# TRANSITION METAL REQUIREMENTS OF SOYBEAN AGGLUTININ

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## 1. Introduction

It has been demonstrated that several lectins require metal ions, usually $Mn^{2+}$ and $Ca^{2+}$, for their activity as measured by sugar binding, polysaccharide precipitation of hemagglutination [1]. Concanavalin A, the lectin of the jack bean (*Canavalia ensiformis*) has been the most thoroughly examined of this group of proteins, and here the importance of metal ions for both activity and stabilization has been demonstrated.

Though soybean agglutinin (SBA) from *Glycine max* has been extensively investigated [1], very little is known about its metal ion requirement. Galbraith and Goldstein [2] reported briefly some years ago that SBA, purified by chromatography on hydroxyapatite [3], contains $Mn^{2+}$ and $Ca^{2+}$ and suggested that these metals are required for the hemagglutinating activity of the lectin.

In this communication we provide further evidence concerning the metal requirements of SBA and also demonstrate that the $Mn^{2+}$ can be replaced by other transition metals to yield active SBA preparations with properties similar to those of the native lectin.

## 2. Materials and methods

Soybean agglutinin was purified by affinity chromatography as described by Gordon et al. [4], except that HCl-treated Sepharose [5] was used for specific binding of the lectin instead of Sepharose–*N*-ε-aminocaproyl-β-D-galactopyranosylamine. All solutions were prepared with double distilled water and demetallized on a column of Chelex 100.

The preparation of demetallized SBA (20 mg in 3 ml saline) was accomplished by four different methods:

(i) By dialysis for 10 days at room temperature against 4 × 250 ml 0.1 M metal-free acetic acid-buffered saline, pH 4.6 containing 0.1 mM $NaN_3$, followed by dialysis for 24 h at 4°C against 0.1 M metal-free Tris-buffered saline, pH 7.2.

(ii) By dialysis for 10 days at room temperature against 4 × 250 ml 0.1 M metal-free citrate-buffered saline, pH 5.0, containing 0.1 mM $NaN_3$ followed by dialysis against 0.1 M metal-free Tris-buffered saline, pH 7.2, as above.

(iii) By dialysis successively for 48 h each at 4°C against 4 × 250 ml 1 M acetic acid and metal-free saline.

(iv) By dialysis against EDTA and acetic acid according to the method of Galbraith and Goldstein [2].

Reactivation of the lectin was attempted in all cases by dialyzing the metal-free SBA for 48 h at 4°C against 4 × 250 ml of a solution containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$ followed by dialysis for 24 h at 4°C against 3 × 250 ml solution of either 0.1 M metal-free Tris-buffered saline, pH 7.2 or metal-free saline.

The metal-exchanged lectins were prepared by dialysis, for 6–8 days at room temperature, 20 mg SBA (7 mg/ml) against 4 × 250 ml, pH 5.4, 0.1 M acetic acid buffer (0.88 M NaCl), containing 0.1 M Ni, Co, Cd or $MnCl_2$. The excess metal chloride was removed by gel filtration on Sephadex G-50, at pH 5.4, and the protein solution was dialyzed for 24 h at 4°C against 3 × 250 ml 0.1 M metal-free Tris-buffered saline, pH 7.2.

SBA concentration was determined by absorbance using the value $E^{1\%}_{280\,nm}$ 12.8 $cm^{-1}$ [6].

Metal content was determined by atomic absorption on a Perkin-Elmer 306 Spectrophotometer. ESR spectra were recorded on Varian E-3 100 kHz Field Modulation Spectrophotometer at 9.5 GHz (X-band).

Hemagglutination, in titer plates [7] of untrypsinized rabbit red blood cells was scored after 3 h at room temperature as the reciprocal of the greatest dilution at which agglutination occurred per mg SBA.

The precipitating activity of SBA was assayed by incubating 1 mg lectin with 1 mg hog gastric mucin in total vol. 1 ml at 25°C for 30 min. The turbidity at 420 nm was then measured in a Coleman 295E spectrophotometer. Blank readings were obtained by adding 10 mg D-galactose which was sufficient to dissolve the glycoprotein–lectin complex. The hog gastric mucin was prepared following the method of Kabat and Mayer [8] with the additional precaution of extensive dialysis against 1 mM EDTA and then against double distilled water.

Polyacrylamide gel electrophoresis was performed in the following systems:

(i) A discontinuous Tris-glycine system, pH 4.3 [9].
(ii) Sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate (SDS), specially pure [10].
(iii) A discontinuous Tris–SDS system, pH 8.8 [11].

The gels were stained for protein with Commassie Brilliant Blue R-250.

Immunodiffusion was performed by the Ouchterlony method [11] in 1% agar noble dissolved in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.9% NaCl.

The antibody to SBA was a gift of Dr Bilha Schechter and Dr Dianna Bowles.

## 3. Results and discussion

Analysis by atomic absorption of the various fractions obtained during the isolation of SBA (table 1) demonstrates that metal contaminants are progressively removed and that only $Ca^{2+}$ and $Mn^{2+}$ remain in the purified product. Different preparations of the purified lectin (mol. wt 120 000, ref. [6]) were found to contain between 3.50 and 4.15 g-atoms Ca/mol protein and between 1.0 and 1.69 g-atoms Mn/mol protein (table 2).

Native SBA (batch No. 9) containing 3.5 g-atoms Ca and 1.1 g-atoms Mn was demetallized using different procedures (fig.1). Dialysis of the native SBA against 1 mM EDTA as the first step of the procedure of Galbraith and Goldstein [2], resulted

Table 2
Metal content of different SBA preparations
(mol metal/120 000 D)

| Batch No. and date | | Mn | Ca |
|---|---|---|---|
| B-3 | 6/76 | 1.20 | 4.00 |
| B-6 | 7/76 | 1.68 | 4.15 |
| B-9 | 12/76 | 1.10 | 3.50 |
| B-13 | 5/77 | 1.60 | 4.00 |
| B-14 | 5/77 | 1.42 | 4.08 |
| Ref. [2][a] | | 0.76 | 14.30 |

[a] Calculated from data of Galbraith and Goldstein [2] who analyzed a sample prepared by the hydroxyapatite procedure of Lis et al. [3]

Results are usually averages of two values, which did not differ by more than 8%.

Table 1
Metal content during SBA isolation (mg/g protein) (Batch B-9)

| Fraction | Ca | Mn | Fe | Cu | Ni | Co | Zn | Mg |
|---|---|---|---|---|---|---|---|---|
| NaCl-extract | 5.8 | 0.19 | 0.21 | 0.05 | 0.04 | 0.01 | 0.22 | 53.0 |
| 30% $NH_4SO_4$, supt. | 27.0 | 0.20 | 0.24 | 0.12 | 0.09 | 0.04 | 0.28 | 88.6 |
| 57% $NH_4SO_4$, ppt. | 1.1 | 0.09 | 0.04 | 0.04 | – | – | 0.05 | 1.75 |
| Dialysis against $H_2O$ | 0.47 | 0.12 | 0.05 | 0.03 | – | – | 0.07 | 0.26 |
| Dialysis against NaCl | 0.29 | 0.09 | 0.04 | 0.02 | – | – | 0.05 | 0.08 |
| Affinity chromatography | 1.39 | 0.52 | – | – | – | – | 0.16 | – |
| Dialysis and lyophilization | 1.12 | 0.45 | – | – | – | – | 0.14 | – |

(–) means less than 0.015 mg/g protein

Fractionation was carried out as described by Gordon et al. [4] except that HCl-treated Sepharose [5] was used for affinity chromatography

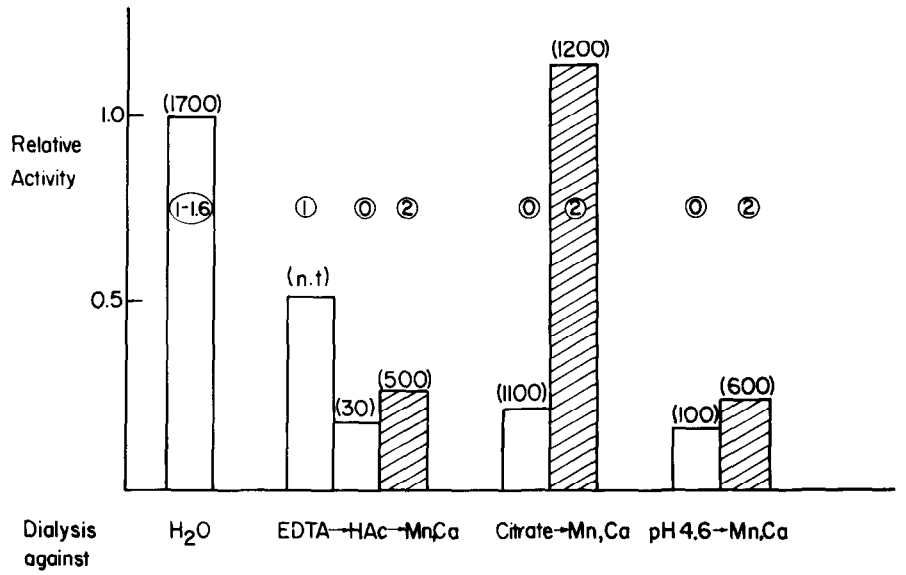


Fig.1. Precipitating and hemagglutinating activities of different preparations of SBA. For each preparation the precipitating activity is indicated by the bar graphs, the hemagglutinating activity by parenthesis and the g-atoms Mn/120 000 D protein within the circle. For experimental details see text.

in a 40–50% loss of activity, which could be fully recovered by dialysis against 1 mM $MnCl_2$ and $CaCl_2$. However, an examination of the EDTA-treated SBA showed no change in the metal content, as compared to the untreated protein. When dialysis against EDTA was followed by dialysis against 1 M acetic acid, further loss of activity was observed (70–80% inactivation), together with loss of both $Mn^{2+}$ and $Ca^{2+}$; the product contained $< 0.1$ g-atom Mn/mol protein and $< 1$ g-atom Ca/mol protein. Dialysis of this preparation against $MnCl_2$ and $CaCl_2$ resulted in only a 10–15% increase in activity even though analysis of the metal content indicated that 3.9 g-atoms Ca and 2 g-atoms Mn were present in the 'remetalized' product. Demetallization with 1 M acetic acid alone (procedure c) gave results similar to those obtained with EDTA and acetic acid.

In order to find milder conditions for 'demetalization', the release of the bound $Mn^{2+}$ from the protein at different pH-values was followed by ESR spectroscopy (fig.2). Using this method it was found that the $Mn^{2+}$ was released from the protein only at pH values below 5. Dialysis of native SBA, at pH 5.4, for 12 days showed no loss of biological activity or metal content, as indicated by both ESR and atomic absorption spectroscopy.

Exhaustive dialysis of SBA, at pH 4.6 (procedure a) afforded an inactive preparation that contained 4.2 g-atoms Ca and no Mn ($< 0.05$ g-atom/mol protein). The activity of this preparation could not be restored by extended dialysis against a solution containing $Mn^{2+}$ and $Ca^{2+}$.

Demetalization procedure (d) in which the SBA was dialyzed against 0.1 M citrate, pH 5.0, resulted in a 75% loss of precipitating activity, but only a 30% loss of hemagglutinating activity. Analysis of the metal content of the citrate-treated SBA gave 4.1 g-atoms/Ca/mol and no Mn. Unlike with the other demetallizing procedures, the precipitating activity could be fully restored in this case by dialysis of the metal-free SBA against a solution of $Mn^{2+}$ and $Ca^{2+}$. This regenerated SBA contained 4.2 g-atoms Ca/mol protein and 1.9 g-atoms Mn/mol protein.

The small loss in hemagglutinating activity of the citrate-treated SBA may be ascribed to the reactivation of the SBA during the time course of the hemagglutinating assay by $Mn^{2+}$ leached from the erythrocytes. Indeed, when the precipitating activity of this

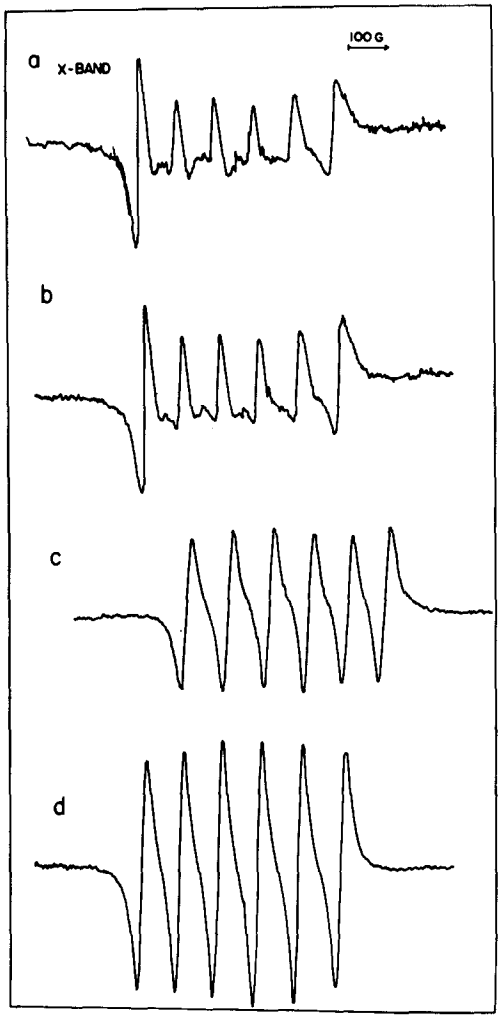


Fig.2. ESR spectra of $Mn^{2+}$ with native SBA at different pH values; (a) 1 mg/ml SBA in 0.1 M Tris-buffered saline, pH 7.0; (b), (c) and (d) 1 mg/ml SBA in 0.1 M acetic acid, buffered saline at pH 5.0, 4.6 and 2.2, respectively. All treatments were for 15 h at room temperature.

preparation was examined in the presence of 0.01 M $MnCl_2$, the lectin was fully active.

The irreversible loss of activity of SBA, at pH < 5.0, suggests that a change in the structure of the protein must occur below this pH, releasing the $Mn^{2+}$ and allowing for its subsequent rebinding without reactivation of the protein. Paulová et al. [12,13] have also demonstrated that the release of $Mn^{2+}$ under acidic conditions from both pea (*Pisum sativum*) and lentil (*Lens esculenta*) lectins resulted in permanent loss of activity, even though 74% of the original $Mn^{2+}$ was rebound by the lentil lectin when dialyzed against $MnCl_2$.

The finding that, at pH 5.0 (in citrate buffer) the $Mn^{2+}$ can be reversibly removed from SBA demonstrates that no irreversible change in the $Mn^{2+}$-binding site has occurred due to this treatment.

The $Mn^{2+}$ could be readily exchanged for $Ni^{2+}$, $Co^{2+}$ and $Cd^{2+}$ by dialysis of the native lectin against 0.1 M solutions of the respective metal chlorides. In all the properties examined the Co- and Cd-SBA were indistinguishable from the native lectin. However, the Ni derivative had a significantly higher hemagglutinating and precipitating activity than native SBA (fig.3).

Ni-, Cd- and Co-SBA contained, respectively, 4 g-atoms Ni, Cd or 3.5 g-atoms Co, in addition to 4 g-atoms Ca. There was no $Mn^{2+}$ present in any of the preparations (fig.3).

The various metal-exchanged SBAs were also compared with native SBA by gel electrophoresis and Ouchterlony plates (figs. 4 and 5). In all cases the properties of the metal-exchanged SBA-s were identical with those of native SBA.

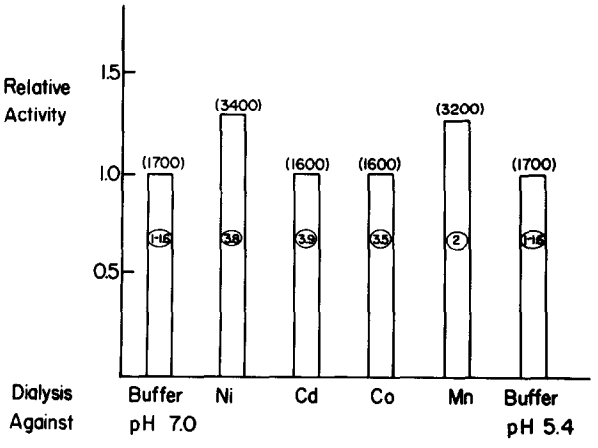


Fig.3. Precipitation and hemagglutinating activities of different metal derivatives of SBA. For each metal derivative the precipitating activity is indicated by the bar graphs, the hemagglutinating activity by parentheses and the g-atoms of metal/120 000 D protein within the circle. For experimental details see text.

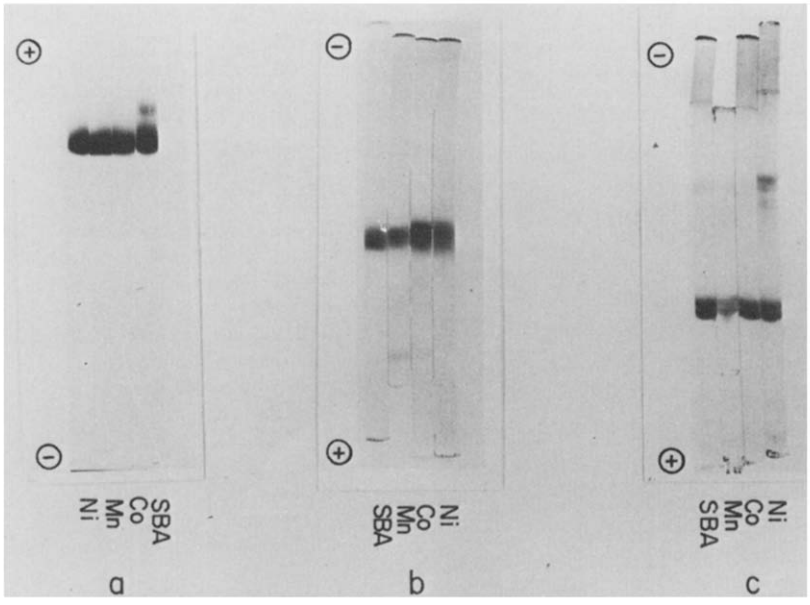


Fig.4. Polyacrylamide gel electrophoresis of different metal derivatives of SBA. The direction of migration was from the top. Native SBA, Ni-, Mn- and Co-SBA (30 μg) were electrophorized, at pH 4.5, in 7.5% acrylamide gel at 2 mA for 4.5 h (A); native SBA, Ni-, Mn- and Co-SBA were electrophoresed according to Weber and Osborn [10] in 10% acrylamide gel containing 0.1% SDS (B), native SBA, Ni-, Mn- and Co-SBA (30 μg) were electrophorized, at pH 8.8 [10], in 10% acrylamide gel at 4 mA for 2 h (C).

In view of the finding that the $Ni^{2+}$, $Co^{2+}$ and $Cd^{2+}$ derivatives of SBA contain 4 g-atoms transition metal/mol protein, an attempt was made to saturate native SBA containing 1 g-atom $Mn^{2+}$/mol using the metal-exchanging procedure, with a 0.1 M solution of $MnCl_2$. The resulting SBA (fig.3) containing 2 g-atoms was indistinguishable from native SBA in Ouchterlony plates and in polyacrylamide gel electrophoresis but was more active both in its precipitating and hemagglutinating activity than native SBA (containing 1 g-atom Mn/mol). It is interesting to note that other lectins, including both pea and lentil [12,13] appear to be depleted in metal as isolated, and activated lectin preparations have been obtained by dialysis against $MnCl_2$. Thus the ability to saturate SBA by dialysis against $MnCl_2$ explains the fluctuation of the Mn content of different SBA preparations.

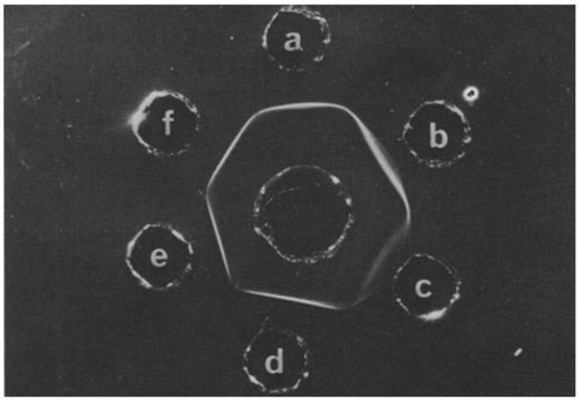


Fig.5. Ouchterlony double diffusion in agar. Center well: anti-SBA (1.3 mg/ml). Outer wells: (a) and (e) native SBA (1.3 mg/ml); (b) Co-SBA (1.3 mg/ml); (c) Cd-SBA (1.3 mg/ml); (d) Mn-SBA (1.3 mg/ml); (f) Ni-SBA (1.3 mg/ml).

The difference in hemagglutinating activity of the SBA preparations saturated by the citrate procedure (1200) and the metal-exchanging procedure (3200) is unclear. However, Jones et al. [14] have recently presented evidence that the method of preparation of a metal-exchanged protein may affect the activity of the final product.

Our findings clearly demonstrate that the $Mn^{2+}$

in SBA is required for activity. Moreover, we show that SBA contains four transition metal sites, two of which have low affinity for $Mn^{2+}$ but only one of the high affinity sites for $Mn^{2+}$ must be occupied to produce a biologically active lectin.

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