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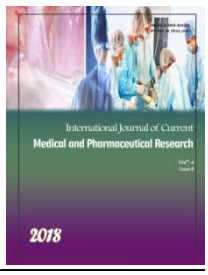

Research Article

DEVELOPMENT OF OLIGOSACCHARIDE/SUGAR COUPLED Fe_3O_4 NANOPARTICLE MATRICES: POTENTIAL USE IN THE PURIFICATION OF LECTINS AND GLYCOPROTEINS

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ABSTRACT

In the present study, magnetite nanoparticles with amine functional groups were synthesized successfully, and were characterized by TEM, XRD and Raman spectroscopy. The size and shape of the amine functionalized magnetite nanoparticles as characterized by TEM, were in the size range of 13-14 nm with spherical shape. The composition and phase purity of magnetite nanoparticles such as crystal phase, particle size and magnetic properties as determined by XRD, showed that the crystal structure formed is corresponding to Fe_3O_4 nanoparticles. The results obtained from the Raman spectrum showed the presence of magnetite with amine groups. Ligand coupled magnetic nanoparticle affinity matrices were developed successfully by coupling the Fe_3O_4 magnetic nanoparticles to phosphomannan (PMC) oligosaccharide and lactose separately using DVS as the spacer arm. The Fe_3O_4-phosphomannan matrix specifically can bind the Cation Dependent mannose 6-phosphate receptor (CD-MPR) and can be eluted using mannose 6-phosphate. The eluted protein migrated as a single band in SDS-PAGE under reducing conditions corresponding to molecular mass of 46 kDa and was recognized by MSC1 antibody. Lactose-specific lectin (LSL) from *Lamellidens corrianus* binds specifically on Fe_3O_4-lactose matrix and can be eluted using 0.3 M lactose. The eluted LSL migrated as a three subunit species with molecular masses of 34 kDa, 30 kDa and 25 kDa under reducing conditions in SDS-PAGE. The three subunits were recognized by a LSL-polyclonal antiserum. These new matrices developed offer a great advantage in the purification of lectins (MPR 46 and LSL) from animals over conventional ligand-coupled Seralose matrices.

INTRODUCTION

Nanoparticles are particles of chemical or biological materials that are in the range of 10 to 1000 nm in size. Among the chemical based nanoparticles, iron, gold and silver nanoparticles are widely used and well-studied. Protein, carbohydrate, lipid and DNA based nanoparticles are well-studied biological nanoparticles, and have recently sparked many applications in various fields of biology and medicine [1]. Magnetic nanoparticles, due to their magnetic field responsive properties, have attracted considerable attention for several biomedical applications such as biosensors and drug delivery systems [2]. Among the metal based nanoparticles, magnetic particles are widely studied, and have tremendous applications in various fields of biology and medicine such as magnetic resonance imaging [3],immunoassays, RNA and DNA isolation [4], and waste water treatment [5, 6]. Superparamagnetic iron oxide nanoparticles (SPION), which can be contacted through external magnets are one of the most promising nanoparticles for these applications. SPION is coated with biocompatible materials and can be functionalized with drugs, proteins or plasmids [4]. Iron oxide nanoparticles are the particles with diameters between 1 and 100 nm. The two main forms are magnetite (Fe_3O_4) and its oxidized form maghemite (γ-Fe_2O_3), among these two forms, magnetite is more magnetic, and attracted extensive interest due to the super magnetic properties and potential applications in many fields. Applications of magnetic nanoparticles are not limited to traditional electrical, optical and magnetic areas but also expanded recently to new applications, including biocatalysis and magnetically assisted bioseparation/affinity purification [7].

Affinity chromatography is a method of selective isolation of macromolecules based on their biological specificity. In traditional affinity chromatography, a ligand is immobilized on to a solid support, the target protein with ligand-specificity is separated based on the affinity; for example antibodies with antigens, enzymes with substrate analogues, nucleic acid with nucleic acid binding proteins, and hormones with receptors. To achieve superior binding and purity by avoiding any steric hindrance for the interaction to occur, normally the ligand is immobilized to a suitable matrix through a spacer arm which facilitates better interaction of the ligand with the biomolecule to be separated and allows a stable interaction between the two [8].

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Lectins are sugar binding proteins of non-immune origin that bind carbohydrate moieties of the glycoconjugates. The interaction of the lectin with particular sugar is as specific as the interaction between those of the antigen and antibody or substrate and enzyme [9]. Lectins are ubiquitous in nature and plant lectins represent the largest and thoroughly studied family of simple lectins. Though much effort has been devoted to study the lectins, the information of the animal lectins is limited. Due to the important roles in cellular events, it is very much essential to purify the animal lectins to gain insights into the structural and functional relationships of animal lectins. Lectins, owing to their unique sugar binding properties, can be purified by affinity chromatography, by immobilizing the specific sugar ligands onto a solid support matrix. Research in our laboratory is focused on developing novel affinity methods to purify lectins from plants and animals with a long term objective to understand their structure function relationships [10, 11]. Lactose-specific lectin is a soluble glycoprotein which plays an important role in the lysosomal targeting in fresh water mussel (unpublished data). Though a very few reports are available on lactose-binding proteins from rat [12], chicken [13] and sponge *Cinachyrella apion*[14], the information about the LSL is limited necessitating the studies on this lectin. In a recent study, we for the first time affinity purified the LSL from *L. corrianus* and made Nanoparticles of the purified lectin and characterized the same [15]. Mannose 6-phosphate receptor 46 (MPR 46) is a type 1 transmembrane glycoprotein and belongs to P-type lectin family, functions in the transport of newly synthesized lysosomal enzymes, defects in which, leads to accumulation of undigested materials in the cells known as lysosomal storage disorders (LSDs) [16-19].

For affinity purification, although agarose is used as support matrix more commonly, a range of other matrix supports, including silica and polyacrylamide are also available. Conventionally, agarose matrices are activated by chemical bifunctional molecules and the ligands are covalently coupled to activated matrices and used as affinity matrix for the purification of lectins [20]. Agarose based affinity matrices such as Sepharose-mannose, Sepharose-galactose, Sepharose-N-acetylglucosamine and Sepharose-lactose were used to purify many lectins from various plant and animal sources [15, 21-25]. MPRs from the animal kingdom were purified by using largely Sepharose as the support matrix for the coupling of PMC (ligand)[26].

In order to develop the alternate methods that will provide purification of lectins and glycoproteins, in the present study magnetic nanoparticles with amino group were synthesized and coupled to phosphorylated manno oligosaccharide and lactose separately. Both matrices were used to purify cation dependent mannose 6-phosphate receptor (MPR 46/CD MPR) from goat liver membrane extracts and lactose-specific lectin from the soluble extracts of the fresh water mussel in a single step. This paper reports an efficient alternative rapid method for the purification of proteins as specified in this study and the magnetic nanoparticles will have potential applications to purify proteins based on their biological specificity.

MATERIALS AND METHODS

Materials

Live fresh water mussels (*Lamellidens corrianus*) were supplied by a local supplier. The soft tissues were collected from the de-shelled mussels and stored immediately at -80°C until use. Goat liver was collected from local slaughter house on ice and was stored at -80°C until use. Divinyl sulfone, 1,6-hexanediamine, mannose 6- phosphate, BCIP/NBT substrate and Triton X-100 were procured from Sigma Aldrich (St. Louis, MO, USA.). Mammalian Synthetic Cytoplasmic tail (MSC1) antibody was a generous gift from Prof. Kurt von Figura, Goettingen, Germany. All other reagents and chemicals used in the study were obtained from Sisco research laboratories, India, and were of the highest purity available.

Methods

Synthesis of amine functionalized Fe_3O_4 nanoparticles

Magnetite nanoparticles with functional amine groups were synthesized by one-pot method according to *Wang et.al.,*[27]. About, 1.0 g of $FeCl_3.6H_2O$, 2.0 g of sodium acetate, and 6.5 g of 1, 6-hexanediamine were added to 30 ml of ethylene glycol and stirred vigorously at 50°C to get a clear solution. This solution was transferred to a Teflon-lined autoclave (50 ml) and incubated at 200°C for 6 h. The magnetic nanoparticles formed were rinsed with deionized distilled water followed by ethanol to effectively remove solvent, and the unbound 1, 6-hexanediamine. During each rinsing step, the nanoparticles were separated from the supernatant by using magnetic force, washed, and the formed magnetic particles were dried at 50°C, and characterized.

Transmission Electron Microscopy

A small drop of alcoholic suspension of the nanoparticles was dispersed on carbon-coated copper grids and the morphology of the magnetic nanoparticles was examined under the transmission electron microscope (Model FEI Technai G2 S-Twin) operated at 100 kV.

Powder X-Ray diffraction

X-ray diffraction (XRD) patterns of magnetic nanoparticles were recorded from 10°C to 80°C using Cu Kα as the X-ray source (λ=1.54 Å). Bruker's AXS Model D8 Advance System was used to carry out the XRD experiments for identification of the crystal phase, particle size and degree of agglomeration, chemical composition, and magnetic properties.

Raman spectroscopy

Raman spectra of the amine functionalized magnetite nanoparticles were recorded with micro-Raman (WiTec ALPHA 300 instrument), with a continuous wave Nd:YAG laser at 532 nm, and laser power of 40 mW was used with an acquisition recording time of 5s. Raman spectra were collected at 5 different positions of the sample and their average has been considered. All spectra were calibrated with Raman peak of silicon wafer at 520 cm^{-1}. In the Micro Raman spectrometer, the laser beam was focused on to the sample using an objective lens (100X), and the theoretical beam waist estimated was ~700 nm. Raman signals were collected in back scattering geometry.

Coupling of Phosphomannan Core to the amine functionalized magnetite nanoparticles

O-Phosphonomannan (a generous gift from Dr. M.E. Slodki, USDA, Peoria IL, USA) was hydrolysed with acetic acid and separated into phosphomannan core (PMC) and pentamannosylphosphate (PMP) as described earlier [28]. About 5 ml suspension of the amine functionalized nanoparticles were washed thoroughly with distilled water and suspended in 0.5 M sodium carbonate/bicarbonate buffer pH

11.0. Divinyl sulfone (0.5 ml) was added and the suspension was gently shaken at room temperature for 70 min, and washed thoroughly with distilled water. The activated particles were washed with 0.5 M sodium/bicarbonate buffer pH 10.0 and the particles were suspended in 5 ml of carbonate buffer pH 10.0, containing 20 mg of PMC, and coupling was allowed to proceed in cold (4°C) for 48h. At the end of coupling, the particle pellet was washed with deionized distilled water and suspended in 0.5 M sodium bicarbonate buffer pH 8.5, containing 50 µL of β-mercaptoethanol and kept for rotation at room temperature for 3 hrs. Finally, the particles were washed with distilled water and stored at 4°C in column buffer until further use. The extent of PMC coupled to magnetite was determined as mannose equivalents as described earlier by *Dubois et al.,*[29].

Extraction of total membrane proteins from goat liver and purification of the MPR46 on Fe_3O_4-NH_2-PMC matrix

Total membrane proteins from the fresh goat liver tissue were extracted as described earlier [30]. To the membrane extract obtained, $MnCl_2$ was added in a final concentration of 10 mM, and used for the purification of MPR 46 employing the magnetic affinity matrix developed. PMC-coupled magnetite nanoparticles were equilibrated with 50 mM imidazole-HCl buffer pH 7.0, containing 150 mM NaCl, 5 mM sodium β-glycerophosphate, 0.05% Triton X-100 and 10 mM $MnCl_2$ (buffer A). In a typical experiment, membrane extract obtained, was mixed with Fe_3O_4-NH_2-PMC nanoparticles that were equilibrated with buffer A and was kept for rotation at 4°C overnight. The following day the magnetite nanoparticles were subjected to magnetic separation using a strong magnet and the unbound protein sample was discarded. The magnetic pellet was washed extensively with the buffer A several times. The bound protein (MPR 46) was eluted by incubating the PMC-magnetite with 5 mM mannose 6-phosphate in buffer A for 1 h in cold.

Coupling of lactose to the amine functionalized magnetite nanoparticles

Lactose was coupled to the activated magnetite nanoparticles as described above except lactose was used as the coupling ligand in place of phosphomannan core.

Extraction of total soluble proteins from L. corrianus and purification of lactose-specific lectin on Fe_3O_4-NH_2-lactose matrix

All operations were carried out at 4°C unless otherwise stated. Fifty grams of the whole animal tissue was homogenized with 10 volumes (500 mL) of 25 mM Tris-HCl, pH 7.0 (buffer B). The homogenate was kept for stirring overnight at 4°C and centrifuged for 30 min at 26,892 X g. The pellet was discarded and the supernatant was collected and re-centrifuged for 15 min to remove any remaining particles. The clear supernatant obtained was subjected to 0-80% ammonium sulphate fractionation for 3 h at 4°C and centrifuged at 26,892 X g for 30 min. The pellet was collected, suspended in minimum volume of 25 mM Tris buffered saline (buffer B) and dialyzed against the same buffer. About 50 ml of the soluble extract was mixed with lactose-coupled magnetite nanoparticles and kept for rotation at 4°C overnight. The following day the particles were separated from the solution by applying magnetic field, washed extensively with buffer B and the bound lectin was eluted with 0.2 M lactose in buffer B.

SDS-PAGE and western blotting

The purity of eluted proteins (MPR 46 and LSL) were analysed by 10% SDS-PAGE under reducing conditions according to *Laemmli*[31], and the proteins were detected by silver staining. The authenticity of the purified MPR 46 was analysed by western blot by probing with MSC1 antibody (MSC1 antibody is MPR46 c-tail peptide antibody which recognizes MPR 46 from molluscs to mammals as tested in our laboratory). Similarly, the purified lactose-specific lectin was detected using the specific primary antibody raised in a rabbit. Both the membranes were incubated separately with goat anti-rabbit IgG conjugated to alkaline phosphatase as the secondary antibody and the blots were developed by incubating with the substrate BCIP/NBT.

RESULTS AND DISCUSSION

Proteins are one of the key elements of cell machinery, and study of proteins is essential for better understanding of the cellular functions. Protein purification using a variety of matrices has been a fundamental requisite in biotechnology. The potentiality of these prepared matrices can be ascertained by their ability to purify proteins. In conventional methods of purification multiple steps are involved to get a pure protein, and in biotechnology, affinity protein purification using antibody-based separation or a matrix with specific tags for binding target protein are commonly used methods for rapid purification of proteins. The main challenge in protein purification is to use a common matrix for purification of different proteins. Magnetic nanoparticles can be used as a novel, versatile and stable forms of matrix for the efficient capture of selected target biomolecules in the presence of other suspended solids even for a small sample volume. The use of magnetic nanoparticles such as superparamagnetic iron oxide nanoparticles for purification of biomolecules in general and proteins in particular have many advantages such as high surface area to volume ratio which allows the efficient binding of desired protein to the surface of the ligand coated particles and get separated using a simple magnetic field [32].

In the present study, Ligand-coupled magnetite nanoparticles intended for affinity purification were designed, synthesized, and characterized successfully. Amine functionalized magnetic nanocrystals were conjugated to sugar ligands and their ability to purify two biologically important and well characterized proteins (MPR46/CD-MPR from goat liver and LSL from *L. corrianus*) were investigated with a long term objective to use the nanoparticle based matrices for the purification of different proteins of interest.

Preparation and characterization of amine-functionalized magnetic nanoparticles

Amine functionalized magnetite nanoparticles were successfully prepared by facile one Pot synthesis using $FeCl_3.6H_2O$ as a sole iron source and 1, 6- hexanediamine as a source of amine groupsand were characterized by TEM, XRD and Raman spectroscopy.

Transmission Electron Microscopy

Size and shape of the prepared nanoparticles were determined by dispersing a small drop of alcoholic suspension of the nanoparticles on carbon-coated copper grids and examining under TEM. The size of the particles were observed to be in the range of 13 nm-14 nm with a spherical shape (Figure. 1).

The size of the nanoparticles obtained in this study and for such similar studies are in the range of 13 nm [33].

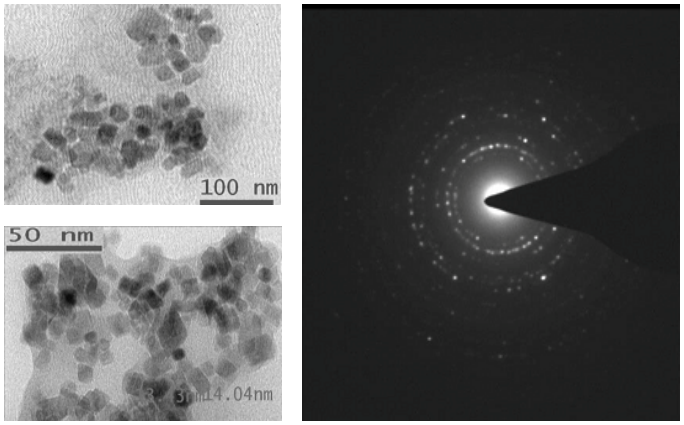

Fig 1 (A) Transmission Electron Micrographs of the amine functionalized magnetite (Fe_3O_4-NH_2) nanoparticles and **(B).** Diffraction pattern in TEM.

Powder X-Ray diffraction (XRD)

The crystalline nature of the nanoparticles was confirmed by subjecting the amine functionalized nanoparticles to XRD studies for the identification of the crystal phase, particles size, and degree of agglomeration, chemical composition, and magnetic properties. X-Ray Diffraction pattern of the prepared magnetite (Fe_3O_4-NH_2) nanoparticles is shown in Figure 2.The peak positions appeared at 30.3°, 35.5°, 43.3°, 57.1° and 62.8° were indexed as (220), (311), (400), (510), (440) crystal planes of Fe_3O_4 respectively. Particles size was calculated by using a Debye- Scherrer equation, by drawing Gaussian fitting for the highest intensity peak and measuring the values of (2θ) and peak width. The analysis was performed using Origin 6 software and the results are in good agreement with the diameters measured from the TEM images.

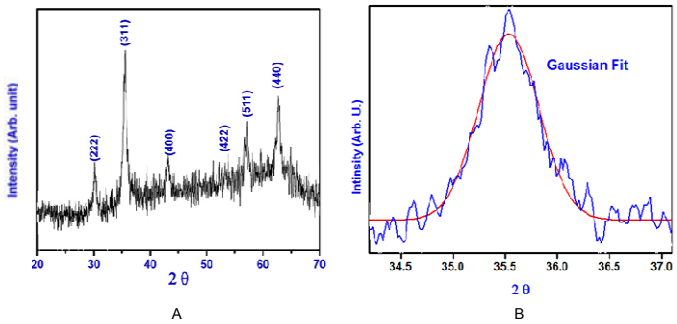

Fig 2 (A). Powder X-Ray diffraction pattern of amine functionalized Fe_3O_4nanoparticles (The peaks correspond to that of magnetite) and**(B).** Gaussian fit.

B(2θ) = 0.57409
K= 0.9
2θ = 35.534
λ = 1.54 nm.

$$d = \frac{k\lambda}{B(2\theta).Cos\theta} = \frac{0.9 * 1.54}{0.57 * \cos(17.76)} = 2.55\ nm$$

B(2θ) is the peak width determined from Gaussian curve and taken at one point on graph. However, the constant of proportionality K (the Scherrer constant), depends on how the width is determined, the shape of the crystal, and the size distribution and usually taken between 0.9-0.94. Clear intense peak from the graph revealed high purity and crystallinity of the nanoparticles with no detectable quantities of impurities in agreement with the previous studies [34].

Raman spectroscopy

To distinguish the iron oxides species, functionalized nanoparticles were subjected to Raman spectroscopy. Raman spectra of the functionalized nanoparticles were recorded as described above. Raman spectra were collected at 5 different position of the sample and their average has been considered. As shown in Figure. 3, the Raman bands at ~300 nm ~400 nm and ~725 nm were observed corresponding to magnetite nanoparticles and at ~1350 and ~1600 nm corresponding to amine groups. The Raman scattering results clearly confirmed the presence of Fe_3O_4-NH_2 crystals which is in consistent with and confirming the TEM and XRD results. The spectra clearly indicates that no other oxides of iron were detected during the preparation of magnetite nanoparticles which is in consistent with earlier studies [34].

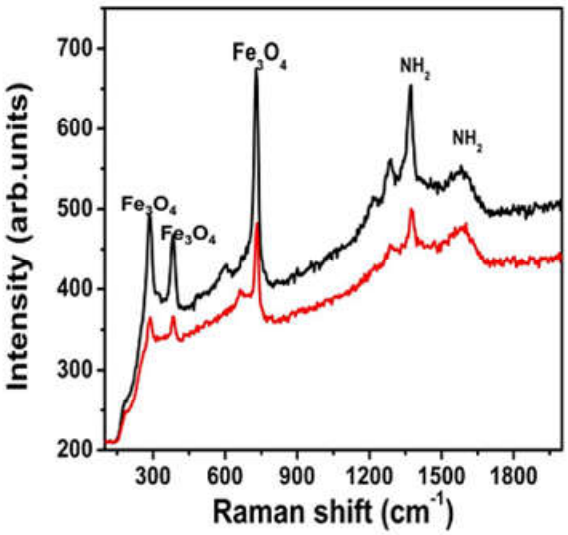

Fig 3 Raman spectra of amine functionalized magnetite (Fe_3O_4) nanoparticles.

Surface functionalization of nanomaterials is crucial for bio-application [35-38]. Modification of the surface of organic molecules is an important event that will allow stabilization of the nanoparticles in biological samples both in terms of pH (7.4) and high salt concentration. The available surface molecules can be further derivatized[4]. The Raman spectra analysis thus provided convincing evidence on the availability of the functional groups on the Nanoparticles prepared.

Affinity purification of goat liver MPR46 using Fe_3O_4-NH_2-PMC matrix

Phosphomannan core obtained from yeast *O*-phosphonomannan has been used as a ligand and immobilized to CNBr activated-Sepharose and Sepharose-divinyl sulfone gel. These affinity matrices have been widely used to purify the CD MPR/MPR 46 protein from different animal species and cell types that allowed us to understand the physiological function of this receptor as well as its evolutionary characteristics [26]. PMC Nanoparticles prepared in our lab have also been shown to bind the Mannose 6-phosphate receptor in specific cell types and thus might have potential applications as drug delivery systems [39]. In the present study PMC has been functionally derivatized to iron nanoparticles for the first time and that the ligand has been immobilized has been confirmed as described above and its ability to purify the CD MPR/MPR 46 in a single step from the membrane extracts

of goat liver has been explored. When the membrane extract from the goat liver tissue was subjected to magnetic affinity separation in the presence of 10 mM $MnCl_2$ for the purification of MPR46 alone as described under methods, receptor protein could be bound specifically and it could be eluted using 5 mM mannose 6-phosphate. From 10 g of acetone powder, about 0.26 mg of purified receptor could be obtained. The eluted protein was analyzed by 10% SDS gel electrophoresis which revealed a band with a molecular mass of ~ 46 kDa, representing the MPR 46 protein (Fig. 4A).The authenticity of the receptor was further confirmed by using an antibody MSC1 (Fig. 4B) that specifically recognizes MPR 46 protein, consistent with our earlier report[40].

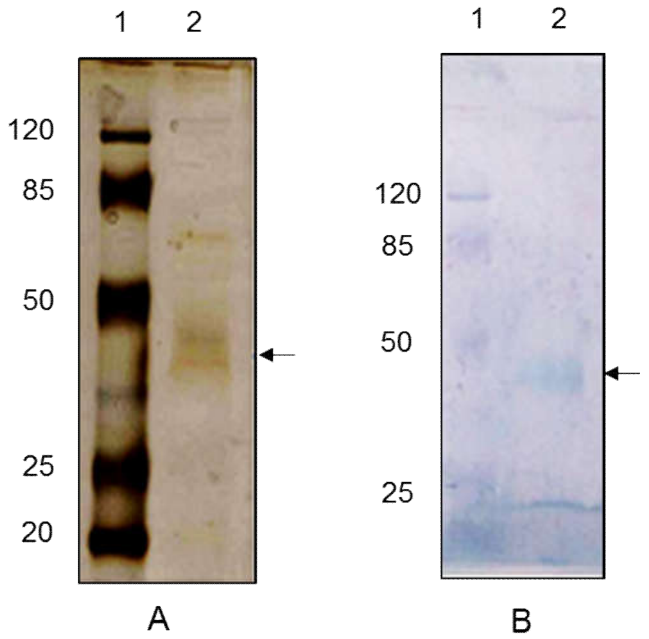

Fig 4 (A).10% SDS-PAGE analysis of purified MPR 46 (lane 1: molecular weight marker and lane 2: purified MPR 46).**(B).** Immunoblot of MPR 46 with MSC1 antibody. (lane1: molecular weight marker and lane 2: MPR46).Arrows indicate the position of receptor.

Purification of lactose specific lectin from L. corrianus using Fe_3O_4-NH_2-lactose matrix

Although several animal lectins with varied sugar specificity have been purified, studies on lactose specific lectins is limited. In a recent study we affinity purified a LSL from the soluble extracts of *L. corrianus* and also prepared the Nanoparticles for this lectin [15]. In the present study to the iron Nanoparticles prepared, the lactose sugar was immobilized and tested for its efficiency for the binding and single step purification of the LSL from *L. corrianus.*

The soluble extract of *L. corrianus* tissue was subjected to magnetic affinity separation. From 80 mg of total crude protein, about 0.8 mg of purified lectin could be obtained. The eluted protein was electrophoresed on 10% SDS gel electrophoresis (Fig. 5A). In a separate experiment the purified lectin was subjected to western blot analysis as described under methods and the LSL detected using the antiserum for the lectin (Fig. 5B). From the western blot it is evident that the three subunits of the lectin seen in SDS-PAGE show immunoreactivity with the antiserum suggesting that they represent the putative lectin.

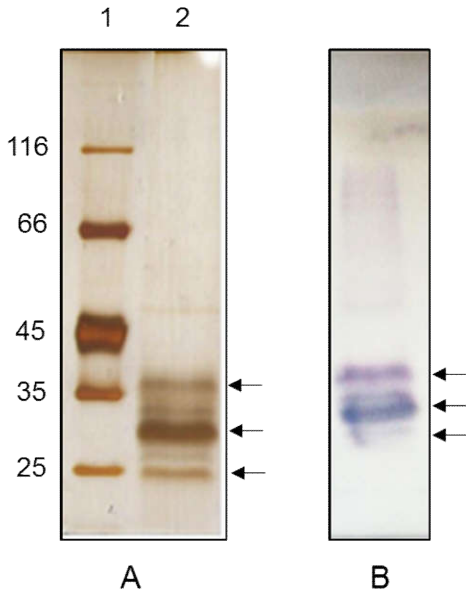

Figure 5 (A) 10% SDS-PAGE analysis of purified lactose-specific lectin (LSL) (lane1: molecular weight marker and lane 2: purified LSL), **(B)**. Immunoblot analysis of LSL with antibody. (lane1: molecular weight marker and lane 2: purified LSL). Arrows indicate the subunits of the purified LSL.

CONCLUSIONS

In summary, the present study is an advancement in the preparation of iron nanoparticles coupled with specific ligands that showed their potential applications to purify two different type of lectins from animal species. Herein, a new method to purify MPR46/CD-MPR and a lactose-specific lectin from goat liver and *L.corrianus* tissue respectively using magnetic nanoparticles is reported. The method is based on the coupling of ligands to amine functionalized magnetic nanoparticles prepared from $FeCl_3.6H_2O$, that are able to capture proteins in a simple and efficient way and which could promote large-scale purification. Both the target proteins were captured onto the magnetic nanoparticles via a ligand interaction mechanism. In contrast to the conventional method, purification of both the proteins suggests a simple and cost effective method for purifying target proteins. Further, the matrix offers several advantages in particular the ease of preparation, high stability and long term storage without affecting its properties. It is proposed that this method be extended to the purification of proteins with similar characteristics and possibly other biomolecules primarily by selecting the ligand that exhibits distinct biological specificity to a target protein. The purified lectins can be used as drug delivery tools which offers a great advantage as biodegradable protein nanoparticles.

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