



Interaction of metal ions with lupin seed conglutin γ

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Abstract

Various metal ions were capable of aggregating and precipitating conglutin γ , an oligomeric glycoprotein purified from *Lupinus albus* seeds, at neutral pH values. The most effective metal ions, at 60-fold molar excess to the protein, were Zn^{2+} , Hg^{2+} and Cu^{2+} ; a lower influence on the physical status of conglutin γ was observed with Cr^{3+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cd^{2+} , Sn^{2+} and Pb^{2+} , while Mg^{2+} , Ca^{2+} and Mn^{2+} had no effect at all. The insolubilisation of the protein with Zn^{2+} , which is fully reversible, strictly depended on both metal concentration and pH, with middle points of the sharp transitions at three-fold molar excess and pH 6.5, respectively. Conglutin γ is also fully retained on a metal affinity chromatography column at which Zn^{2+} and Ni^{2+} were complexed. A drop of pH below 6.0 and the use of chelating agents, such as EDTA and imidazole, fully desorbed the protein. A slightly lower binding to immobilised Cu^{2+} and Co^{2+} and no binding with Mg^{2+} , Cd^{2+} and Mn^{2+} were observed. The role of the numerous histidine residues of conglutin γ in the binding of Zn^{2+} is discussed. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Lupin seed conglutin γ is an abundant oligomeric glycoprotein of still unknown function. However, several unusual features make this protein an interesting one to investigate both from the molecular and functional point of view. Conglutin γ accounts for about 5% of the total proteins of mature lupin seeds. It is a tetrameric protein, each monomer consisting of two disulphide-linked polypeptides of 29 and 17 kDa (Restani et al., 1981). The heavy chain is glycosylated (Duranti et al., 1981). Conglutin γ is deposited in the protein bodies along with the storage proteins, but it is not cleaved at germination (Duranti et al., 1994a) and is actively secreted upon heat-treatment of mature seeds (Duranti et al., 1994b). Moreover, this protein has been localised in the extracellular spaces of germinating cotyledons (Duranti et al., 1994a) and in the seedling radicles (Duranti et al., 1996). Amino acid sequence of *Lupinus albus* conglutin γ deduced from the nucleotide sequence of the corresponding gene (Scarafoni et al., 1998) has proved to be homologous to the 7S basic globulin

(Bg7S) of soybean, which showed protein kinase activity (Komatsu et al., 1994) and receptor-like properties, being capable of binding insulin-related peptides (Komatsu and Hirano, 1991) and leginsulin, a hormone-like peptide purified from soybean seeds (Watanabe et al., 1994). Apart from this sequence homology, which is also partially shared with other scarcely characterised plant proteins (Satoh et al., 1992; Chung et al., 1997), conglutin γ did not show any similarity with known catalytic or biologically active proteins.

In the search of a function of this protein in the seeds and other tissues where it has been located, we have undertaken a study aimed at evidencing some molecular property which might help understanding its role. In this work we present the results obtained by treating purified conglutin γ with various divalent and trivalent metal ions and report its preferential interaction with zinc ions.

2. Results

The effect of a number of divalent and trivalent metal ions on the solubility of purified conglutin γ in buffered neutral solutions at fixed protein and metal concentrations has been tested as a preliminary approach to

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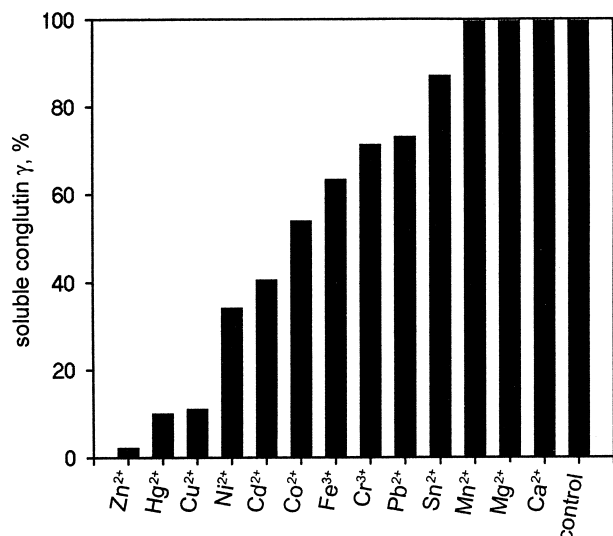


Fig. 1. Solubility of conglutin γ in the presence of metal ions. Conglutin γ (17 μ M) was incubated with each metal at a final concentration of 1 mM, as detailed in the Methods. Soluble conglutin γ in the supernatants was determined spectrophotometrically. Each result, is expressed as percent of soluble conglutin γ in the control without metals and represent the average of two separate assays.

evaluate the interaction of the protein with these ions (Fig. 1). Three metal ions, namely Mg^{2+} , Ca^{2+} and Mn^{2+} , showed no effect on the physical status of conglutin γ . Other metals, namely Cr^{3+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cd^{2+} , Sn^{2+} and Pb^{2+} , to various extent decreased conglutin γ solubility. However, the most striking effects were observed with Zn^{2+} , Hg^{2+} and Cu^{2+} which caused an almost complete insolubilisation of the protein. When conglutin γ precipitated by these metals was resuspended in a buffered solution containing 50 mM EDTA, the protein became soluble again with a yield $\geq 90\%$, suggesting an almost complete reversibility of the process. Thus, metal-induced insolubilisation did not implicate denaturation of conglutin γ , which would have prevented a complete resolubilisation of the protein.

Since the main aggregation effect was observed with Zn^{2+} , we decided to investigate in greater detail the modalities of interaction of conglutin γ with this ion. To this purpose, the dependence of conglutin γ insolubilisation on Zn^{2+} concentration at two pH values, which will be shown to be critical for the phenomenon to occur, has been studied (Fig. 2). While at acidic pH no significant difference from the control experiment was observed at any Zn^{2+} concentration, at pH 7.5 conglutin γ insolubilisation was sharply dependent on Zn^{2+} concentration. When the Zn^{2+} concentration was equimolar to the protein, solubility was scarcely affected, but already at five-fold metal excess, precipitation of conglutin γ was almost complete. The mid point of this transition occurred at about three-fold molar excess to the protein.

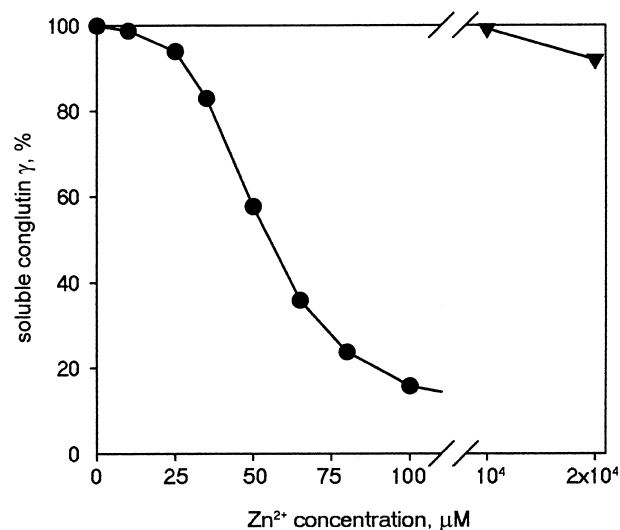


Fig. 2. Dependence of conglutin γ insolubilisation on Zn^{2+} concentration. Conglutin γ (20 μ M) was treated with various Zn^{2+} concentrations at pH 7.5 (●) and 4.5 (▼) and the amount of soluble fraction, spectrophotometrically assessed, was expressed as a percentage of the control without Zn ions. Results are the average of two separate assays.

Loss of solubility of conglutin γ by Zn ions was also strongly pH dependent (Fig. 3), in that a relatively small pH change dramatically altered the physical status of conglutin γ in the presence of the metal, being its insolubilisation negligible at pH 4.5, but quantitative at pH 7.5. The mid point of this transition was around pH 6.5.

Since metal-induced protein insolubilisation is a relatively undetermined phenomenon which may not necessarily reflect the occurrence of a specific interaction, we decided to investigate the binding of conglutin γ to a Metal Affinity Chromatography (MAC) matrix by coupling various divalent metal ions. The results obtained with MAC were fairly consistent with the solubility trials, in that most, but not all, of the metals causing a significant loss of solubility of conglutin γ at a fixed metal concentration, also bound the protein to the stationary phase (Fig. 4). Again, we focussed on the interaction of conglutin γ with Zn^{2+} and studied the dependence of the protein binding to the Zn^{2+} -coupled matrix (ZnAC) under different experimental conditions. Conglutin γ binding was quantitative at neutral pH, while at acidic pH, complete desorption of the protein took place, as shown by the corresponding elution profile in Fig. 5. The transition was as sharp as in the insolubilisation trials, with a mid point very close to pH 6.0. In addition to the drop of pH below 6.0, two different metal chelating agents, namely EDTA and imidazole, did quantitatively elute the bound protein (Fig. 5). Non-precipitating Zn^{2+} concentrations in the elution buffer, that is around 5 μ M in the conditions used for ZnAC, did not desorb the protein, seemingly because they were far too low to compete with the concentration of the

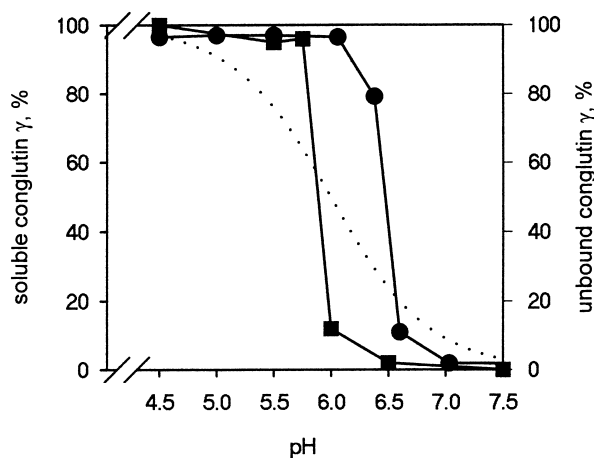


Fig. 3. Dependence of conglutin γ insolubilisation and binding to ZnAC on the pH. Conglutin γ (20 μ M) was treated with 1 mM Zn^{2+} at the pH indicated. The amount of soluble fraction, spectrophotometrically assessed, was expressed as percent of the control without Zn ions (●-●). Results are the average of two separate assays. The dependence of conglutin γ binding to ZnAC on pH is expressed as percent of the unbound conglutin γ with respect to the total loaded protein (■-■). The titration of histidine imidazole group (···) is also reported as percent of positively charged amino group.

metal bound to the matrix, claimed to be in the range of 30–40 mM by the manufacturers. However, when conglutin γ pre-treated with equimolar concentrations of Zn^{2+} , was loaded on the ZnAC column, the binding of the protein was reduced to 15% of the control (not shown).

3. Discussion

It has long since been known that the interaction of metal ions with proteins can lead to aggregation and consequently precipitation of the protein molecules (Hughes and Klotz, 1956). This phenomenon has been used to get selective precipitation of a protein from a mixture. This was the case of hemoglobin, which has been shown to precipitate from the serum with various metal ions, including Zn^{2+} and Cu^{2+} as the most effective agents (Frantzen et al., 1997). In some cases metal precipitation of the proteins is accompanied by denaturation, which usually significantly reduce their solubility (Adhikari et al., 1998). However, this does not seem to be the case of conglutin γ , which immediately becomes again soluble by simply complexing the precipitating metal with EDTA.

The finding that conglutin γ underwent aggregation and precipitation with various metals, in particular with those affecting also the solubility of hemoglobin, prompted us to investigate further the modalities of this interaction, especially with Zn ions, which had showed the greatest effects on the solubility of the protein. Indeed, pH dependence of both conglutin γ precipitation by

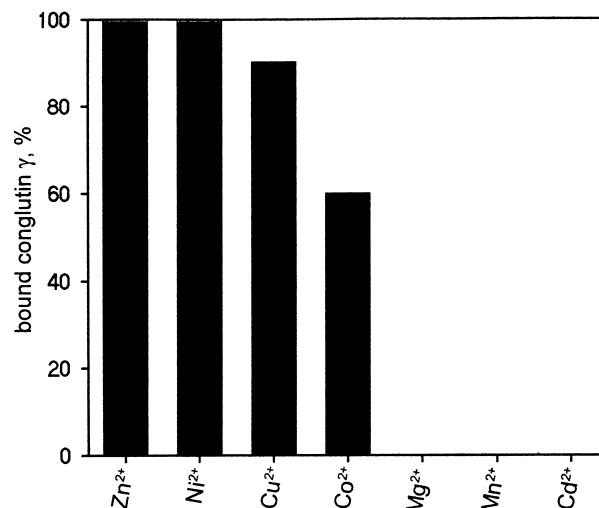


Fig. 4. Effect of various metal ions on the binding of conglutin γ to MAC. Conglutin γ (13 μ M) was loaded on a column of iminodiacetic acid immobilised on cross-linked 4% beaded agarose complexed with the metal ions indicated. The amount of bound conglutin γ is expressed as percent of the total loaded protein.

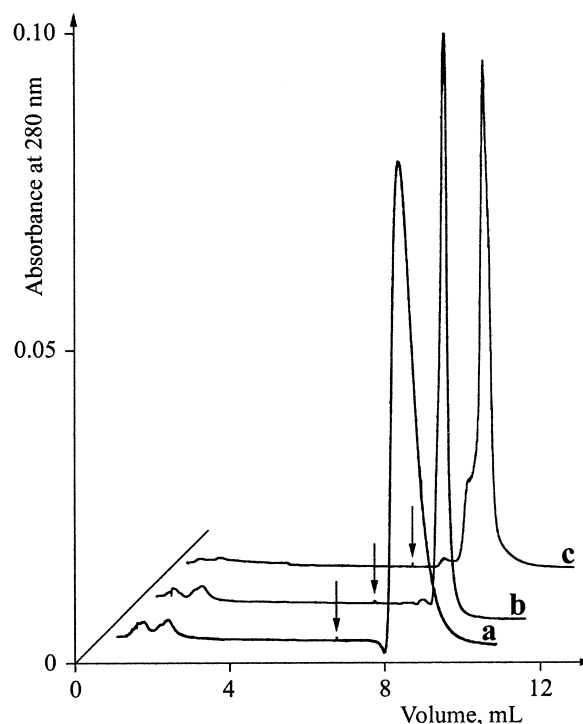


Fig. 5. ZnAC elution profiles of conglutin γ . The protein (400 μ g) was loaded on the column as described in the section 4.4. After washing with 50 mM Tris-HCl, pH 7.5, containing 500 mM NaCl, the following elution buffers were applied (arrows): sodium acetate, pH 4.5 (a); washing buffer containing 250 mM imidazole (b); washing buffer containing 10 mM EDTA (c).

Zn^{2+} and binding to ZnAC matrix appeared to be very strict and both transitions closely corresponded to the pK_a of 6.0 of histidine side chain amino group (Fig. 3). Although this is an indirect evidence of the involvement

of histidine side chains in the interaction of conglutin γ with Zn ions, and possibly with the other metals too, these two independent results strongly suggest that this is the case. The involvement of these side chains in the binding of Zn ions has already been reported for various proteins, including bovine prolactin (Permyakov et al., 1997), carbonic anhydrase (Hunt et al., 1999), histatin-5 (Melino et al., 1999) and retinal-binding protein (Müller and Skerra, 1994). Indeed, *Lupinus albus* conglutin γ is relatively rich of histidine residues (19 out of 419 amino acid residues) (Scarafoni et al., 1998). On the other hand, the involvement of -SH groups in the interaction of conglutin γ with Zn^{2+} can be excluded since all the 12 cysteine residues of the protein are involved in disulphide bridges (Scarafoni et al. 1998; Duranti, M., unpublished results), and the alkaline pK_a of the thiol groups is far from the pH at which the observed transitions occur.

With the notable exception of Cd^{2+} which, despite aggregating about 60% of conglutin γ in our experimental conditions, did not bind the protein in MAC, the other metals tested behaved similarly both in MAC and as precipitating agents, with Zn^{2+} , Ni^{2+} and Cu^{2+} as the most effective ligands, Co^{2+} as less good ligand, Mn^{2+} and Mg^{2+} as non-ligands nor precipitating agents. This finding suggests that, although the two distinct phenomena not obviously relate to each other, they might have a common molecular basis.

The quantitative desorption of conglutin γ from the ZnAC column promoted by acidic pH and metal-chelating agents indicates a fully reversible binding of the protein to the matrix, as it was observed in the insolubilisation trials. The possibility that conglutin γ precipitates in the column, in analogy to the observed metal-induced insolubilisation of the free protein, cannot be excluded. However, if this is the case, the complete reversibility of the binding suggests that insolubilisation is not likely to involve conglutin γ unfolding, which, according to other studies (Duranti et al., 2000), would prevent prompt resolubilisation and desorption of the protein to take place.

Although the present results do not provide sufficient information to shed light on the physiological significance of the metal binding by conglutin γ , the possibility of a specific function of the metal could not be excluded. Further studies, aimed to assess the binding parameters, the stability of the apo- and holo-protein and the molecular determinants of the metal interaction with conglutin γ , are in progress.

Moreover, since some of the metals causing significant aggregation of conglutin γ belong to the class of toxic heavy metals, such as Cr^{2+} , Cd^{2+} , Hg^{2+} and Pb^{2+} , the possibility of exploiting the conglutin γ properties described in this paper for detoxification processes could be envisaged and is also currently being investigated.

4. Experimental

4.1. General procedures

The concentration of the conglutin γ solutions was determined by measuring the absorbance at 280 nm assuming an absorption coefficient of one for a 1 mg ml^{-1} solution (Duranti et al., 2000). Molar concentrations of conglutin γ were calculated considering the protein in its monomeric form, that is with M_r 46,000.

Chromatographic elutions were followed at 280 nm using a LKB Uvicord monitor.

All the metals used were in the form of chloride salts.

4.2. Purification of conglutin γ from mature lupin seeds

Conglutin γ was purified essentially as described by Duranti et al. (1994b). The purified conglutin γ was found to be homogeneous by SDS-PAGE (not shown). Purified conglutin γ was precipitated with ammonium sulphate (80% saturation) and then resuspended in 50 mM Tris-HCl, pH 7.5 containing 50 mM EDTA in order to remove any metal ion present in the preparations. Removal of salts and metal-EDTA complexes was carried out by gel filtration using a G25 Sephadex column.

4.3. Metal ion-induced insolubilisation of conglutin γ

Solutions of conglutin γ , from 17 to 20 μM in 50 mM Tris-HCl buffer, pH 7.5 have been used for the insolubilisation assays at room temperature with 1 mM concentrations of the various metal ions. The resulting mixtures were stirred and then centrifuged at $10,000 \times g$ for 20 min. Protein concentration in the supernatants was spectrophotometrically determined against a blank containing the corresponding metal concentration in the buffer. Assays with Zn ions at different pH values were carried out by using the following buffers: 50 mM sodium acetate, pH 4.5; 50 mM 2-morpholinoethanesulfonic acid (MES), pH from 5.5 to 6.5; 50 mM Tris-HCl, pH 7.5.

4.4. Metal affinity chromatography (MAC)

A column (2 cm $\times$ 1 cm) of iminodiacetic acid immobilized on cross-linked 4% beaded agarose (SIGMA) was set up and equilibrated in 50 mM Tris-HCl buffer, pH 7.5 containing 500 mM NaCl (binding buffer) and 2 mg ml^{-1} of the desired metal ion. The metal excess was washed with 10 volumes of the binding buffer. One millilitre of 13 μM conglutin γ solution was applied to the column. Adsorbed conglutin γ was eluted with 50 mM sodium acetate buffer, pH 4.5 containing 500 mM NaCl, and with the binding buffer containing either 10 mM EDTA or 250 mM imidazole.

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