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An alternate high yielding inexpensive procedure for the purification of concanavalin A

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Abstract
Concanavalin A (Con A) purification method was developed by using calcium alginate cellulose beads containing cobalt (II) ions. The yield of Con A obtained by calcium alginate cellulose beads containing cobalt (II) ions was 6.8% as compared to 3.65% yield obtained by Sephadex G-50 method. Purified Con A agglutinated trypsin-treated rabbit red blood cells. FTIR spectra showed the presence of β sheet structure of purified Con A. AFM and SEM images confirmed the morphology of purified Con A. Con A precipitin reaction with dextran and ovalbumin was investigated by the UV-visible spectrophotometer. The turbidity measured for the binding of dextran and ovalbumin was decreased in the presence of these salts. These findings lead us to conclusion that our purification method by using calcium alginate cellulose beads containing cobalt (II) ions is better than the classical method of sephadex in terms of protein yield.

Keywords: Concanavalin A; FTIR; AFM; SEM; calcium alginate cellulose beads.

Abbreviations: AFM, Atomic force microscopy; SEM, Scanning electron microscopy; FTIR, Fourier transform infrared spectroscopy; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Introduction

Lectins are widely utilized as a tool in the field of carbohydrate biochemistry. Immobilized lectin columns are useful to separate the sugar compounds on the basis of slight structural differences. Con A is a lectin from jack bean, and it is well studied protein because of its molecular structure and much medical application. Con A specifically reacts with α-D-mano and α-D-glucopyronoside residues with unsubstituted hydroxyl groups at C-3, C-4 and C-6 positions or with polysaccharides, glycoproteins. Con A was the first among the lectins to be isolated in the pure form (Sumner, 1919; Sumner and Howell, 1936). Con A is composed of four identical protomers held together by polar interactions, hydrogen bonds and electrostatic interactions (Recke *et al*., 1975; Hardman and Ainsworth, 1976; Hardman and Goldstein, 1977).

Con A binds to the membrane glycoproteins of a variety of cell types leading to various biological responses such as mitogenesis in lymphocytes (Sharon and Lis, 1972). Several studies have shown that cells transformed by RNA or DNA containing tumor viruses are agglutinated by Con A at the concentrations which fail to agglutinate non-transformed cells. Polymer-induced heteronucleation, a powerful technique that has been used for the discovery of two new forms of Con A from the jack bean (Foroughi *et al*., 2011). Prefibrillar oligomers of proteins are suspected to be the primary pathogenic agents in several neurodegenerative diseases. Con A can be used as model protein to study protein aggregation in cells (Vetri *et al*., 2011). Carbohydrate-functionalized oligothiophene hybrids reveal specific binding with the model lectin from *Canavalia enisformis* (Con A) (Schmid *et al*., 2011).

Commercial Con A contained three types of protein, which were separated by affinity chromatography on Sephadex G-50. When a protein is aggregated by chemical crosslinking inside sephadex beads of appropriate pore size, it gets trapped inside the beads. At other hand, sephadex is very expensive if it is used for the purification of Con A. During the purification of Con A by sephadex column, blocking of column take place but this problem does not exist in calcium alginate-cellulose beads column.

Present paper is aimed to work out an inexpensive and high yield procedure for the purification of Con A by using cobalt (II) into calcium alginate-cellulose beads. This procedure increase the protein yield compared to sephadex based method and in terms of cost cheaper than sephadex based method.

Materials and Methods

Materials

Jack-bean meal, Sephadex G-50 and dextran were purchased from Sigma Chemicals Co. (MO, USA). Ovalbumin and SDS were brought from SRL Chemicals Co. (Mumbai, India), [2-amino-2-(hydroxyl methyl) propane-1, 3-diol] (Tris) was obtained from Qualigens Fine Chemicals Co. (Mumbai, India). All other chemicals and the reagents used in this study were of analytical grade.

Isolation and purification of Con A from jack bean meal

Con A was isolated from 10% jack bean meal extract. Firstly, jack bean meal was soaked in 0.5 M NaCl at 4 °C for 4 h and was filtered through four layers of cheese cloth. The homogenate thus achieved was centrifuged by REMI cooling centrifuge at 6000 rpm for 30 minutes. Solid ammonium sulphate was added to the supernatant to achieve 30% saturation and than 80% saturation. After 12 h, the precipitate was collected by centrifugation and dialysed in 0.01 M Tris/HCl buffer containing sodium chloride, manganese chloride and magnesium acetate. Further purification of Con A was carried out by using Sephadex G-50, calcium-alginate cellulose beads and calcium-alginate cellulose beads containing cobalt (II) in separate batch processes.

Preparation of calcium alginate cellulose beads containing cobalt ions

An aqueous mixture of sodium alginate (2.5%), cobalt (0.3%) and cellulose (3%) was made in buffer. The resulting mixture was slowly extruded as droplets through a 5.0 ml syringe with attached needle into 0.2 mol/L calcium chloride solution. The formation of calcium alginate-cellulose beads was instantaneous and the solution was further gently stirred for 2 h. The beads were than washed and stored at 4 °C in 0.1 M acetate buffer (pH 5.6) for further use.

Haemagglutination activity

Haemagglutinating activity of Con A was detected by using trypsinized erythrocytes. The lectin solution was serially diluted in microtiter V plate and then further mixed with 50 µl of 4% suspension of rabbit erythrocytes. Haemagglutination was observed after 1 h at room temperature.

FTIR spectra of purified Con A

FTIR spectrum of purified Con A was monitored with INTERSPEC 2020 model FTIR instrument, USA. The calibration was done by polystyrene film. The samples were injected by Hamiet 100 µL syringe in ATR box. The syringe was first washed by acetone followed by distilled water. FTIR analysis was done to monitor the functional groups of the compounds.

AFM and SEM of purified Con A

Tapping mode AFM of purified Con A from calcium-alginate cellulose beads containing cobalt (II) was performed using commercial etched silicon tips as AFM probes with typical resonance frequency of ca. 300Hz (RTESP, Veeco). SEM analysis of the surface and cross-section of freeze dried sample of purified Con A was performed with Philips-515 scanning electron microscope (USA). The membrane samples were mounted on an aluminium sample mount and the sputter was coated with gold to minimize surface charging. The specimens were observed at a 15 kV accelerating voltage.

Determination of protein concentration

Protein concentration was routinely determined by the method of Lowry *et al.* (1951).

SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli *et al.* (1970) in 10% acrylamide gel.

Influence of salts on the interaction of Con A with polysaccharides and glycoprotein in presence of lactose and arabinose

The influence of three monovalent salts namely NaCl, KCl and NaBr on the precipitin reaction between Con A and dextran (or ovalbumin) in presence of lactose (0.01 M) and arabinose (0.01 M) was studied by turbidimetric method.

Results

Purification of Con A

Ammonium sulphate fractionation used for the isolation of Con A. Dialysed homogenate achieved was incubated with Sephadex G-50, calcium alginate-cellulose beads and calcium alginate-cellulose beads containing cobalt (II) was carried out in separate beakers. Separation of Con A was achieved by adsorption on Sephadex G-50 and calcium alginate-cellulose beads containing transition metal. Elution of adsorbed Con A on the Sephadex G-50 and the

calcium alginate-cellulose beads was achieved by 0.2 M glucose solution. Eluted Con A contained bound glucose. Extensive dialysis of eluted Con A in Tris/HCl buffer containing sodium chloride, manganese chloride and magnesium acetate remove bound glucose. The protein yield and the degree of purification obtained at various stages of isolation of Con A have been summarized in table 1. Table 1 represent the Con A purified with the help of Sephadex G-50, calcium alginate-cellulose beads and calcium alginate-cellulose beads containing cobalt (II). The protein yield of Con A purified by calcium alginate-cellulose beads containing transition metal ions (cobalt) was 6.8% as compared to 3.65% achieved by Sephadex G-50 procedure.

Haemagglutination activity

The crude homogenate of jack bean meal agglutinated trypsin treated rabbit red blood cells. Purified Con A by the procedure of calcium alginate-cellulose beads containing cobalt (II) ions demonstrated higher titer with respect to Con A purified by Sephadex G-50 purification method. The titer of the tested lectin was expressed as the reciprocal of the highest dilution showing agglutination of trypsinized rabbit erythrocytes.

SDS-PAGE

SDS-PAGE of crude jack bean meal extract, Con A purified by Sephadex G-50 and calcium alginate-cellulose beads containing cobalt (II) ions are shown in Fig. 1. Crude extract after incubation with Sephadex was shown in lane 1; it appears like a smear due to presence of variety of proteins in the extract. Lane 2 presented the similar pattern in smear represents the crude extract after incubation with calcium alginate cellulose beads containing cobalt (II) ions. A band of Con A purified with the help of Sephadex G-50 procedure and calcium alginate-cellulose beads containing cobalt (II) procedure has been represented in lane 3 and 4 respectively in which the position of purified Con A is same as to commercially available Con A in lane 5. Electrophoretic patterns of the purified and the commercially obtained Con A was similar in nature suggested that the purified Con A was homogenous in nature with respect to commercial Con A.

FTIR analysis

The lectin purified by calcium alginate-cellulose beads containing cobalt (II) ions is Con A was further confirmed by FTIR spectroscopy. FTIR analysis revealed the presence of amide I (1635.76), amide II (1535.04), bands as demonstrated by Fig. 2. Although the native-state of Con A have secondary structure and it predominantly β-sheet with no α-helix, the sequence must have some helical propensity. It manifests that the main structure is consisted of β sheet forms, thus it shows that, the lectin purified is Con A (Alvarez *et al*., 1987).

AFM and SEM analysis

Fig. 3a presents the AFM image of Con A purified by calcium-alginate cellulose beads containing cobalt (II) ions and the 3 dimensional image with corresponding height are presented in Fig. 3b. Clearly, the lateral size of the protein imaged by the AFM is large enough due to the convolution effect of the tip, while the height in the photograph should reflect the protein size more accurately.

SEM observations further confirmed the AFM results, Fig. 4 showed SEM image of the Con A purified by calcium alginate-cellulose beads containing cobalt (II) ions and it clearly demonstrates the Con A in crystal like structures. Each bunch of crystal was composed of closely packed Con A forming a radiating structure resulting from freeze-drying of the sample. SEM and AFM showed the morphological homogeneity of the purified Con A by calcium alginate-cellulose beads containing cobalt (II) ions.

Effect of salts on the interaction of Con A with polysaccharides and glycoprotein in presence of lactose and arabinose

The results obtained with the two ligands (dextran and ovalbumin) were qualitatively dissimilar. It is to be noted that the precipitin reaction could not be studied with precision in TM buffer, pH 7.4, alone, since the stability of Con A was very low in the absence of neutral salt. Effect of NaCl, NaBr and KCl on the interaction of Con A with dextran and ovalbumin in presence of lactose and arabinose has been shown in Fig. 5 and 6. At 2M concentration of NaCl, the precipitin reaction of Con A with ovalbumin was significantly inhibited where as in case of Con A with dextran, the precipitin reaction inhibition increases with increase in NaCl concentration. In figure 6, reveals that 2.5 M KCl the concentration which highly inhibited the precipitin reaction. 2.5 M NaBr was the concentration exhibited the similar inhibition to the precipitin reaction between Con A and

ovalbumin, as by 2.5 M KCl. The non carbohydrate moieties of glycoprotein considerably enhanced the affinity of Con A for the specific ligand. Thus it can be said that the carbohydrate moiety was the primary site for interaction and subsequent stabilization of Con A-ovalbumin complex because of the presence of non-saccharide moieties in the ovalbumin. The precipitation reaction of Con A with dextran was influenced by both the nature and the concentration of the salts. It has been observed that increase in salt concentration causes a decrease in precipitin reaction in all of the three investigated salts for dextran and ovalbumin in the presence of lactose and arabinose, which does not inhibit the interaction between Con A and dextran or ovalbumin.

Discussion

Purified Con A by the procedure of calcium alginate-cellulose beads containing cobalt (II) has higher protein yield as compared to Sephadex G-50 procedure in batch process. Increase in the yield of Con A purified by calcium alginate-cellulose beads containing transition metal ions such as cobalt (II) as compared to only calcium alginate-cellulose beads is due to the affinity of histidine and tryptophan present in Con A towards the transition metal ions (Michele *et al*., 1988; Khan *et al*., 2010). Purification yield of Con A was more when purified by using the transition metal ion cobalt than nickel. Purification cost of Con A by calcium alginate cellulose beads support was quiet low than the Con A purified by Sephadex G-50 procedure. Purification procedure of Con A developed by using the calcium alginate-cellulose bead support has superiority over Sephadex in terms of storage, simplicity, cost and protein yield.

The data on the effect of salts on the precipitin reaction of Con A with complex and large ligands, e.g. polysaccharides, glycoproteins and cells, are too sparse to lead to any definite conclusion (Hardman and Goldstein, 1977). These considerations prompted us to investigate systematically the effect of three different neutral salts, namely NaCl, KC1 and NaBr, on the precipitin reaction of Con A with two specific large ligands, namely dextran and ovalbumin in the presence of lactose and arabinose. These salts caused significant inhibition to the precipitin reactions of Con A with dextran and ovalbumin implicating that lactose and arabinose have no role in the reaction, however these two sugars do not inhibit the interaction between Con A and ovalbumin (or dextran). Chemical and structural differences among the two macromolecular ligands may account for the marked differences in the dependence of their reactions with Con A on the salt concentrations. Unlike the structures of dextran, that of ovalbumin would be sensitive to salts, especially at high ionic strength. Thus, the electrostatic free energy of ovalbumin corresponds to 13 negative charges/molecule at pH 7.4. Consequently, the polar interactions between Con A and polysaccharides are likely to be more extensively under operation during the reaction of the lectin with dextran.

Multiangle light-scattering, highlighted the onset of the condensation process which gives rise to formation of compact fractal aggregates of Con A. AFM microscopy observed thin fibrils of Con A, formed in the early stage of aggregation, which further interact to form larger structures with a netlike spatial organization (Carotta *et al*., 2011). Con A has shown a remarkable antiproliferative effect on human melanoma A375 cells. Also, there was a link between the antiproliferative activity of Con A and its sugar-binding activity. And the interesting aspect of this interaction is that Con A can induce human melanoma A375 cell apoptosis in a caspase-dependent manner (Liu *et al*., 2009). The affinity of Con A for manno β-septanoside 7 was found to be higher than any of the previously reported mono-septanoside ligands confirmed by isothermal titration calorimetry (ITC) in conjunction with docking simulations (Duff *et al*., 2011)

The aim of the present study was to develop the simple and inexpensive procedure for the purification of Con A was achieved by introducing the cobalt (II) ions in calcium alginate-cellulose beads. This procedure is better than the Sephadex G-50 procedure as the inexpensive procedure is superior in terms of cost and protein yield. Since salts would influence non-covalent interactions, including polar interactions, their pronounced inhibitory effect on Con A-dextran (or Con A-ovalbumin) was not unexpected.

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Table 1 and Figures follow……

Table 1. Purification table of Con A with the help of Sephadex G-50, calcium alginate-cellulose beads and calcium alginate-cellulose beads containing cobalt (II).

Step fraction	Total protein (mg)*	Total activity (titer)#	Specific activity (titer/mg)	Purification fold	Protein yield (%)
Dialysed homogenate (common to all)	400	15200	38	1	100
Purification of Con A by using Sephadex G-50					
Elution by 0.2 M glucose solution	14.6	6200	424.6	11.17	3.65
Purification of Con A by using calcium alginate-cellulose beads					
Elution by 0.2 M glucose solution	18.84	7800	414.0	10.8	4.7
Purification of Con A by using calcium alginate-cellulose beads containing cobalt ions					
Elution by 0.2 M glucose solution	27.3	9000	329.6	8.67	6.8

The titer of the tested lectin is expressed as the reciprocal of the highest dilution showing agglutination of trypsinized rabbit erythrocytes.
* Determined by the method of Lowry *et al* (1951).

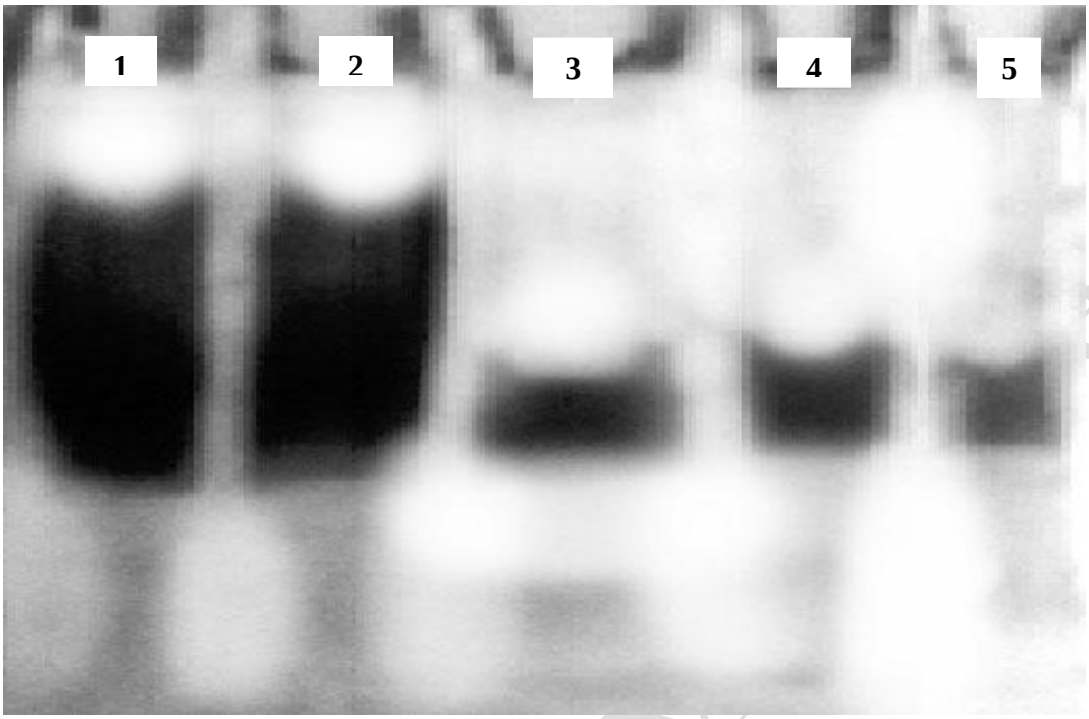

Figure 1: SDS-PAGE profile of Con A purification (10% gel, stained with Coomassie Blue R250). Lane 1: (Crude after incubation with sephadex); 2 (Crude after incubation with cellulose beads containing metal ions); 3 (Purified Con A from Sephadex); 4 (Purified Con A from cellulose beads containing metal ions); 5 (Commercially purified Con A).

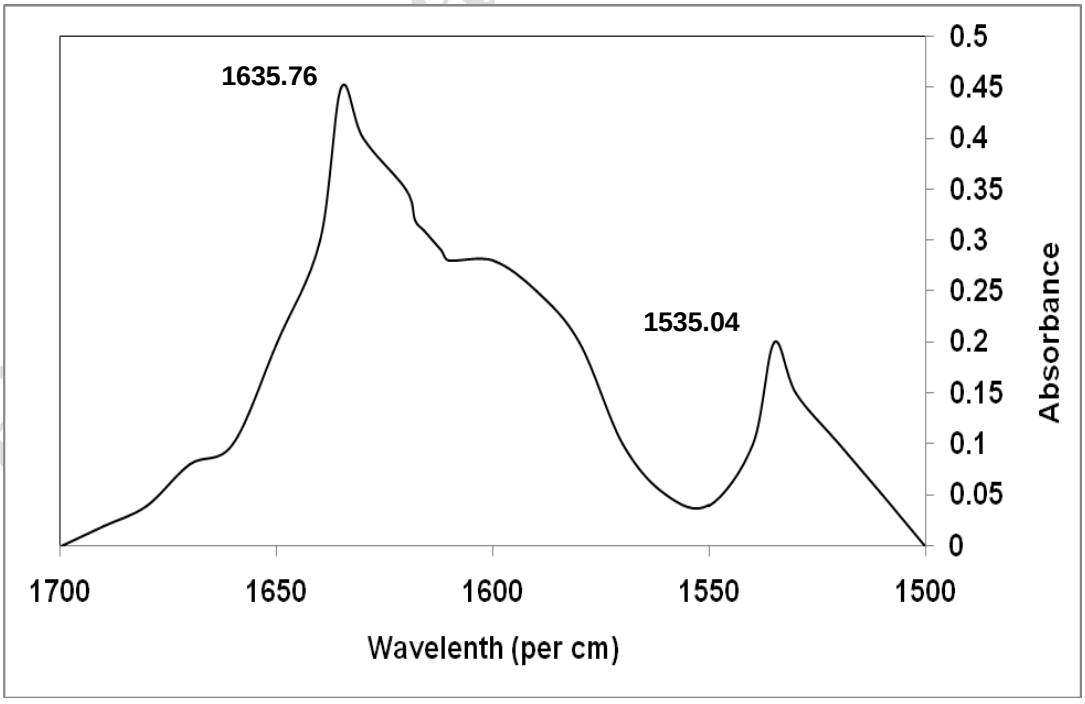

Figure 2: FTIR spectra of purified Con A.

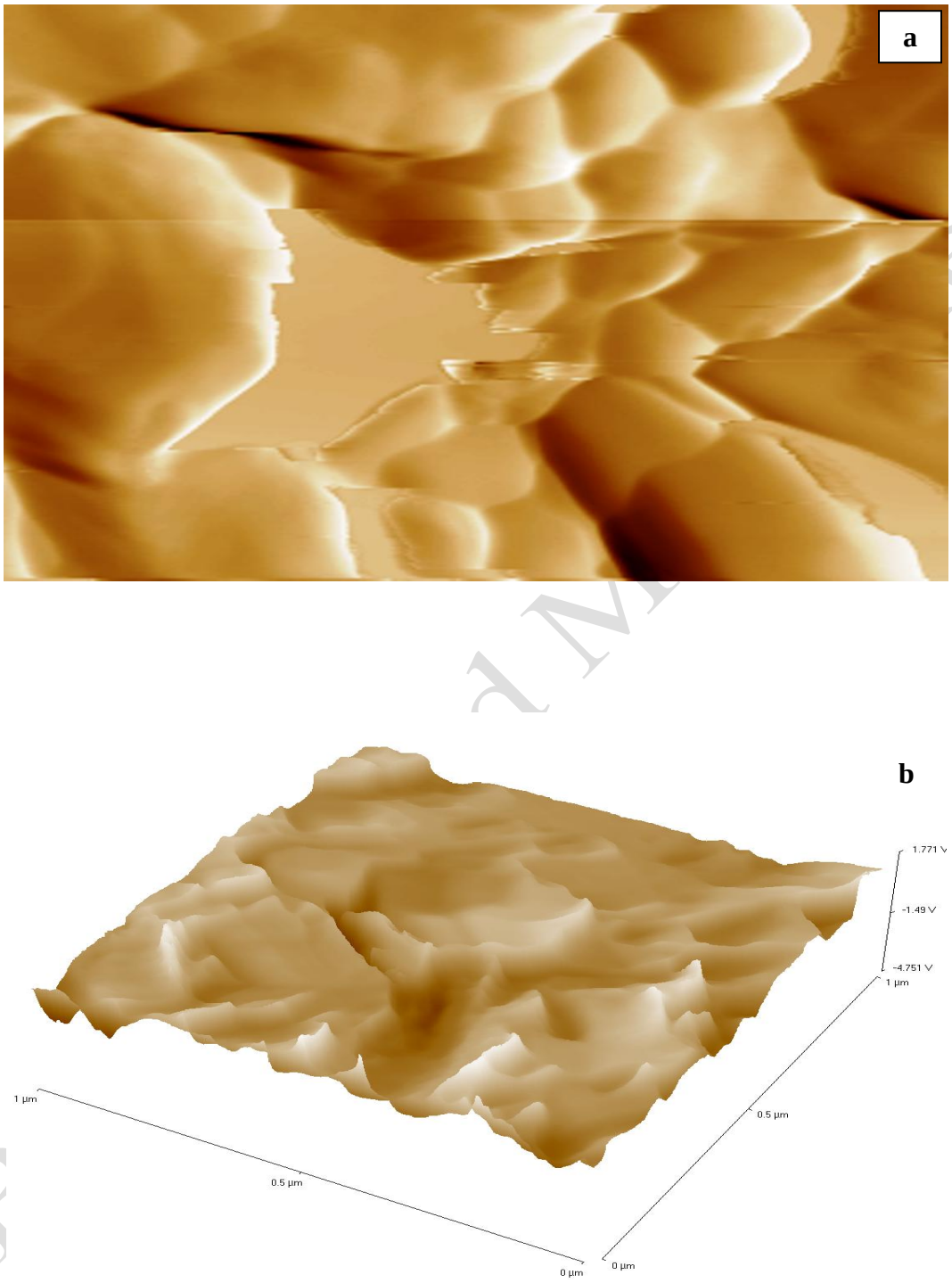

Figure 3: AFM micrographs of purified Con A (a) 2D image of Con A (b) 3D image of Con A.

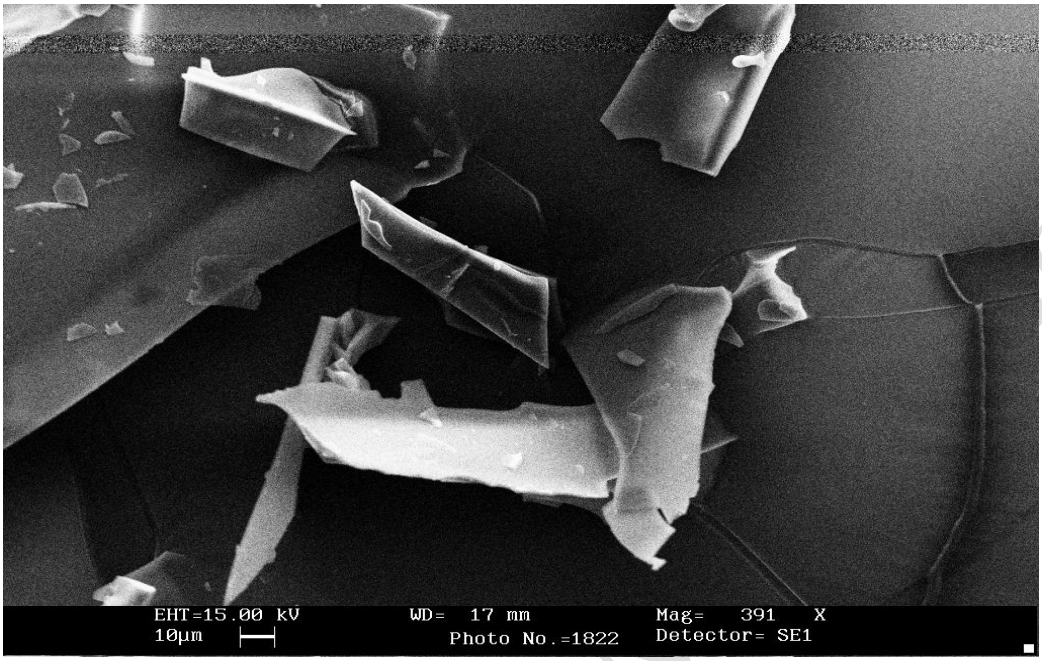

Figure 4: SEM image of purified Con A.

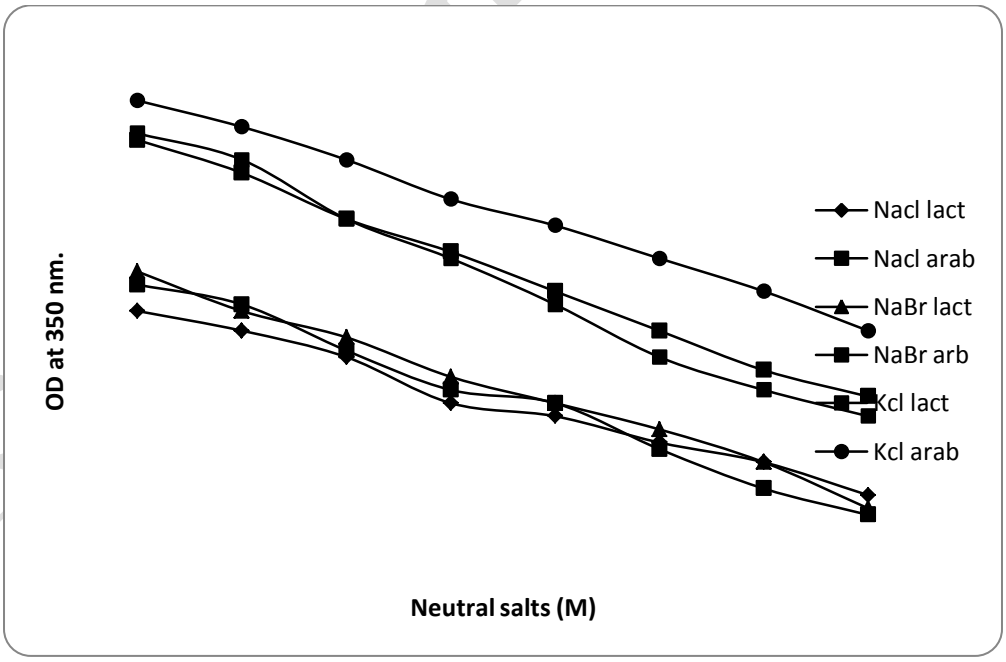

Figure 5: The influence of NaCl, NaBr and KCl concentration on Con A-dextran precipitin reaction in the presence of lactose and arabinose. A fixed concentration of Con A (0.5 mg/ml) mixed with lactose and arabinose separately, than added different concentration of NaCl, NaBr and KBr followed by fixed concentration of dextran (2.5 mg). After incubation for one hour at room temperature, the turbidity of the reaction mixture was measured at 350 nm.

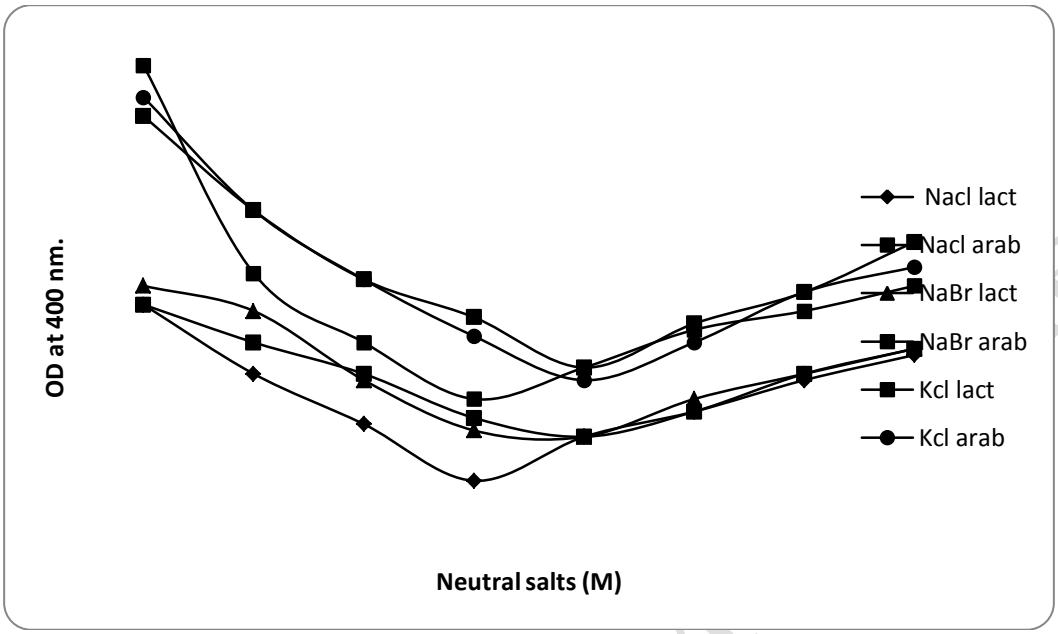

Figure 6: The influence of NaCl, NaBr and KCl concentration on Con A-ovalbumin precipitin reaction in presence of lactose and arabinose. A fixed concentration of Con A (1.5 mg/ml) mixed with lactose and arabinose separately, then added different concentrations of NaCl, NaBr and KCl followed by fixed concentration of ovalbumin (2.2 mg/ml). After incubation for three and a half hours at room temperature, the turbidity of the reaction mixture was measured at 400 nm.