Purification and characterization of serum $\alpha_2$-globulin binding protein from Alocasia macrorhiza tuber

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Received 2 August 1999; revised 6 December 1999

A protein capable of precipitating serum $\alpha_2$-globulin was purified from Alocasia macrorhiza tuber. The protein, designated as Alocasia protein was heat labile and was found to exist in four isomeric forms, each having four subunits. The serum $\alpha_2$-globulin binding activity of the Alocasia protein was not altered by the action of proteolytic enzymes like trypsin, chymotrypsin and pepsin. Unlike the naturally occurring lectins, the Alocasia protein failed to agglutinate erythrocytes, leukocytes and the microbial organisms. In addition, different sugars and sugar derivatives did not prevent the complex formation between the Alocasia protein and $\alpha_2$-globulin of serum. Divalent metal ions and SH agents did not affect the activity of the protein. Preliminary studies indicated that haptoglobin and $\alpha_2$-macroglobulins were the major, if not the exclusive proteins that are responsible for interaction with the purified Alocasia protein.

Many plant proteins have been known to interact with biologically unrelated animal proteins. Notable among them are proteins that are inhibitors of animal digestive enzymes, lectins which bind with a variety of animal glycoproteins and antibody-antigen system and these interactions lead to the formation of soluble or insoluble complexes. Our preliminary studies showed the presence of significant amount of serum protein interacting factor in Alocasia macrorhiza tuber as evidenced by precipitation behaviour on agar gel media. It is quite possible that one or more proteins present in both the media can contribute in complex formation. Isolation of these proteins to homogeneity is essential to study their chemical nature and characteristic properties. Here we describe the purification and characterization of serum $\alpha_2$-globulin binding protein from the tuber of Alocasia.

Materials and Methods
Carboxymethyl (CM) cellulose, diethylaminoethyl (DEAE) cellulose, triethylaminoethyl (TEAE) cellulose and Affigel-10 were obtained from Bio-Rad Laboratories, USA. Sephadex G-75, G-100 and phenyl Sepharose CL-4B were procured from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine trypsin (EC 3.4.21.4), salt free and twice crystallized, porcine pepsin (EC 3.4.4.1) and bovine $\alpha$-chymotrypsin (EC 3.4.21.1) were the products of Sisco Research Laboratories, India. Cytochrome c (horse heart) and myoglobin (horse heart) were purchased from Sigma Chemical Co., USA. Bovine serum albumin, ovalbumin, $\alpha$-chymotrypsinogen A (bovine) and blue dextran were procured from Pharmacia Fine Chemicals, USA. The radial immunodiffusion (RID) plates and partigen ruler for measuring haptoglobin and $\alpha_2$-macroglobulin were obtained from Serotec Laboratories, Germany. The amino acids, sugars and their derivatives were obtained from Sisco Research Laboratories, India. All other reagents were of analytical grade.

Assay of the serum protein binding activity of the Alocasia tuber protein

At different stages of purification and characterization, the activity of the Alocasia extract protein was determined based on its serum protein precipitation action on serum coated agar plate. The plate was prepared by boiling agarose (50 mg) in 4.8 ml of 0.2 M Tris-HCl buffer, pH 7.0 containing 0.02% sodium azide. The gel was allowed to cool for about 50-60°C and 0.2 ml of human serum was added with constant mixing. The gel was then poured onto a circular transparent plate and after cooling, uniform
wells of 3 mm diameter were created. The Alocasia extract (5 μl) was added to the wells and the plate was kept at room temperature for 18 hr. The precipitin ring formed was taken as an index of complex formation between serum protein and the presumably proteinaceous component present in the extract. To maintain uniformity throughout the study, properly preserved pooled serum sample was used for preparing agar gel plates. The square of the precipitin ring diameter in mm was calculated using the radial immuno-diffusion (RID) method and partigen ruler. The unit of activity was defined as the amount of protein (in μg) required to form 10 square mm ring after 18 hr.

**Other assay methods**

Protein was determined by the method of Lowry et al. with bovine serum albumin as standard. Carbohydrate content was estimated by the phenol sulfuric acid method using galactose as standard. The unit of carbohydrate was expressed as galactose equivalent and its percentage per protein content was calculated.

**Immobilization of trypsin on affigel-10**

Trypsin (100 mg in 5 ml) was immobilized with 10 ml of affigel-10 at pH 7.6 using 0.1 M borate buffer.

**Preparation of the crude extract**

All operations were carried at room temperature unless stated otherwise. The freshly peeled Alocasia tuber (100 g) was homogenized with 170 ml of 0.01 M HCl, pH 2.0, kept for 2 hr with occasional stirring and centrifuged at 15,000 g for 20 min. The residue was reextracted with 30 ml of 0.01 M HCl. Both the supernatants were pooled and the pH was adjusted to 7.0 using 0.2 M Tris solution and the final volume was made up to 250 ml with 0.2 M Tris-HCl buffer, pH 7.0.

**Purification**

The crude extract was subjected to 50% saturation with (NH₄)₂SO₄ by adding 78.25 g of the salt with constant stirring at 4°C. The cloudy solution was allowed to stand for 18 hr at 4°C. The precipitate was separated by centrifuging at 15,000 g for 20 min and dissolved in a minimum volume of 0.02 M Tris-HCl buffer, pH 7.0. The solution was dialyzed against 100 volumes (v/v) of the same buffer for 18 hr at 4°C. The dialyzate was centrifuged at 15,000 g for 20 min to remove the insoluble particles. The clear supernatant, designated as the (NH₄)₂SO₄ fraction (15 ml) was treated with 0.1 M CaCl₂ (40 μl CaCl₂ per ml of (NH₄)₂SO₄ fraction) and was kept at 4°C for 2 hr. The precipitate formed was removed by centrifuging at 15,000 g for 20 min at room temperature. The clear supernatant was dialyzed against 0.02 M Na₂HPO₄ buffer, pH 7.0 and designated as the CaCl₂ treated fraction.

**Chromatography**

The CaCl₂ treated fraction was applied to a column of TEAE-cellulose (1.5 x 22.6 cm, bed volume 30 ml), equilibrated with 0.02 M Na₂HPO₄ buffer, pH 7.0. The column was washed with the equilibration buffer. The bound proteins were eluted with equilibration buffer containing 0.1 M NaCl at a flow rate of 10 ml/hr. All the fractions were assayed for protein content. In each case 3 ml fractions were collected. The fractions showing maximum serum protein interacting activity (numbers 18-21, Fig. 1A) were pooled, dialyzed against deionized water and concentrated by lyophilization. This was designated as TEAE-cellulose fraction (1.5 ml). The TEAE-cellulose fraction was first dialyzed against 0.02 M Na₂HPO₄ buffer, pH 7.0 and passed through an trypsin-affigel-10 column (0.9 x 15 cm, bed volume 10 ml), equilibrated with 0.02 M Na₂HPO₄ buffer, pH 7.0 at 4°C. The column was washed with the equilibration buffer maintaining the flow rate of 3 ml/hr and 3 ml fractions were collected. The bound proteins were eluted with 0.01 M HCl at a flow rate of 5 ml/hr. The fractions of washings that showed serum protein precipitating activity (numbers 3-8, Fig. 1B) were pooled together and designated as "trypsin affigel-10 washing fraction". Similarly the HCl eluted fractions (numbers 13-16) were assayed for the serum protein interacting activity. Besides, these fractions were tested for antitryptic and antichymotryptic activities as well by the method as described by Sumathi and Pattabiraman. Trypsin-affigel-10 washing fraction was concentrated to 1.0 ml and dialyzed against 0.02 M Na₂HPO₄ buffer, pH 7.0 containing 0.15 M NaCl. The fraction was then subjected to gel chromatography on a Sephadex G-100 column (1.5 x 62.2 cm, bed volume 120 ml), equilibrated and eluted with the above mentioned buffer. Fractions (3 ml) were collected at a flow rate of 6 ml/hr. The fractions with high protein content as well as maximum activity (numbers 17-23, Fig. 1C)
Fig. 1—Chromatography of the Alocasia protein at different steps of purification. (A): Purification step 3: Elution profile of calcium chloride treated fraction from the TEAE-cellulose anion exchange column; column size 1.5 x 22.6 cm, flow rate 10 ml/hr, fraction size 3 ml, elution with 0.02 M Na₂HPO₄ buffer containing NaCl, pH 7.0, fractions 18-21 were pooled. (B): Purification step 4: Resolution of TEAE-cellulose fraction by affinity chromatography on trypsin bound affigel-10; column size 0.9 x 15 cm, flow rate 5 ml/hr, fraction size 3 ml, washing with 0.02 M sodium phosphate buffer, pH 7.0, fractions 3-8 were pooled. (C): Purification step 5: Resolution of trypsin affigel-10 washing fraction by gel chromatography on Sephadex G-100; column size 1.5 x 62.5 cm, flow rate 6 ml/hr, fraction size 3 ml; fractions 17-23 were pooled. (D): Purification step 6: Rechromatography of Sephadex G-100 fraction on Sephadex G-100; fractions 18-23 were pooled. The protein content of all the eluates were estimated by the method of Lowry et al (1951). (□-□): protein; (O-O): serum protein interacting activity.
were pooled and dialyzed against deionized water and concentrated to 1.0 ml. The concentrated solution was designated as "Sephadex G-100 fraction". The Sephadex G-100 fraction (1.0 ml) was subjected to rechromatography on the same column, as mentioned above. The fractions (numbers 18-23, Fig. 1D) that exhibited maximum protein content as well as activities were pooled, dialyzed against deionized water and concentrated to 1.0 ml by lyophilization. This fraction named as "Alocasia protein" was used for all the subsequent characterization studies.

**Electrophoresis**

The Alocasia protein was subjected to agarose gel electrophoresis with 0.05 M sodium barbitone buffer, pH 8.6. The protein band was stained with amido black.

Cellulose acetate electrophoresis of the Alocasia protein was performed on cellulose acetate strip using buffer media, namely, (a) 0.1 M sodium acetate buffer, pH 5.5, (b) 0.05 M Na₂HPO₄ buffer, pH 7.0 and (c) 0.05 M sodium barbitone buffer, pH 8.6. The protein band was stained with amido black.

Polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE in the presence of β-mercaptoethanol were performed by the procedure of Laemmli using 10% gel (w/v). The protein band was stained with Coomassie brilliant blue R-250. Molecular mass of Alocasia protein was determined by SDS-PAGE using following markers: cytochrome c 12,384 Da, ribonuclease 13,700 Da, myoglobin 17,000 Da, chymotrypsinogen 25,000 Da, ovalbumin 43,000 Da, bovine serum albumin 67,000 Da and lactate dehydrogenase 1,40,000 Da.

**Detection of Alocasia protein interaction with human serum albumin and globulins**

In order to check whether the Alocasia protein causes precipitation of albumin or any of the globulins, assays were carried out using serum albumin or serum globulin coated agar plates separately. For this purpose albumin was separated from globulins through affinity chromatography on blue Sepharose as described by Travis.

**Effect of temperature and pH**

The effect of temperature on the stability of Alocasia protein was studied by subjecting 560 µg protein in 100 µl deionized water, in each case, to heat treatment at 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C and 100°C for 10 min. The cooled solutions were centrifuged to remove precipitate if any, formed. The percentage change in the serum protein interacting activity was determined in each case.

In order to study the effect of pH, Alocasia protein (200 µg in each case) was mixed with 0.1 ml of 0.01 M HCl, pH 2.0 and 0.02 M sodium bicarbonate buffer, pH 11.0. After incubation for 20 hr at 4°C, the contents were dialyzed against deionized water and assayed for the serum protein binding activity.

**Effect of sugars**

The sugars and their derivatives, which were used to study the effect on protein-protein interactions, included mannose, glucose, fructose, fucose, N-acetyl-L-glucosamine and N-acetyl-L-galactosamine. Agar plates coated with human serum protein, was prepared as described under general methods with the only difference that one of the sugars or sugar derivatives (0.3 M) was included while preparing the plate in each case. Aliquots (5 µl) containing varying concentrations of Alocasia protein (5 to 25 µg) were added to the wells. The precipitin rings formed were measured and compared with that of control having no sugar or sugar derivatives.

The precipitation behavior of the Alocasia-serum protein complex was also studied in aqueous medium using 0.1 ml of Alocasia protein (800 µg), 0.1 ml of serum (containing 7.0 mg total protein) and 0.5 M sugar or sugar derivatives.

**Agglutination using different cells**

Erythrocytes of all the four major blood groups (A, B, AB and O) were separated from human blood and washed four times with saline. In each case 10 µl of washed erythrocytes were suspended in 20 µl of 0.1 M phosphate buffered saline (PBS), pH 7.4 and incubated with 20 µl of Alocasia protein (240 µg) at 37°C for 2 hr. The protein extract of red kidney bean was used as a positive control because of its agglutination behavior with erythrocytes. Microscopic examination of erythrocytes was done to find out either presence or absence of agglutination behavior of Alocasia protein.

In a separate study, 1 ml of either trypsin treated (1% trypsin) or albumin treated (0.5% albumin) erythrocyte suspension was incubated with 1 ml of Alocasia protein (300 µg) at 37°C for 2 hr. The erythrocytes were observed for agglutination. Similarly,
Alocasia protein (125 μg in 15 μl) was treated with 15 μl of leukocytes suspended in normal saline and observed for agglutination after 90 min.

The Alocasia protein (125 μg in 15 μl) was added to *Staphylococcus aureus* medium, which was suspended in normal saline and observed for agglutination. The same procedure was repeated with *Streptococcus pyogenes*, *Escherichia coli*, *Candida albicans* and *Cryptococcus neoformans*.

**Selective precipitation of serum protein by complexing with Alocasia protein**

Alocasia protein (800 μg in 0.1 ml 0.02 M Tris-HCl buffer, pH 7.0) was treated with 0.1 ml of serum containing 7.0 mg of total protein of which 2.8 mg was globulin. The mixture was kept at 4°C for 12 hr. The precipitate formed as a result of complex formation was removed by centrifugation at 10,000 g for 10 min. The precipitate free serum was subjected to cellulose acetate or agar gel electrophoresis at pH 8.6. For the purpose of comparison electrophoresis on cellulose and agar gel were also run with control serum, diluted 1-2 with 0.02 M Tris-HCl buffer, pH 7.0.

**Detection of haptoglobin, α₂-macroglobulin and ceruloplasmin activity**

The precipitate free serum (5 μl each) obtained from above procedure was applied to RID agar plates coated with anti-haptoglobin and anti α₂-macroglobulin separately. The presence or absence of haptoglobin and α₂-macroglobulin activity was assessed by the appearance of precipitin ring around the wells. The precipitate free serum (60 μl) was also used for the assay of ceruloplasmin.

**Results and Discussion**

**Purification**

A protein, capable of interacting with serum proteins, was isolated from the tuber of *Alocasia macrorhiza*. The details of purification are summarised in Table 1. The protein, designated as "Alocasia protein", was purified 8-fold with 45% recovery of the interacting activity.

The purified Alocasia protein exhibited a single band when subjected to electrophoresis on agar gel and cellulose acetate (Fig. 2A,B). PAGE in presence of SDS also showed single protein band (Fig. 3A). However, four protein bands were seen when subjected to PAGE in the absence of SDS (Fig. 3B). It is quite unlikely that the four bands obtained on PAGE is due to the existence of monomeric, dimeric and higher oligomeric forms of protein, since gel chromatography showed no sign of any extra protein peaks. The presence of these four protein bands could be attributed to the existence of isomeric forms of Alocasia protein. Such isomeric forms of plant

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**Table 1—Purification of α₂-globulin binding protein from Alocasia tuber**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Activity (units)</th>
<th>Sp. activity (per mg protein)</th>
<th>Fold purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>142.0</td>
<td>3550</td>
<td>25.00</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>81.0</td>
<td>3115</td>
<td>38.46</td>
<td>1.53</td>
<td>87.74</td>
</tr>
<tr>
<td>CaCl₂ treated</td>
<td>46.0</td>
<td>3066</td>
<td>66.66</td>
<td>2.66</td>
<td>86.39</td>
</tr>
<tr>
<td>TEAE fraction</td>
<td>29.8</td>
<td>2421</td>
<td>111.10</td>
<td>4.40</td>
<td>56.33</td>
</tr>
<tr>
<td>Affigel-10</td>
<td>13.2</td>
<td>1884</td>
<td>142.80</td>
<td>5.70</td>
<td>53.11</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>10.0</td>
<td>1666</td>
<td>166.00</td>
<td>6.64</td>
<td>46.92</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>8.0</td>
<td>1600</td>
<td>200.00</td>
<td>8.00</td>
<td>45.07</td>
</tr>
<tr>
<td>(rechromatography)</td>
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</table>

[One unit of interacting activity is defined as the amount of protein required to form 10 sq mm precipitin ring]
Molecular weight

The possibility of Alocasia protein existing in isomeric forms notwithstanding, the molecular weights are identical or close to each other around 51,300 as determined by chromatography on Sephadex G-100 column. Each of them is likely to be made up of four subunits having molecular weight around 12,600 as evidenced by SDS-PAGE. Presence of isomeric forms having identical or nearly similar molecular weight has been also reported in the case of plant lectins and proteaseous enzyme inhibitors\textsuperscript{14,19-22}.

Precipitation on agar plate and quantitation

Precipitation behavior on serum coated agar plate was taken as an index to quantitate Alocasia protein either in crude or purified state (Fig. 4). This method is comparable to RID method currently used in the field of clinical chemistry for the determination of immunoglobulins and complement proteins. On plotting the square of ring diameter (in mm) against concentration of Alocasia protein ranging from 5 to 25 \(\mu\)g a linear graph was obtained (Fig. 5). No precipitation zone was formed when Alocasia protein was added to agar plate coated with purified human serum albumin or bovine serum albumin.

Effect of proteolytic enzymes

The activity of Alocasia protein remained unchanged when treated (200 \(\mu\)g) with proteolytic

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Fig. 3—(A): SDS-polyacrylamide gel electrophoresis of Alocasia protein. (B): Polyacrylamide gel electrophoresis of Alocasia protein without SDS. [The electrophoresis was performed with 80 \(\mu\)g of protein in both the cases].

proteins exhibiting similar biological activities have been identified among lectins like soybean agglutinin\textsuperscript{12} and fucose binding lotus protein\textsuperscript{13}, and among protease inhibitors as well, which include \(\alpha_1\)-trypsin inhibitors of sweet potato and taro (Colocasia antiquorum) tubers\textsuperscript{14,15}.

The possible isomeric forms of purified Alocasia protein could not be separated further by techniques such as ion exchange chromatography using CM-cellulose or DEAE-cellulose, chromatography on phenyl Sepharose and gel chromatography on Bio Gel P-100.

A significant amount of protein, devoid of serum protein precipitating activity, was removed by insolubilization using \(\text{CaCl}_2\) in the early stage of purification. The same effect was also observed when either crude extract or \((\text{NH}_4)_2\text{SO}_4\) fraction was treated with \(\text{MgCl}_2\). The effect of divalent cations on precipitating the unwanted proteins, observed in the present study, is comparable to the precipitation of paracasein as calcium paracaseinate\textsuperscript{16} and polymerization and precipitation of actin in presence of \(\text{Mg}^{2+}\) (ref. 17). \(\text{Mg}^{2+}\) is also known to play significant role in the precipitation of high density lipoprotein in serum\textsuperscript{18}.

The isoelectric point (pI) of the Alocasia protein seems to be less than 7.0, as the protein moved towards anode, when cellulose acetate electrophoresis was performed at that pH. Moreover, absence of any mobility of protein on electrophoresis at pH 5.5 indicated that the pI may be around 5.5.

Fig. 4—Precipitation ring of Alocasia protein on serum coated agar gel plate.
enzymes such as pepsin (300 μg, pH 2.0), trypsin (200 μg, pH 7.6) and chymotrypsin (200 μg, pH 7.6).

Effect of temperature, pH and metal ions
The Alocasia protein was found to be stable up to 60°C when subjected to heat treatment for 10 min. However, there was a decrease in % serum protein binding activity to 70%, 36.6%, 20% and 10% at 65°C, 70°C, 75°C and 80°C, respectively. Complete loss of activity was observed at 90°C and above (Fig. 6). In contrast, the protein was found to be stable in both acidic (pH 2.0) and alkaline pH (pH 11.0) when incubated for 20 hr at 4°C. The activity of Alocasia protein remained unchanged in the presence or the absence of divalent metal ions like Mn²⁺, Ca²⁺, Cu²⁺, Zn²⁺ or Pb²⁺.

Other characteristics
Purified preparation of Alocasia protein did not exhibit any antitryptic or antichymotryptic activities, although Alocasia tuber has been reported to be a good source for trypsin/chymotrypsin inhibitors. The possibility of the purified Alocasia protein being a proteinase inhibitor was ruled out by removing all the trypsin/chymotrypsin inhibitors using immobilized trypsin on affigel-10 column. It did not show α-amylase inhibitory activity (assayed as described by Shivaraj and Pattabiraman). The Alocasia protein was found to be a glycoprotein containing 4.0% carbohydrates, expressed as galactose equivalent.

Influence of sugars and sugar derivatives
None of the sugars and sugar derivatives used in the present study, prevented the complex formation between the Alocasia protein and the serum proteins. Addition of these sugars even at high concentration did not dissociate the complex either. Moreover, the Alocasia protein could not be immobilized or precipitated by any of the polysaccharide matrices used. This is in contrast to lectin-glycoprotein complexes which have been known to be dissociated by use of specific sugars or sugar derivatives.

Agglutination
The erythrocytes or leukocytes obtained from all the four major blood groups did not show any agglutination when treated with Alocasia protein. Prior treatment of erythrocytes with trypsin or albumin did not result in any agglutination either, unlike reported in the case of certain other lectins. Similar negative results were obtained when agglutination test was carried out with Alocasia protein and certain strains of microorganisms like

Fig. 5—Square of precipitin ring diameter in mm against protein concentration of Alocasia protein.

Fig. 6—Effect of temperature on stability of Alocasia protein. Values are means of three experiments.
and haptoglobin 29 of human serum origin. But another agar plates coated with purified α2-macroglobulin 28 cut decrease in the density of activities. This property was also confirmed by using complex); Lane 2; control serum].

obtained after precipitating the Alocasia protein-serum protein (Fig. 7). The precipitate free serum also showed excess. Cellulose acetate electrophoresis of the precipitate free serum (supernatant obtained after precipitating the Alocasia protein-serum protein complex). Care was taken to avoid Alocasia protein in the presence or absence of β-mercaptoethanol revealed similar band, suggesting that the probable subunits of the protein are not linked by -SH groups.

Selective precipitation
Treatment of serum with Alocasia protein resulted in precipitation of Alocasia protein-serum protein complex. Care was taken to avoid Alocasia protein in excess. Cellulose acetate electrophoresis of the precipitate free serum (supernatant) showed a clear-cut decrease in the density of α2-globulin band (Fig. 7). The precipitate free serum also showed marked decline in α2-macroglobulin and haptoglobin activities. This property was also confirmed by using agar plates coated with purified α2-macroglobulin 28 and haptoglobin 29 of human serum origin. But another prominent protein of α2-globulin family, namely ceruloplasmin, remained unaltered. This suggests that haptoglobin and α2-macroglobulin are the two prominent α2-globulins involved in binding and precipitation with Alocasia protein. However, the possibility of other members of α2-globulin participation cannot be ruled out.

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Fig. 7—Cellulose acetate electrophoresis of precipitate free and control serum. [Lane 1, the precipitate free serum (supernatant obtained after precipitating the Alocasia protein-serum protein complex); Lane 2; control serum].

Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Candida albicans and Cryptococcus neoformans. All these results suggest that the Alocasia protein may not be a lectin. Further investigation is needed to substantiate this contention.

Effect of β-mercaptoethanol and cysteine
Presence of β-mercaptoethanol or cysteine did not change the serum protein precipitation by Alocasia protein. This possibly indicates that -SH group may not be present or even if present, does not affect the serum protein binding activity of Alocasia protein. SDS-PAGE of the purified protein in the presence and absence of β-mercaptoethanol revealed similar band, suggesting that the probable subunits of the protein are not linked by -SH groups.