

# ESR studies on the effect of ionic radii on displacement of $Mn^{2+}$ bound to a soluble $\beta$ -galactoside binding hepatic lectin

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**Abstract** Binding of divalent metal ions to hepatic soluble  $\beta$ -galactoside binding lectin was studied using electron spin resonance (ESR) spectroscopy. The  $Mn^{2+}$  bound to hepatic lectin could be displaced by  $Mg^{2+}$ ,  $Cu^{2+}$ ,  $Ni^{2+}$  and  $Ca^{2+}$  but not by  $Sr^{2+}$ . As the ionic radii of  $Mg^{2+}$  (0.65 Å),  $Cu^{2+}$  (0.73 Å) and  $Ni^{2+}$  (0.72 Å) are appreciably smaller than  $Ca^{2+}$  (0.99 Å), it appears that the  $Mn^{2+}$  binding site is more accessible to  $Mg^{2+}$ ,  $Cu^{2+}$ , and  $Ni^{2+}$  as compared to  $Ca^{2+}$ , the ionic radius of  $Mn^{2+}$  being 0.80 Å.  $Sr^{2+}$  with an ionic radius of 1.13 is thus unable to displace bound  $Mn^{2+}$ . Surprisingly, the presence of specific sugars like  $\alpha$ -lactose, or  $\alpha$ -D-galactose facilitated the displacement of bound  $Mn^{2+}$  by metal ions whereas non-specific sugars, i.e.  $\alpha$ -D-glucose,  $\beta$ -D-fructose and  $\alpha$ -D-ribose had no effect. It appears that minor perturbations in the saccharide binding site significantly affect the ability of the metal binding site to ligate bivalent metals.

**Key words:**  $\beta$ -Galactoside binding lectin; Metal binding; EPR

## 1. Introduction

Lectins are generally considered to be non-enzymatic and non-immune proteins which selectively bind to a specific carbohydrate structure [1]. The animal lectins are broadly classified into two major categories, the C type ( $Ca^{2+}$ -dependent) animal lectins which are structurally related to the asialoglycoprotein receptor while the S type (thiol-dependent) animal lectins form a distinct group which are generally  $\beta$ -galactoside binding [2]. Embryonic and differentiating tissues of various vertebrates from teleosts [3,4], to amphibians [5], birds [6,7] and mammals [8–10], contain a  $\beta$ -D-galactoside binding lectin that agglutinates trypsinized rabbit erythrocytes and is specifically inhibited by  $\beta$ -D-galactopyranosyl  $\beta$ -D-thiogalactopyranoside and lactose. The major  $\beta$ -galactoside binding lectin occurring in vertebrate tissues is a dimer with a subunit mass of about 14 kDa and requires thiol but not divalent metal ions for its haemagglutination activity, and exhibits considerable sequence homology among various species [11].

Besides the 14 kDa lectin, other  $\beta$ -galactoside binding lectins have been found in mammalian tissues which include sheep, goat and buffalo liver having molecular weights of 18, 22 and 24 kDa, respectively [12]. Of these three  $\beta$ -galactoside binding hepatic lectins, only sheep lectin required 2-mercaptoethanol for its haemagglutination activity; in addition it requires  $Ca^{2+}$ . In contrast to major soluble  $\beta$ -galactoside specific lectins, goat and buffalo soluble hepatic lectins show haemagglutination activity against trypsinized rabbit red blood cells only in the presence of  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Ni^{2+}$  or  $Sr^{2+}$  [12] thus differing

significantly in cofactor requirement from the known major mammalian  $\beta$ -galactoside specific lectins [13,14]. We thus find it important to confirm the presence of a metal binding site of these lectins. This paper describes to our knowledge for the first time the ESR characterization of binding of the metal ions to a soluble  $\beta$ -galactoside specific lectin from goat liver. The importance of this study lies in the fact that the majority of the known soluble  $\beta$ -galactoside specific lectins are independent of metal ion requirement.

The ESR spectrum of  $Mn^{2+}$  is highly responsive to changes in coordination symmetry [15]. We have thus used ESR spectroscopy to investigate changes in the transition metal site of soluble  $\beta$ -galactoside specific goat hepatic lectin on binding to various metal ions in the presence or absence of specific and non-specific sugars. The ESR spectrum was first used for the study of low molecular weight  $Mn^{2+}$  complexes of biochemical interest. The method was then used for the investigation of an interaction of  $Mn^{2+}$  with proteins [15–19]. In most of these cases, the phenomenon of the absence of any observable ESR signal of the bound form of  $Mn^{2+}$  to protein was used for the determination of the dissociation constant of the binding site and their number. The observed ESR signal served as a measure of the equilibrium concentration of free  $Mn^{2+}$  ions [15–19].

## 2. Materials and methods

### 2.1. Reagents

Neuraminidase, sugar substrates, phenylmethyl-sulfonyl fluoride (PMSF) and fetuin-agarose were purchased from Sigma Chemical Co., MO, USA. The glass capillaries used were from TOP Syringe Manufacturing Co., Bombay, India. All the other reagents were of analytical grade.

### 2.2. Isolation of soluble hepatic lectin

The soluble goat hepatic lectin was isolated essentially as described by Ali and Salahuddin [12] with some modifications. Goat liver (25 g) was homogenised in 10 mM sodium phosphate buffer pH 7.5, containing 2 mM PMSF. The debris was removed by centrifugation and the clear supernatant thus obtained was incubated with DEAE-cellulose gel equilibrated in the same buffer. After washing unbound protein, the bound protein was eluted with 0.5 M NaCl. This was then applied on an asialofetuin-agarose column (1.5 × 10 cm) equilibrated in 10 mM Tris HCl buffer (pH 7.5) containing 150 mM NaCl, and 5 mM  $CaCl_2$ . The bound protein was specifically eluted with 0.5 M lactose. The lectin was then rechromatographed on a fresh asialofetuin-agarose column. The eluted lectin was first dialysed against 10 mM EDTA in 0.01 M Tris HCl buffer pH 7.5 to remove bound metal ions and then extensively dialysed against 0.01 M Tris HCl buffer pH 6.8 to remove EDTA. The lectin thus obtained was used throughout the study. The lectin exhibited a single band on SDS-PAGE thus indicating its purity. To prepare asialofetuin-agarose column, fetuin-agarose was treated with neuraminidase as described by Ali and Salahuddin [12].

### 2.3. ESR spectroscopy

In an experiment, 100  $\mu$ l of soluble goat hepatic lectin (0.5 mg/ml as determined spectrophotometrically taking absorbance at 280 nm) and

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MnCl<sub>2</sub> (final concentration 0.25 mM) in 0.01 M Tris HCl buffer pH 6.8 (50  $\mu$ l) and 50  $\mu$ l of the same buffer were mixed and ESR spectra recorded on a Varian ESR spectrometer model E 104A at 100 kHz field modulation. Instrument parameters were as follows: field 3237 gauss, scan range 1000 gauss, modulation amplitude 2 gauss, receiver gain  $2.5 \times 10^3$ , time constant 0.5 s, scan time 2 min, microwave power 10 mW and frequency 9.01 GHz. The peak-to-peak amplitude of the fourth peak from the low field side of the spectrum was measured for Mn<sup>2+</sup> concentration [15]. Nanomoles of Mn<sup>2+</sup> can be detected easily without the distortion of peaks. The peak amplitude shows a linear increase with increase in concentration of Mn<sup>2+</sup> (calibration curve not shown). All measurements were made at room temperature. For displacement studies, either CuSO<sub>4</sub>, MgCl<sub>2</sub>, NiCl<sub>2</sub> (final concentration 0.3 mM to 0.8 mM) or CaCl<sub>2</sub> (final concentration 25 mM to 60 mM) and SrCl<sub>2</sub> (final concentration 100 mM) were also added. To study the effect of inhibitory and non-inhibitory sugars,  $\alpha$ -lactose,  $\alpha$ -D-galactose,  $\alpha$ -D-glucose,  $\beta$ -D-fructose and  $\alpha$ -D-ribose (45 mM final concentration) were also added, adjusting the volume accordingly. The amount of sugars added was 45 fold higher than that required to produce 50% reduction in haemagglutinating activity.

### 3. Results and discussion

The spectra of Mn<sup>2+</sup> ion bound to the goat hepatic soluble  $\beta$ -galactoside specific lectin in aqueous solution are presented in Fig. 1, a–p. Spectra were recorded as described above. Taking into consideration the broadening of the lines, the signal intensity of the same molar concentration of Mn<sup>2+</sup> in hepatic lectin was grossly lower (almost flat spectra) than that of Mn<sup>2+</sup> in aqueous solution. It has been suggested that the disappearance of the hyperfine structure of the complex indicates the formation of a covalent bond [15]. It could also be a function of the tumbling rate and the size of anisotropy in the resonance and this would provide a case for ‘immobilization’ of Mn<sup>2+</sup> by the protein moiety [16]. This immobilization would be analogous to those observed in spectra of organic free radicals bound to proteins in solutions [15]. These spectra approach the spectra observed in polycrystalline and powder samples because of the incomplete averaging of anisotropic interactions resulting from a slow molecular tumbling of protein bound ions. In some cases this ‘immobilization’ is the dominant effect of the macromolecules in the changes of the Mn<sup>2+</sup> EPR spectra [15].

Addition of different metal ions (each added independently in increasing concentrations) resulted in an increase in the intensity of the resolved spectra as well as narrowing of the components due to release of Mn<sup>2+</sup> from the metal binding site (Fig. 1, a–p). The amount of Mn<sup>2+</sup> displaced from the metal binding site was found to be different for different metal ions (Fig. 2). Addition of Mg<sup>2+</sup> (0.03 mM to 0.875 mM final concentration) released 69% of the bound Mn<sup>2+</sup>, i.e. 0.172 mM out of 0.25 mM added. Addition of Ni<sup>2+</sup> (0.03 mM to 1.2 mM final concentration) released 84% of the bound Mn<sup>2+</sup>. Addition of Cu<sup>2+</sup> (0.03 mM to 1.25 mM final concentration) released 96% of the bound Mn<sup>2+</sup>. In all these cases total amount of bound Mn<sup>2+</sup> was not displaced even if higher concentrations of metal ions were used. On the contrary Ca<sup>2+</sup> release 100% of the bound Mn<sup>2+</sup> on addition of 25 to 50 mM of CaCl<sub>2</sub>. However, the final concentration of Ca<sup>2+</sup> added (i.e. 50 mM) to displace 100% of the bound Mn<sup>2+</sup> is 40 fold higher than that of Cu<sup>2+</sup> (1.25 mM) and about 57–61 fold higher than the final concentration of Mg<sup>2+</sup> (0.875 mM) and Ni<sup>2+</sup> (0.813 mM) used respectively, for displacement of bound Mn<sup>2+</sup>.

This differential release of Mn<sup>2+</sup> by various metal ions used could be directly correlated with the ionic radii of these ions.

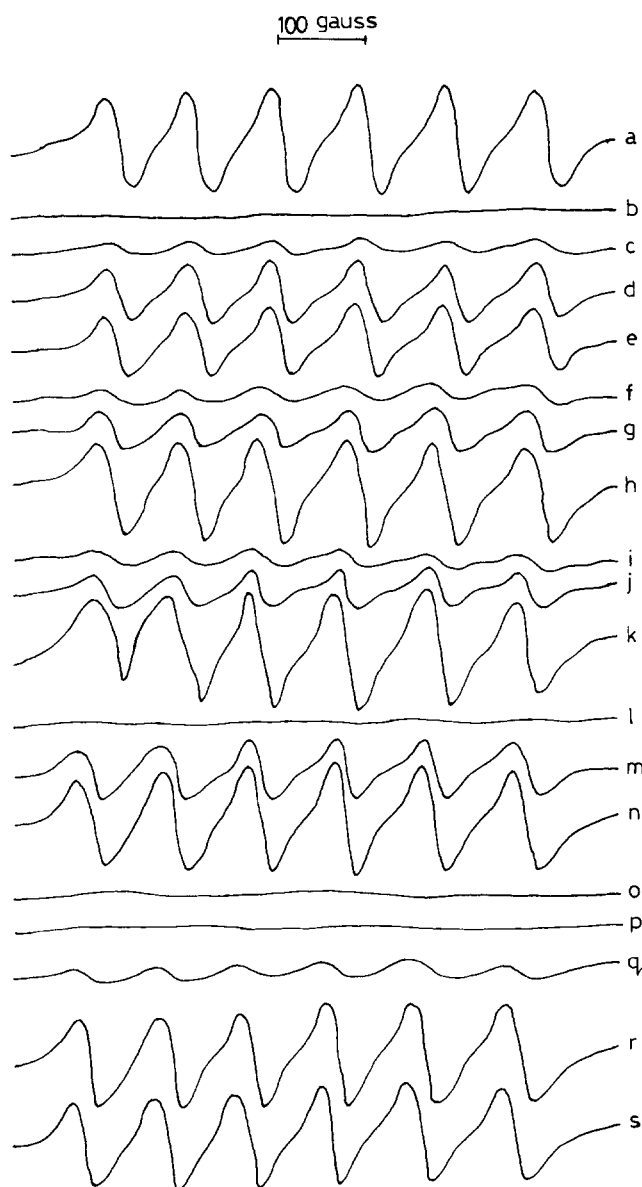


Fig. 1. Electron spin resonance (ESR) spectra of Mn<sup>2+</sup> bound to soluble  $\beta$ -galactoside binding goat hepatic lectin. Instrument parameters are as described in section 2. MnCl<sub>2</sub> (0.25 mM final conc.) was added to soluble  $\beta$ -galactoside binding goat hepatic lectin (0.5 mg/ml final conc. as determined spectrophotometrically). Increasing concentration of various metal ions were then added to displace bound Mn<sup>2+</sup>. (a) Free MnCl<sub>2</sub>, 0.25 mM; (b) MnCl<sub>2</sub> bound to hepatic lectin. Displacement by addition of (c) 0.063 mM MgCl<sub>2</sub> (d) 0.25 mM MgCl<sub>2</sub> (e) 0.875 mM MgCl<sub>2</sub> (f) 0.063 mM NiCl<sub>2</sub> (g) 0.25 mM NiCl<sub>2</sub> (h) 0.813 mM NiCl<sub>2</sub> (i) 0.063 mM CuSO<sub>4</sub> (j) 0.25 mM CuSO<sub>4</sub> (k) 0.813 mM CuSO<sub>4</sub> (l) 25 mM CaCl<sub>2</sub> (m) 40 mM CaCl<sub>2</sub> (n) 50 mM CaCl<sub>2</sub> (o) 25 mM SrCl<sub>2</sub> (p) 100 mM SrCl<sub>2</sub> (q) 0.028 mM MgCl<sub>2</sub> in presence of 45 mM  $\alpha$ -lactose (r) 0.227 mM MgCl<sub>2</sub> in presence of 45 mM  $\alpha$ -lactose (s) 0.875 mM MgCl<sub>2</sub> in presence of 45 mM  $\alpha$ -lactose. Spectra in presence of other varying concentrations of metal ions or sugars are not shown due to lack of space.

The ionic radius of Mn<sup>2+</sup> is 0.80 Å [20], Mg<sup>2+</sup>, Ni<sup>2+</sup> and Cu<sup>2+</sup> with ionic radii of 0.65 Å, 0.72 Å and 0.73 Å [21] displace 67%, 84% and 96% of bound Mn<sup>2+</sup>, respectively. Ca<sup>2+</sup> with an ionic radius of 0.99 Å which is larger than that of Mn<sup>2+</sup> displaces 100% of bound Mn<sup>2+</sup>. However, as 40 to 60 fold higher concentrations of Ca<sup>2+</sup> are required as compared to Mg<sup>2+</sup>, Ni<sup>2+</sup> and

$\text{Cu}^{2+}$ , it appears that the binding site is more accessible to these ions as compared to  $\text{Ca}^{2+}$ . This possibility is confirmed by the fact that  $\text{Sr}^{2+}$  with an ionic radius of 1.13 Å is totally unable to displace the bound  $\text{Mn}^{2+}$ , even when added to 100 mM final concentration. Although  $\text{Mg}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Ca}^{2+}$  were able to displace  $\text{Mn}^{2+}$ , it is clear that the binding site is more specific for  $\text{Mn}^{2+}$  in comparison to these ions as 3.5, 3.25, 5 and 200 fold higher concentrations than  $\text{Mn}^{2+}$  were required, respectively, to displace bound  $\text{Mn}^{2+}$  which was added in a final concentration of 0.25 mM. This is also confirmed by the fact that addition of displacing metals ( $\text{Mg}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$  or  $\text{Ca}^{2+}$ ) prior to the addition of  $\text{MnCl}_2$  to hepatic lectin or after the addition of  $\text{MnCl}_2$  to hepatic lectin does not make any difference in their ability to displace  $\text{Mn}^{2+}$  (data not shown).

As the earlier report suggested that presence of divalent metal ions was necessary for the lectin to exhibit haemagglutination activity [12], we studied the effect of the presence of inhibitory and non-inhibitory sugars on the displacement of bound  $\text{Mn}^{2+}$  from the hepatic lectin. The spectra for the displacement of  $\text{Mn}^{2+}$  by  $\text{Mg}^{2+}$  in the presence of  $\alpha$ -lactose is given in Fig. 1, q–s. The ESR spectra for displacement of  $\text{Mn}^{2+}$  by  $\text{Mg}^{2+}$  in presence of  $\alpha$ -D-galactose,  $\alpha$ -D-glucose,  $\beta$ -D-fructose and  $\alpha$ -D-ribose are not shown. The amount of  $\text{Mn}^{2+}$  displaced by  $\text{Mg}^{2+}$  in the presence of various sugars is depicted in Fig. 3. Surprisingly it was found that presence of an inhibitory sugar  $\alpha$ -lactose or  $\alpha$ -D-galactose (45 mM final concentration) facilitated the release of bound  $\text{Mn}^{2+}$ . In the absence of sugars, addition of  $\text{Mg}^{2+}$  (0.028 mM to 0.875 mM final concentration) displaced about 69% of the bound  $\text{Mn}^{2+}$  (i.e. 0.153 mM out of 0.22 mM bound). Presence of non-inhibitory sugars like  $\alpha$ -D-glucose,  $\beta$ -D-fructose or  $\alpha$ -D-ribose made no difference and approximately 69% of the bound  $\text{Mn}^{2+}$  was released in their presence also. However, presence of  $\alpha$ -D-galactose resulted in the displacement of 86% of bound  $\text{Mn}^{2+}$  and presence of  $\alpha$ -lactose resulted in displacement of 91% of the bound  $\text{Mn}^{2+}$  by  $\text{Mg}^{2+}$ . The experiments on the displacement of  $\text{Mn}^{2+}$  by  $\text{Mg}^{2+}$  in the presence of non-inhibitory and inhibitory sugars were repeated three times and the difference was statistically significant ( $P < 0.001$ ).

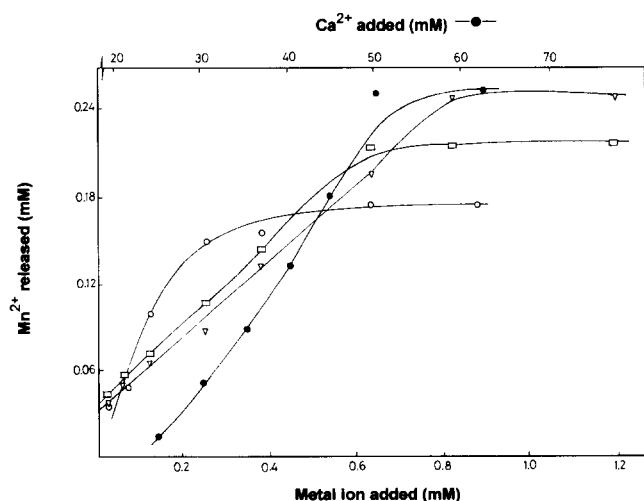


Fig. 2. Release of  $\text{Mn}^{2+}$  bound to soluble  $\beta$  galactosidebinding goat hepatic lectin on addition of subsequent concentrations of  $\text{MgCl}_2$  (○),  $\text{NiCl}_2$  (□),  $\text{CuSO}_4$  (▽) and  $\text{CaCl}_2$  (●). The peak to peak amplitude of the fourth peak from the low field side of the spectra shown in Fig. 1 were measured for determining  $\text{Mn}^{2+}$  concentration.

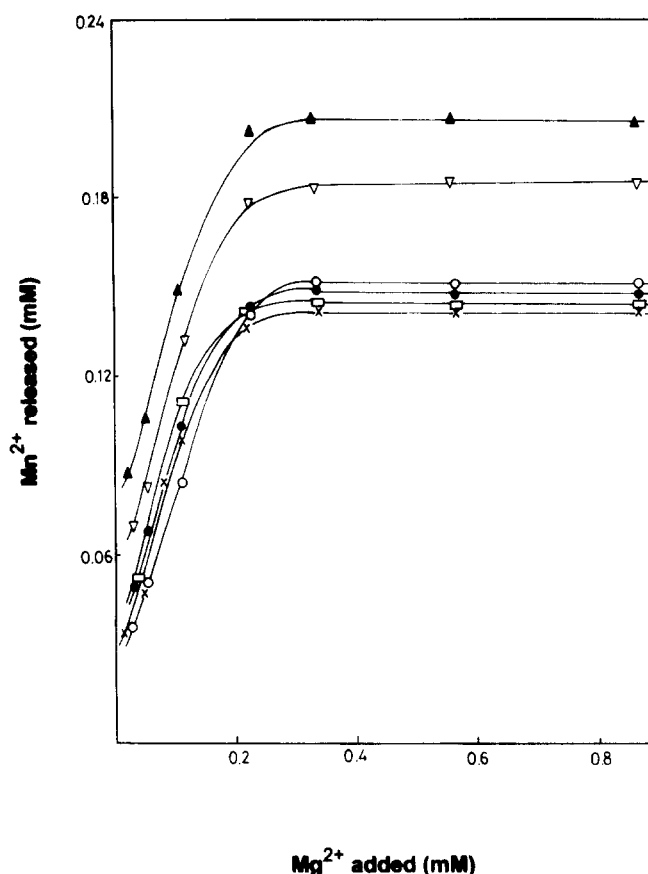


Fig. 3. Release of  $\text{Mn}^{2+}$  bound to soluble  $\beta$ -galactosidebinding goat hepatic lectin on addition of subsequent concentrations of  $\text{MgCl}_2$  (●) in the presence of  $\alpha$ -D-ribose (○),  $\beta$ -D-fructose (□),  $\alpha$ -D-glucose (X),  $\alpha$ -D-galactose (▽) and  $\alpha$ -lactose (▲).

Thus the soluble goat hepatic  $\beta$ -galactoside lectin was found to be different from goat peripheral blood lymphocyte lectin where presence of inhibitory sugar made no difference in the release of bound  $\text{Mn}^{2+}$  [22]. The data also suggest that the saccharide binding site is likely to be in close proximity to the metal binding site in the soluble goat hepatic  $\beta$ -galactoside lectin. It is concluded that minor perturbations in the saccharide binding site significantly affect the ability of metal binding site to ligate bivalent metals.

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