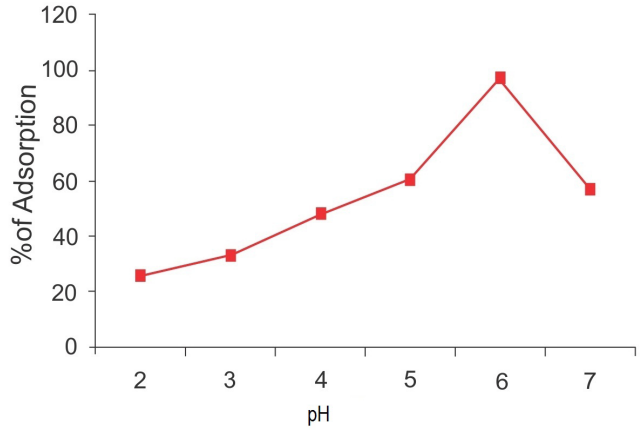

Figure 2. Effect of pH on binding of cadmium to MoCP showing maximum binding at pH 6.

Effect of time on binding of cadmium to MoCP

Time is an important parameter to determine the binding efficiency of metals to the adsorbent. The binding efficiency of cadmium to MoCP was analysed at different time intervals (5, 10, 20, 30, 60, 180, 360, 480 and 600 minutes respectively). The maximal binding was observed at 240 min. The cadmium adsorption increased with increasing the contact time, the maximum removal of cadmium as is evident from the (**Fig. 3**). From the figure it is clear that at 180 min there is maximum removal of cadmium from the solution beyond which there is no significant change.

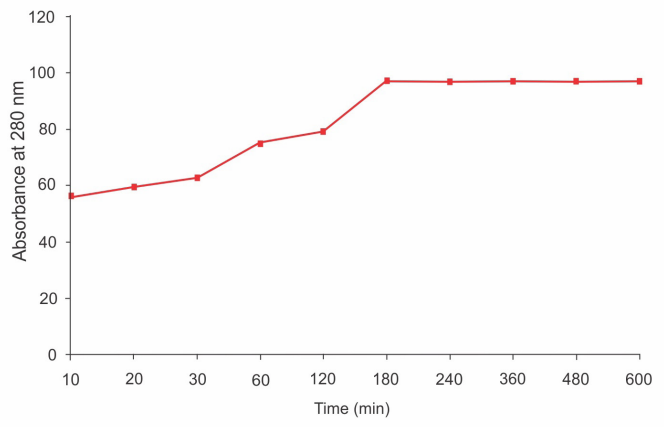

Figure 3. Effect of time on binding of cadmium to MoCP showing maximum binding at 180 min.

Effect of metal concentration on its binding to MoCP

The binding efficiency of different concentrations of the metal ions (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 and 110 mg/l) to MoCP was analysed and 30 mg/l was found to be the optimal concentration (**Fig. 4**).

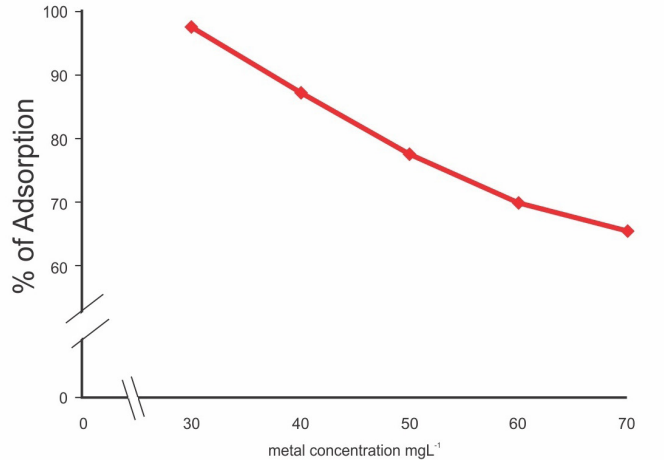

Figure 4. Effect of metal concentration on binding to MoCP.

Adsorption kinetics

The equilibrium metal uptake qe (mg.g-1) and the sorption efficiency (%) was calculated according to the mass balance equations:

Amount of adsorption: q_e (mg.g-1) = [(c_o - Ce) v] / m (1)

Sorption efficiency: % = [(c_o - c) 100] / c_o (2)

Where co and ce are the initial and equilibrium concentrations (mg.l-1), V the volume of solution (L), m is the weight of the protein used (g), and the c is the solution concentration at the end of the sorption process. The information on the kinetics of solute uptake is necessary to select the optimum operating conditions for full-scale batch process. In this study we have used two different models to study the mechanism of cadmium biosorption. This was studied using two models pseudo first order and pseudo second order. The data was better fitted with pseudo second order. The pseudo first order model derived by Lagergren and is one of the most widely used models for the biosorption of solutes from a liquid solution. It is expressed in the following equation:

Log (qe −qt) = log (qe) −Ks_1 / 2.303×t; where qe and qt are the amount of metal ions adsorbed on the adsorbent at equilibrium and at any time t (mg g−1) respectively. Ks1 (min−1) is the Lagergren constant of the pseudo first order biosorption. The pseudo second order model assumes that biosorption follows a second order mechanism, so that the rate of occupation of biosorption sites is proportional to the square of the number of occupied sites. The pseudo second order model expressed as t /qt = t /qe + 1 /$k_2q^2_{e;}$ where k2 is the equilibrium rate constant of the second order biosorption (g mg−1 min−1). The pseudo-second order rate constant k2 and the value of qe were calculated from the plot of t/qe versus t (**Fig. 5**)

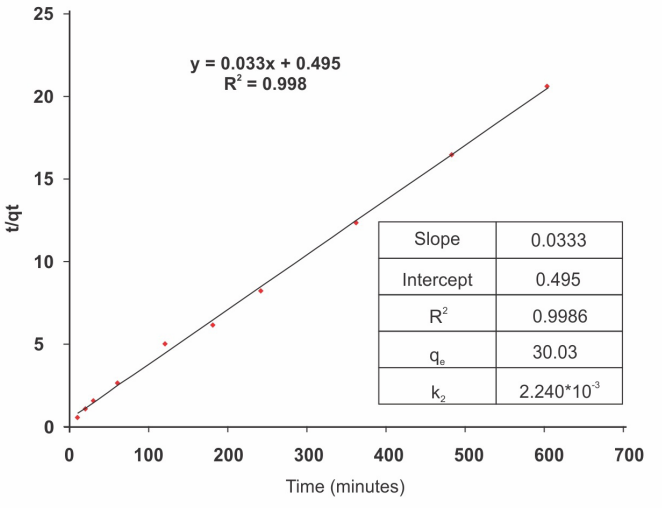

Figure 5. Pseudo second order kinetic model for adsorption of Cadmium to MoCP.

Adsorption isotherms

The results suggest that a plot of log q_e versus log C_e in figure 6 represents a measure of linearity involved. The data was better fitted in Freundlich equation (**Fig. 6**).

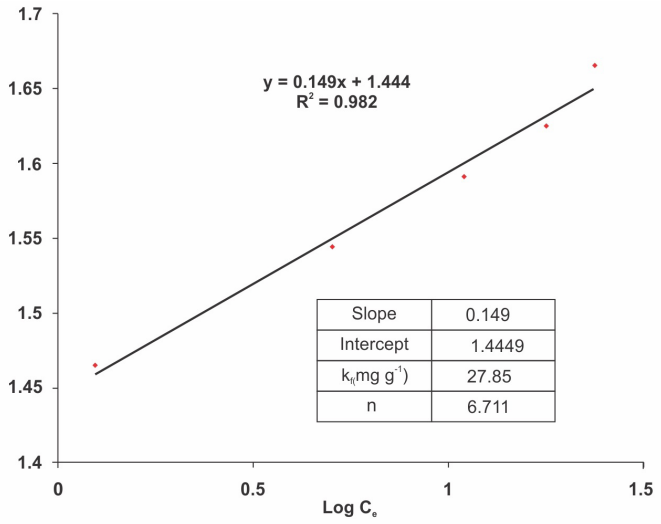

Figure 6. Freundlich isotherm of cadmium binding to MoCP.

Preparation and characterization of MoCP nanoparticles

For the preparation of MoCP nanoparticles, methods standardized in our laboratory for protein nanoparticle preparation was used (40).

Scanning Electron Microscopy (SEM)

MoCP nanoparticles were prepared and characterized. For SEM analysis the nanoparticles were prepared as described under methods. The results are shown in (**Fig. 7a**). From this it is evident that different size of MoCP nanoparticles could be obtained in the study in the range of 70±5nm. In order to confirm the authenticity of these nanoparticles, these were separated on a SDS-PAGE along with purified MoCP and the proteins transferred to a nitrocellulose membrane as described under methods. The fact that the nanoparticles also showed a immunoreactivity with the MoCP antibody suggests that the nanoparticles indeed are those of MoCP (**Fig 7b**).

However, the presence of additional bands (starred) in addition to the MoCP band seen is possibly because of the aggregated nature of nanoparticles obtained during the preparation.

Discussion

The importance of *M. oliefera* plant and its seed material as an interesting source to study various aspects of phytochemistry, proteins and medicine has been growing over the past few years (45). Our laboratory also has isolated and purified from the seeds of this plant a coagulant protein MoCP and also raised an antibody for the same (38) additionally a α-mannosidase was also purified from the seeds and biochemically characterized (46).Our recent interest has also been to explore and identify what components in some seed materials are involved in removing metals from aqueous solutions. As a first step towards this, we recently characterized the proteins and polysaccharides from *Strychnos potatorum* seeds (31). We further found that the proteins isolated from these seeds specifically were able

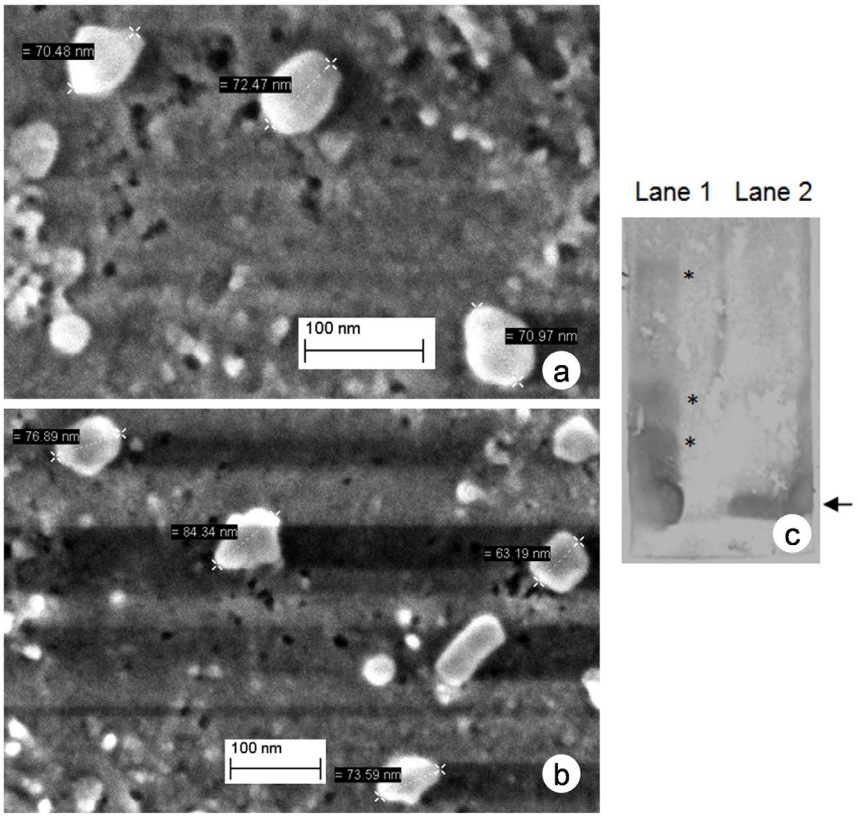

Figure 7. a and b). SEM analysis of MoCP nanoparticles. c). Immuno-reactivity of the MoCP nanoparticles (lane 1) and isolated MoCP (lane 2) with an antibody to MoCP. *represents possible aggregates of nanoparticles reactive to antibody.

to remove cadmium from aqueous solutions (31).The present study was initiated to understand the role of MoCP in binding of cadmium from aqueous solutions. MoCP has been studied by various researchers and has been designated as a coagulant protein that clarifies turbidity from suspensions (37). Cadmium toxicity has been identified as one of the important health hazard for humans as well as for the plants. Therefore identifying the components that can effectively remove cadmium from solutions and prepare appropriate methods for their utility for usage both in vivo and in vitro is important. Having isolated the MoCP, we attempted to prepare nanoparticles, as recent studies using seed powder, protein loaded nanoparticles in various fields of research gained considerable attention in this field (38, 39). Thus this investigation t not only showed that MoCP can bind and remove heavy metals and the nanoparticles prepared showed effective nanosize dimensions that have been confirmed to be MoCP by immune reactivity with the antiserum. Additional investigations are initiated to check the efficacy of these nanoparticles potential to apply in water purification (e.g coupling protein nanoparticles to gels, immobilizing them over a glass which will have wider applications.

The binding of cadmium to nanoparticles synthesized and the nanoparticles should be immobilized to affigel and analysis of the adsorption kinetics of cadmium will be our future direction of work. In summary, this is the first report to show that MoCP effectively binds cadmium and helps in removing the same from aqueous solutions. Furthermore, the purified MoCP prepared as nanoparticles for the first time were authenticated by their size and immune-reactivity with specific antibody giving enough scope to use these nanoparticles for further evaluating their potential as possible protein sensors for detection of heavy metal contamination in aqueous solutions which is the future direction of work in our laboratory.

Conclusion

Seeds of *M. oleifera* and different parts of the plant have been shown to contain a wide variety of biologically important components and one of the seed proteins that has been of interest is the coagulant protein. In an earlier study we isolated this protein and also raised a polyclonal antiserum for this in a rabbit. In the present work MoCP was isolated in order to study its role in the cadmium binding and the results indicate immobilized MoCP has a potential role in cadmium adsorption and this is dependent on the pH, time of incubation as well as temperature. Maximum adsorption for cadmium was at pH 6.0 and the equilibrium was attained at time. Adsorption processes differ depending on the matrix used, the processing of the biomass and solution chemistry (1). Adsorption types are differentiated as "physiosorption" if the attraction between the solid surface and the adsorbed molecules is physical in nature. Generally, in physical adsorption the attractive forces between adsorbed molecules and the matrix surface are van der Waals forces and they being weak result in reversible adsorption. On the other hand if the attraction forces are due to chemical bonding, it is called as "chemisorption". In view of the higher strength of the bonding in chemisorption, it is difficult to remove chemisorbed species from the surface of the matrix. Preliminary results on the nanoparticle preparation revealed that the nanoparticles are indeed of the MoCP and our future we would like to extensively characterize these nanoparticles for their efficiency in cadmium binding and their possible uses as biosensors for cadmium detection at optimal and under toxic conditions.

Acknowledgments

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Conflict of interest statement

The authors declare there is no conflict of interest.

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