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Single Step Immobilized Metal Ion Affinity Precipitation/Chromatography Based Procedures for Purification of Concanavalin A and *Cajanus cajan* Mannose-Specific Lectin

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Abstract—Concanavalin A and a mannose-specific lectin could be precipitated specifically from extracts of jack bean and *Cajanus cajan* seeds, respectively, using metal charged EGTA. Single step purification of the lectins was also possible using iminodiacetic acid-Sepharose charged with metal ions. Nondenaturing electrophoresis in polyacrylamide gel and that performed in presence of SDS ascertained homogeneity of the isolated lectins. The migration behavior of the purified lectins was comparable with those of the lectins purified using alternative procedures.

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Immobilized metal ion adsorption (IMA) is a collective term used to include all kinds of adsorptions whereby metal ions immobilized on polymers cause or dominate interaction at the solution site [1-3]. Currently IMA chromatography (IMAC) is widely used in the fractionation/purification of recombinant proteins bearing specific histidine affinity tails [4, 5]. A number of amino acid residues contribute to binding of proteins to chelated metal ions, but histidine residues are known to form particularly strong complexes with Cu^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}. Affinity tails containing $(His)_{2-9}$, (His-Tyr), or (His-Gly-His) have all been employed with varying success [6-8].

Although IMAC, like other column chromatographic procedures, is excellent for the purification of proteins on an analytical scale, problems are encountered when large volumes of the protein extracts are to be handled. Protein precipitation strategies, on the other hand, provide convenient alternatives in such a situation. The precipitation procedures suffer from poor specificity resulting in low to moderate purification. The affinity purification procedure introduced by Larsson and Mosbach [9], using bifunctional ligands addressed the issue with reasonable success, although ligands that can specifically precipitate various proteins are not always available. Carlsson [10] improved and further evaluated the potential of affinity precipitation of recombinant proteins carrying histidine tails using EGTA charged with transition metals. Such precipitation requires the presence of at least two metal ion-binding sites on the proteins to facilitate the formation of large insoluble aggregates. Apparently, oligomeric proteins with at least one oligohistidine tail in each subunit have considerable potential for specific immunoprecipitation. A number of native proteins have also been shown to bind to metal chelated supports through their exposed surface histidines; IMAC has been used with varying success in their purification [11-13].

Concanavalin A (Con A) is a very widely used lectin and has been purified from the jack bean by various procedures including those that employ its affinity for glu-

Abbreviations: IDA) iminodiacetic acid; IMA) immobilized metal ion adsorption; IMAC) IMA chromatography; Con A) concanavalin A.

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cose and mannose. Con A is tetrameric and has been shown to possess unusually strong affinity for Cu^{2+}-iminodiacetic acid (IDA) [14-19]. This is related to the presence of six histidine residues including His24, which is exposed to the surface in each of the subunits. Specific precipitation of the lectin using metal charged EGTA was therefore envisaged. The possibility of purifying the lectins using IDA-Sepharose in a single step was also explored. Attempts were also made similarly to purify a related but dimeric glucose/mannose specific lectin from *Cajanus cajan* seed extracts [20] that contains eight histidine residues in each subunit using IMAC.

MATERIALS AND METHODS

Materials. Jack bean meal and IDA-Sepharose were purchased from Sigma (USA). *Cajanus cajan* seeds were purchased from a local grocery shop.

Preparation of crude Con A and *C. cajan* seed extract. Ten grams each of commercial jack bean meal and *C. cajan* seeds were suspended separately in 100 ml of 0.1 M phosphate buffer, pH 7.5, containing 0.2 M NaCl. The suspension was stirred for 7 h at room temperature and centrifuged at 10,000*g* for 30 min. The clear supernatant thus obtained was dialyzed against the buffer.

Metal affinity precipitation. The metal chelate complex EGTA$(Me)_2$ was prepared by mixing 2.5 mmol EGTA and 5.2 mmol K_2CO_3 in 50 ml of distilled water. EGTA is assumed to coordinate two metal ions but was added in excess to avoid possible inhibitory action by free metal ions. Thus, by addition of $ZnCl_2$ or $CuCl_2$ to the EGTA, 96% of binding sites are expected to be occupied. Two volumes of crude extract was mixed with one volume of EGTA$(Me)_2$, which was added drop by drop. The solution was incubated on ice for 20 min, pelleted by centrifugation, and washed twice with ice-cold sodium acetate buffer, pH 6.0. The pellet was finally dissolved in 0.1 M Tris HCl buffer, pH 7.5, containing 30 mM EDTA.

Purification using IDA-Sepharose. The metal chelate complex IDA:Me(II) was prepared by mixing 0.5 ml of IDA-Sepharose with 0.1 M $CuCl_2$ or $ZnCl_2$. Unbound salts were washed off, and the support was incubated with the crude extracts for 4 h at 4°C, washed, and eluted with 30 mM EDTA [21].

Protein estimation. Protein concentrations were determined either spectrophotometrically or using the procedure of Lowry et al. [22]. The absorbance of protein solution at 280 nm was measured on a Hitachi (Japan) single beam spectrophotometer using cuvettes of 1 cm path length. Light absorption measurements were performed in the visible range on an AIMIL Photochem-8 colorimeter.

Determination of molecular weights by SDS-PAGE. Electrophoresis was carried out in 15% polyacrylamide gels using a Tris-glycine buffer (pH 8.3) containing 0.025 M Tris, 0.192 M glycine, and 0.1% SDS according to the procedure of Laemmli [23]. Protein samples were prepared to given a final concentration of 1% (w/v) SDS, 0.5% (v/v) 2-mercaptoethanol, 0.25 M Tris-HCl, 10% (v/v) glycerol, and traces of bromophenol tracking dye. Samples were treated in boiling water for 3-5 min prior to electrophoresis. The gels were stained with Coomassie brilliant blue R-250. Marker proteins used included glucose oxidase (80 kD), ovalbumin (45 kD), α-chymotrypsinogen (20 kD), ribonuclease A (13.69 kD), and cytochrome *c* (11.7 kD).

Hemagglutination. The activity of Con A and *C. cajan* lectin was detected using trypsinized erythrocytes. For trypsinization, 10 mg of trypsin was incubated with 0.1 ml of packed cells in normal saline for 10 min at 37°C. The trypsinized cells were washed twice with normal saline and used further.

RESULTS AND DISCUSSION

Purification of Con A and *C. cajan* lectin by the metal ion affinity based technique. EGTA charged with Cu^{2+} or Zn^{2+} was used to precipitate the lectins from 10% (w/v) seed extracts of jack bean or *C. cajan*, as described in the methods section. The precipitate was washed with 0.1 M phosphate buffer containing 0.2 M NaCl, finally dissolved in a solution containing 30 mM EDTA, and dialyzed extensively. Ninety-one milligram of protein was thus isolated from the extract prepared from 10 g jack bean seeds, while the same amount of *C. cajan* seeds yielded 53 mg of purified protein.

Both the Con A and *C. cajan* lectin isolated by immobilized metal ion affinity precipitation/chromatography readily gave visible agglutination of the trypsinized rabbit erythrocytes (data not shown).

PAGE of precipitate obtained from jack bean extract with EGTA(Zn) and EGTA(Cu) complexes. In order to examine the purity of the lectin precipitated from jack bean extract with EGTA(Zn) and EGTA(Cu) complexes, PAGE was performed under non-denaturing conditions. As shown in Fig. 1a, the precipitated material migrated as a single major band comparable with pure Con A. For comparison the crude extract of jack bean meal and supernatant remaining after precipitation with EGTA were also subjected to PAGE. While both the preparations revealed the presence of multiple band on staining with the dye, staining in the region corresponding to Con A in the lane bearing the supernatant was faint, indicating the precipitation of most of the lectin in presence of EGTA charged with the metal ion. Both Cu^{2+} and Zn^{2+} appeared to be equally effective in the specific precipitation of Con A from the jack bean extract. Specific precipitation of Con A could also be ascertained by carrying out PAGE in presence of 15% SDS. Precipitates obtained using either Zn^{2+} or Cu^{2+} charged EGTA migrated as a single major

band with mobility comparable with that of pure Con A (Fig. 1b).

Purification of Con A and *C. cajan* lectin using IDA-Sepharose. Using a slightly modified strategy, attempts were also made to purify Con A from jack bean extract also in a single step. The procedure involved incubation of the extract with IDA-Sepharose charged with either Zn^{2+} or Cu^{2+}, washing off the unbound proteins, and elution of the bound proteins with EDTA as described in the text. Both non-denaturing PAGE (Fig. 2a) or that performed in presence of SDS (Fig. 2b) suggested that, among the proteins of the jack bean extract only, Con A binds strongly to IDA-Sepharose and can be conveniently eluted in a homogenous form.

The *C. cajan* lectin could also be similarly purified using the strategy and as shown in Fig. 3. The migratory

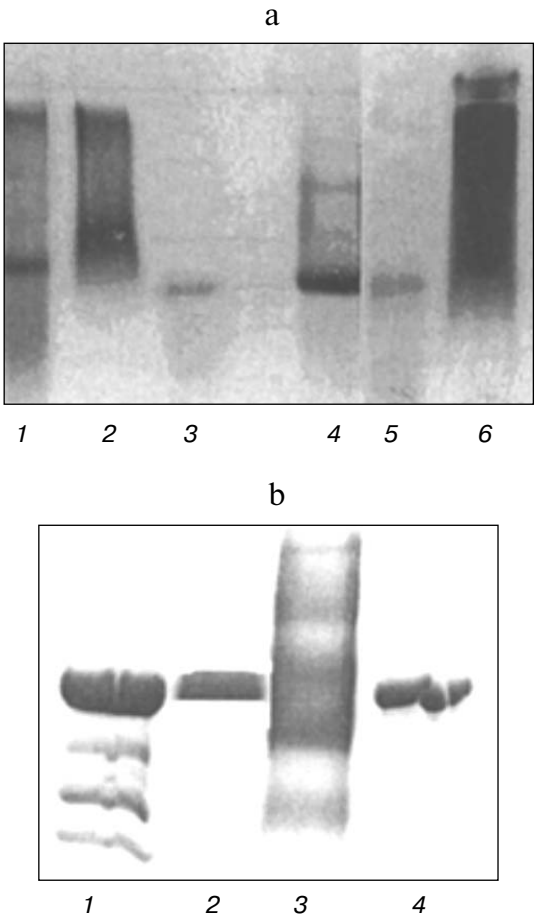

Fig. 1. Polyacrylamide gel electrophoresis of buffer-solubilized Con A obtained from jack bean extract with EGTA(Zn) and EGTA(Cu) complex. a) SDS-free 7.5% gel at pH 8.8. Lanes: *1*) buffer solubilized Con A; *2*) supernatant obtained after precipitation from jack bean extract with EGTA(Zn); *3*) complex-dissociated (EGTA-Zn) Con A; *4*) commercial preparation of Con A; *5*) complex-dissociated (EGTA-Cu) Con A; *6*) supernatant obtained after precipitation from jack bean extract with EGTA(Cu). Each well was loaded with 25 μg protein. b**)** SDS-containing 15% gel at pH 8.3. Lanes: *1*) commercial preparation of Con A; *2*) complex-dissociated Con A obtained from metal affinity precipitation with Zn^{2+}; *3*) buffer-solubilized Con A from jack bean; *4*) complex-dissociated Con A obtained from metal affinity precipitation with Cu^{2+}. Each well was loaded with 15 μg protein.

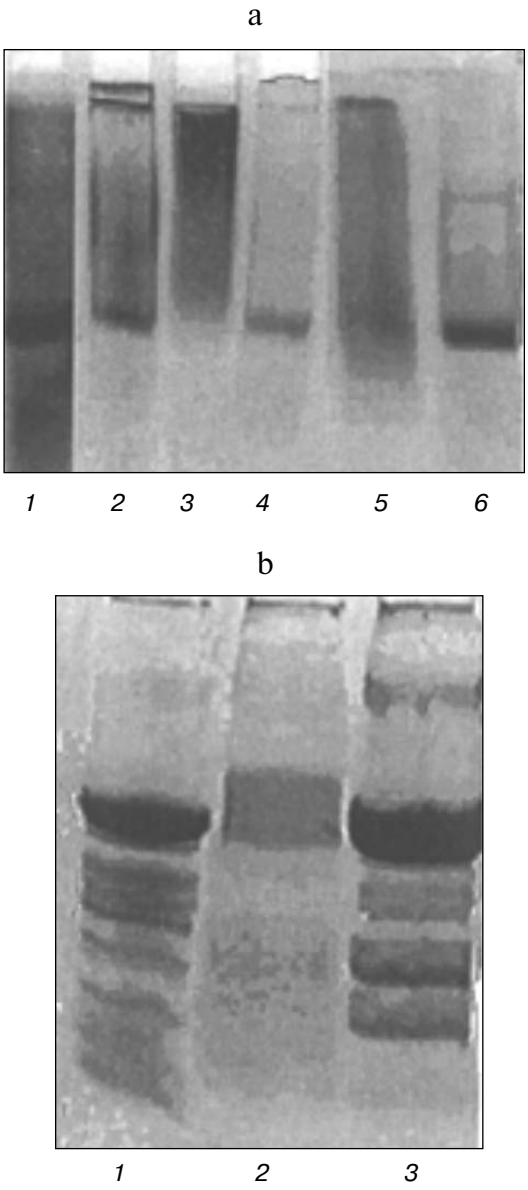

Fig. 2. Polyacrylamide gel electrophoresis of the eluate obtained from jack bean extract with IDA(Cu) and IDA(Zn) complex. a) SDS-free 7.5% gel at pH 8.8. Lanes: *1*) buffer solubilized Con A from jack bean; *2*) complex-dissociated Con A obtained with IDA(Cu) complex; *3*) supernatant obtained after binding of jack bean on (IDA)Cu; *4*) commercial Con A; *5*) supernatant obtained after binding of jack bean on (IDA)Zn; *6*) commercial preparation of Con A. Each well was loaded with 25 μg protein. b**)** SDS-containing 15% gel at pH 8.3. Lanes: *1*) complex-dissociated Con A obtained with metal affinity chromatography using Cu^{2+}; *2*) complex-dissociated Con A obtained with metal affinity chromatography using Zn^{2+}; *3*) commercial Con A. Each well was loaded with 15 μg protein.

Fig. 3. SDS-PAGE of *C. cajan* lectin in 15% gel. Lanes: *1*) purified *C. cajan* lectin obtained with EGTA metal affinity chromatography using Zn^{2+}; *2*) purified *C. cajan* lectin obtained with IDA metal affinity chromatography using Zn^{2+}. Each well was loaded with 15 μg protein.

behavior of the lectin purified by IMAC using Zn^{2+} was identical with that of the lectin isolated by the earlier standardized procedure from this laboratory.

Molecular weight determination of the purified lectins. Molecular weights of the purified lectins were determined using PAGE performed in the presence of 0.1% SDS. Molecular weights of the lectin subunits were determined by comparing their mobility with those of standard proteins and were found to be 25.7 and 18.5 kD for the Con A and the *C. cajan* lectin, respectively. These values are in excellent agreement with the subunit molecular weights of the lectins determined by alternative procedures. The Con A sample used revealed the presence of some fragments presumable resulting from cleavage of the lectin during storage, and the migration of the major band was considered to represent Con A monomer.

These data thus strongly suggest the possibility of purifying Con A from jack bean extract and *C. cajan* lectin from its seeds in a single step using metal affinity precipitation.

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