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# Self-aggregation of legume seed storage proteins inside the protein storage vacuoles is electrostatic in nature, rather than lectin-mediated

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**Abstract Conglutins are multisubunit, glycosylated, major storage proteins present in *Lupinus* seeds that self-aggregate in a calcium/magnesium-dependent manner. Two of these globulins exhibit lectin activity. The 210 kDa globulin derived from β-conglutin that accumulates in *Lupinus* cotyledons during germination was used as a model protein to establish whether the self-aggregation process is electrostatic in nature or lectin-mediated. This protein binds in a very strong manner to chitin and recognizes a variety of glycoproteins including immunoglobulins G. Several compounds were tested for their inhibitory effect on the cation-dependent self-aggregation process. Sialic acid and phytin were the most effective whereas chitin and mucin were totally ineffective. The inability of the oligosaccharidic side chains of the 210 kDa protein, β-conglutin and immunoglobulin G to interfere with the aggregation strongly supports the view that Ca/Mg are electrostatically involved in the in vitro self-aggregation of *Lupinus* globulins. The results suggest that calcium and magnesium ions are also electrostatically involved in vivo in the macromolecular aggregation of legume seed storage proteins, ensuring their efficient packing inside the protein storage vacuoles. This mechanism is responsible for the typical insolubility of legume globulins in water.**


*Key words:* Legume; Seed; Storage protein; Lectin; *Lupinus albus*

## 1. Introduction

Globulins represent the predominant type of storage proteins in legume seeds [1]. On the other hand, legume lectins constitute a large family of homologous proteins that resemble each other in their physicochemical properties but differ in their carbohydrate specificities [2]. These two families of proteins accumulate in large amounts in mature seeds. They are both synthesized during seed development and deposited in densely packed storage protein deposits termed protein storage vacuoles by an intracellular secretory process [1,3]. A number of biological roles have been assigned to legume lectins. Apart from their patterns of synthesis, accumulation and degradation, which parallel those of the reserve proteins, they are sequestered with the storage proteins in sufficient concentrations to constitute auxiliary storage proteins. In some legume seeds, they can account for 10% or more of the total protein [4]. However, very little is known about how storage proteins are organized within the protein storage vacuoles, although this organization may well be important in ensuring efficient use of storage space and facilitating mobilization of storage proteins during germination [5]. The common localization of storage proteins and lectins and the interaction between them led Einhoff et al. [6] to suggest a biological role for the seed lectins: during seed maturation they may act as a packaging aid for storage proteins and enzymes into developing protein storage vacuoles. Lectins may thus contribute to an ordered construction and degradation of protein storage vacuoles.

Although *Lupinus* seeds have been reported to lack lectins [7,8], two recent studies have questioned these reports. Duranti et al. [9] suggested that γ-conglutin, a minor storage protein from *Lupinus albus* seeds, exhibits a lectin-type activity. Ramos et al. [10] reported the accumulation in germinating cotyledons of a 20 kDa polypeptide with lectin activity that is derived from β-conglutin, the major storage protein present in *Lupinus* seeds. Both these lectin activities are associated with seed storage globulins, an observation that questions their possible homology with the well characterized family of legume lectins [2]. Legume seed storage globulins (including γ-conglutin and β-conglutin from *Lupinus*) self-aggregate in a $Ca^{2+}/Mg^{2+}$-dependent manner [11]. On the other hand, legume lectin interaction with carbohydrates has long been known to require tightly bound $Ca^{2+}$ and $Mg^{2+}$ (or another transition metal) [2].

Globulins are defined as proteins that are insoluble in water or low-salt aqueous solutions but readily soluble in solutions of high ionic strength [5]. The characteristic insolubility in low-salt solutions of the legume seed storage globulins is due to their self-aggregation in a divalent cation-dependent manner, forming very large molecular mass aggregates [11]. This process may be envisaged as an adaptation to ensure an efficient packaging of the proteins inside the protein storage vacuoles. This divalent cation-dependent, self-aggregation process is highly dependent on the ionic strength and on the pH of the surrounding medium, and may be explained by two alternative and extreme hypotheses: (1) Electrostatic interactions which, in their simplest form, would involve the divalent cations acting as bridges between adjacent, negatively charged protein molecules; (2) lectin-mediated, with the subunits of multivalent lectins binding simultaneously, in a cation-dependent manner, to the oligosaccharidic side chains of several storage glycoproteins. From the theoretical point of view, these two alternative hypotheses, which may also operate in

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combination, seem equally likely. Bridging of calcium ions between negatively charged protein molecules is observed, for example, in the cross-linking between milk submicelles, where the calcium ions form bridges between the negatively charged phosphate groups of α- and β-casein molecules present in adjacent submicelles [12]. The self-aggregation of glycosylated, multivalent lectins has also been described. A lectin isolated from the bark of *Sophora japonica*, for example, is self-aggregatable due to the binding activities of all its four subunits, which enable them to recognize and bind *N*-linked oligosaccharide chains on three of the four subunits. The lectin is soluble in the presence of 5 mM methyl α-mannoside, but insoluble in the absence of the sugar, and precipitates after centrifugation. After removing the supernatant, the precipitated lectin becomes soluble upon addition of buffer containing 5 mM methyl α-mannoside [13].

The observation that the main storage proteins present in *Lupinus* seeds are all glycosylated globulins [14] and the evidence indicating that two of them exhibit lectin activity [9,10] suggest that a lectin-mediated aggregation of the storage proteins may be involved in the efficient packaging of these globulins inside the protein storage vacuoles. Alternatively, the packaging of the storage globulins may be electrostatic in nature.

To address this question, a 210 kDa glycosylated, lectin-like globulin that is a stable, intermediate breakdown product of β-conglutin (the vicilin-like, major globulin present in *Lupinus* cotyledons) catabolism [10] was used as a model protein. The protein was allowed to self-aggregate in vitro, in the presence of $Ca^{2+}$ and $Mg^{2+}$ ions [11]. A number of compounds were subsequently tested for their inhibitory effect on the self-aggregation process, including monosaccharides, amino acids, single oligosaccharides and oligosaccharides extracted from several glycoproteins.

## 2. Material and methods

### 2.1. Biological material

Dry seeds of white lupin (*L. albus* L.) cv. Lublanc were obtained from a local market. When appropriate, the seeds were germinated for 8 days [14]. In all cases, the seed coats were removed and the intact cotyledons dissected from the axes and stored frozen at −80°C until required.

### 2.2. Purification of β-conglutin

β-Conglutin, the major globulin present in the seeds of *Lupinus* species, was extracted and purified as described before [11]. The dry cotyledons were first milled and the total globulin fraction isolated from the resulting meal after deffating with *n*-hexane and removal of the albumins. The total globulins were subsequently fractionated and β-conglutin isolated by fast protein liquid chromatography (FPLC) anion exchange chromatography on a Q-Sepharose column (∅ = 1 cm; $h = 8$ cm; flow rate = 1.5 ml/min) [11]. The pure protein was desalted into 10 mM Tris–HCl buffer, pH 7.5.

### 2.3. Purification of the 210 kDa glycosylated globulin with lectin activity

The 210 kDa glycosylated globulin with lectin activity is a major, stable, intermediate breakdown product of β-conglutin catabolism that accumulates in very high levels between days 4 and 12 after the onset of germination. The 210 kDa protein was extracted and isolated from the cotyledons of 8 day old seedlings. Following isolation of the total globulin fraction, the protein corresponding to β-conglutin was purified by FPLC anion exchange chromatography as explained above and subsequently subjected to FPLC gel filtration on the Superose 12 HR 10/30 column previously equilibrated in 50 mM Tris–HCl buffer, pH 7.5. This last purification step does not affect the polypeptide pattern of the purified fraction, but removes unidentified low molecular mass compounds that appear to interfere with the protein biological activity (results not shown). The native protein exhibits an apparent molecular mass of 210 kDa (when estimated by gel filtration), being composed of a major 20 kDa polypeptide and at least three lower molecular mass polypeptides and several higher molecular mass polypeptides, when assayed by SDS–PAGE. The pure protein was desalted into 10 mM Tris–HCl buffer, pH 7.5.

### 2.4. Purification of goat IgG

Goat IgG was purified from goat blood by affinity chromatography on a protein G-Superose column as previously described [11]. The pure proteins were desalted into 10 mM Tris–HCl buffer, pH 7.5.

### 2.5. Proteolytic digestion of glycoproteins and fractionation of the resulting fragments by gel filtration

10 mg of each pure glycoprotein under study (β-conglutin from *L. albus* dry cotyledons, the 210 kDa protein from 8-day germinated *L. albus* cotyledons, and goat IgG) was desalted into water, lyophilized and resuspended in 10 mM Tris–HCl buffer, pH 7.5 (2 ml). The glycoproteins were digested by addition of pronase (Boehringer) (0.4 mg/ml) and $NaN_3$ (0.02% w/v) and incubation at room temperature for 10 h, followed by a second addition of pronase (0.2 mg/ml) and a further 36 h incubation at room temperature. 1 ml of each sample was loaded into the Superdex peptide, FPLC gel filtration column, previously equilibrated with 10 mM Tris–HCl buffer, pH 7.5. The fractions (1 ml) eluting from the column were continuously monitored for $A_{280}$ and assayed for the presence of carbohydrates as described below.

### 2.6. Turbidity measurements

Turbidity measurements of protein solutions were made spectrophotometrically at 600 nm in 1 ml silica cuvettes, following the procedure described by Okubo et al. [15]. Measurements were made at 25°C, in 10 mM Tris–HCl buffer, pH 7.5, using a protein concentration of 0.5 mg/ml and an incubation period of 5 min. The stock solutions utilized were: Ca (1 M)+Mg (1 M); glucose, galactose, mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, glucosamine, galactosamine, glucuronic acid, galacturonic acid, glycine, lysine, arginine, histidine and phytin (1 M); lactose (0.58 M); raffinose (0.79 M); sialic acid (0.5 M); glutamic acid (0.3 M); bovine submaxillary mucin (1 mg/ml); chitin (0.1% w/v). The final pH of all solutions was always adjusted to 7.5.

### 2.7. Electrophoresis and affinoblotting

Electrophoresis was performed in polyacrylamide slab gels under denaturing, reducing conditions (R-SDS–PAGE) as described by Rosa et al. [16].

Detection of glycopolypeptides on Western blots was performed essentially by the concanavalin A/peroxidase method developed by Faye and Chrispeels [17].

### 2.8. General assays

Protein was measured by a modification of the Lowry method [18].

The presence of carbohydrates in the fractions eluting from the Superdex peptide gel filtration column was detected in microtiter plates using the phenol–sulfuric acid hexose assay [19]. Raffinose was used as the standard.

## 3. Results and discussion

The characteristic insolubility in water or low-salt solutions of the major legume storage proteins, which led to their classification as globulins, results precisely from their calcium/magnesium-dependent self-aggregation. Indeed, a simple extraction of the legume seed proteins with a low-salt aqueous solution produces the globulins in a highly insoluble form. However, the extraction of the storage proteins under high ionic strength conditions produces a highly soluble protein solution. Under these conditions the storage proteins are freely soluble even if desalted into pure water, but self-aggregate or become highly insoluble upon the addition of calcium and/or magnesium in the mM range (results not shown).

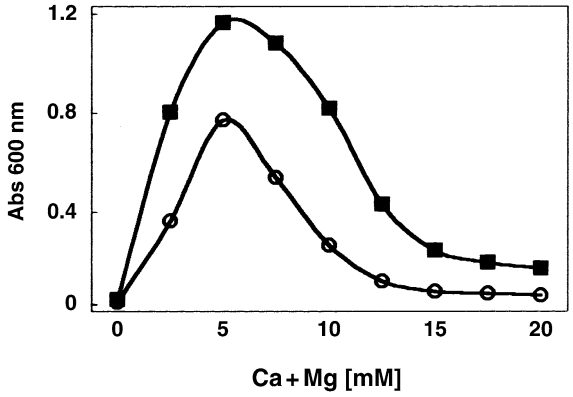


Fig. 1. Effect of calcium+magnesium on the self-aggregation of β-conglutin and the 210 kDa protein. β-Conglutin (0.5 mg $ml^{-1}$; ○) and the 210 kDa protein (0.5 mg $ml^{-1}$; ■) were purified from dry seed or 8-day germinated, respectively, cotyledons of *Lupinus*. Turbidity was measured as described in Section 2.

Treatment of the insoluble globulins with ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to sequester the large amounts of divalent cations that are present in the seeds also produces a highly soluble protein solution.

Two glycosylated proteins were purified from *Lupinus* cotyledons and used in this work: β-conglutin from dry seeds, the major seed storage protein, and a 210 kDa protein that accumulates at high levels in *Lupinus* cotyledons after the 4th day of germination, which is a stable breakdown product of β-conglutin catabolism and contains a major 20 kDa polypeptide with lectin activity [10,14,20]. The data presented in Fig. 1 illustrate the effect of the presence of calcium and magnesium on the self-aggregation of β-conglutin and the 210 kDa protein. In a previous study it was demonstrated that the divalent cation-dependent increases in turbidity (measured by the absorbance at 600 nm) correlate with the degree of self-aggregation of these proteins [11]. Self-aggregation of each of the proteins under study increases markedly with increasing concentrations of Ca+Mg, reaching a maximum that depends on the protein considered [11] – 5 mM calcium+5 mM magnesium in the case of β-conglutin and the 210 kDa protein. Higher concentrations of Ca+Mg produce a gradual decrease in turbidity, an observation that may be tentatively explained by the effect of increasing ionic strength or by saturation of the negatively charged, calcium binding sites on the proteins.

Divalent cation-promoted self-aggregation of β-conglutin has been shown to be highly dependent on protein concentration, ion strength and pH [11]. In addition, the 20 kDa polypeptide component of the 210 kDa protein recognizes and binds to a variety of glycoproteins, including peroxidase, alkaline phosphatase and immunoglobulins G [10] and binds, in a very strong manner, to chitin. Other vicilin-type legume seed storage proteins have been reported to bind to chitin [21]. To try to elucidate the nature of the interactions between the individual protein molecules in the build-up of the macromolecular aggregates, namely if they involve electrostatic (with the divalent cations acting as bridges between the negatively charged proteins) or lectin-mediated interactions, a number of experiments were performed.

A buffered solution (10 mM Tris–HCl, pH 7.5) containing the 210 kDa protein (0.5 mg $ml^{-1}$), 5 mM $CaCl_2$ and 5 mM $MgCl_2$ (cation concentrations that produce maximum self-aggregation; see Fig. 1) was incubated in the presence of increasing concentrations of several compounds and the resulting effects on turbidity detected by spectrophotometry (Fig. 2). The compounds tested included negatively charged, positively charged and neutral carbohydrates and amino acids. Glucose, galactose, mannose, fucose, lactose, raffinose, *N*-acetyl-glucosamine and *N*-acetyl-galactosamine did not affect the aggregation state of the protein (results not shown). However, other carbohydrates (glucuronic acid, glucosamine, galactosamine, galacturonic acid and sialic acid; Fig. 2A), amino acids (glycine, lysine, arginine, histidine and glutamic acid) and phytin (Fig. 2B) interfered with the aggregation process, leading to decreased values of turbidity. Particularly sharp were the effects of sialic acid and phytin, both of which produced, at very low concentrations, marked reductions in the aggregation state of the 210 kDa protein. Phytin, with its negatively charged phosphate groups, may interact with divalent cations and thus interfere with the electrostatic interactions between the negatively charged molecules. On the other hand, lectins exist that are specific for sialic acid [22,23]. However, this negatively charged monosaccharide is apparently absent from plant glycoproteins [24] and, for this reason, could not

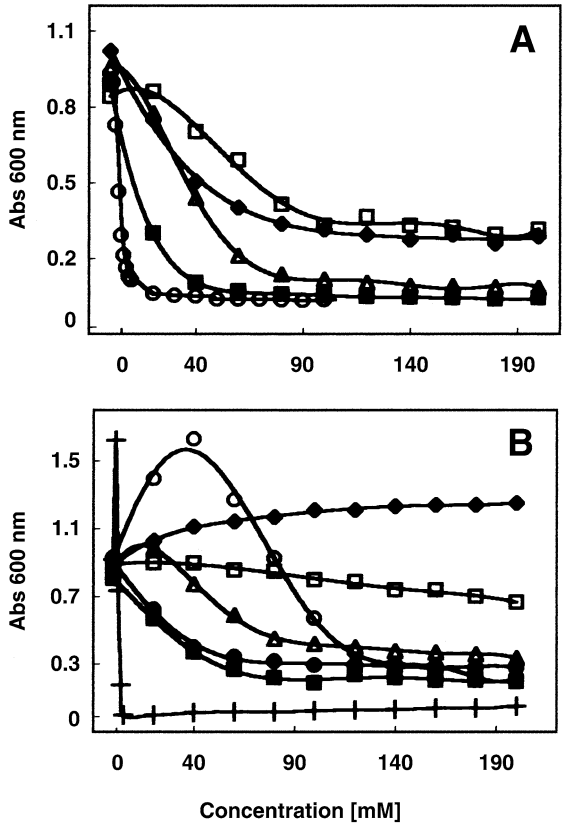


Fig. 2. Effect of carbohydrates (A) and amino acids and phytin (B) on the Ca+Mg-induced self-aggregation of the 210 kDa protein. A buffered solution (10 mM Tris–HCl, pH 7.5) containing 0.5 mg $ml^{-1}$ of the 210 kDa protein, 5 mM $CaCl_2$ and 5 mM $MgCl_2$ was incubated in the presence of increasing concentrations of selected compounds and the effects on turbidity determined as described in Section 2. A: ◆, glucuronic acid; □, glucosamine; △, galactosamine; ■, galacturonic acid; ○, sialic acid. B: ◆, buffer (control); □, glycine; ■, lysine; ●, arginine; △, histidine; ○, glutamic acid; +, phytin.

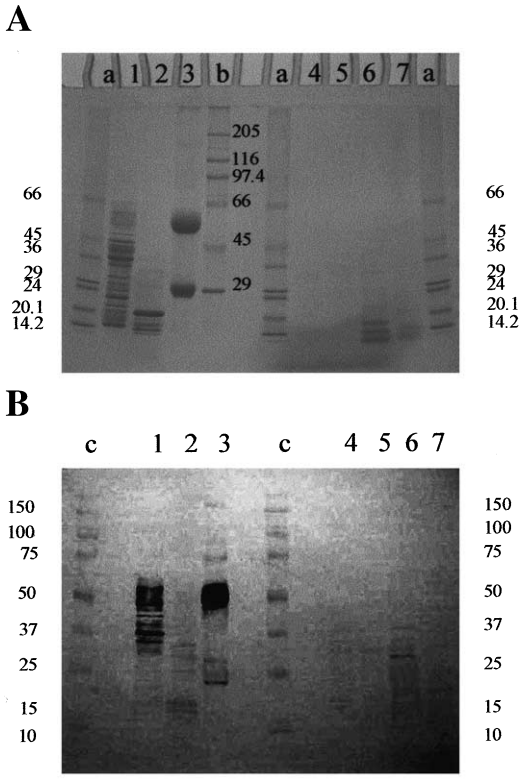


Fig. 3. Digestion of three glycoproteins with pronase. 10 mg each of β-conglutin (lane 1), 210 kDa protein (lane 2) and goat immunoglobulin G (lane 3) was digested with pronase (lanes 4, 5 and 6, respectively) as described in Section 2. A: Total polypeptides were visualized by SDS–PAGE. B: Glycoproteins were detected by affinoblotting. Lane 7, pronase (20 μg); lanes a and b, molecular mass standards (kDa); lane c, prestained molecular mass standards (kDa).

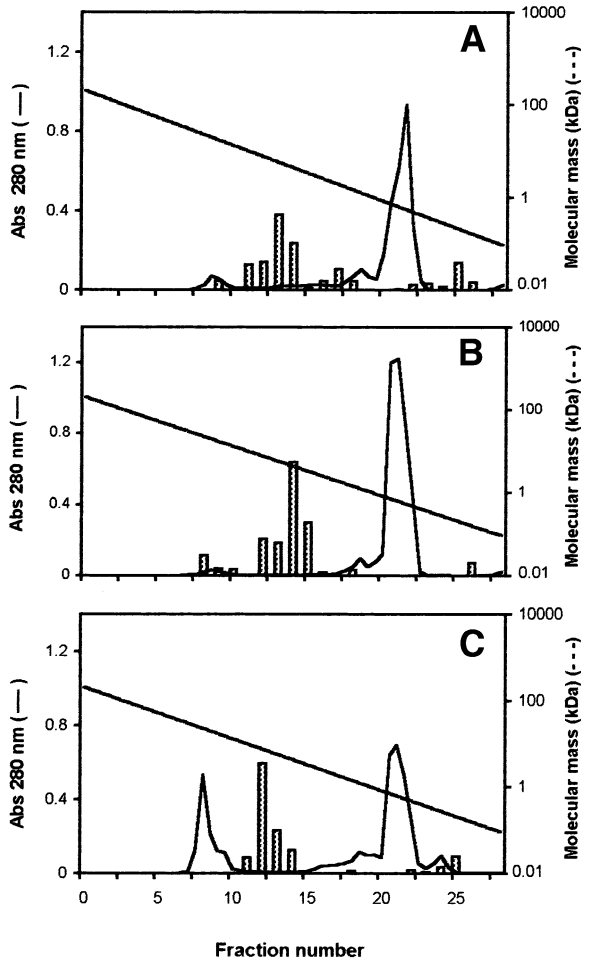


Fig. 4. Fractionation by gel filtration on a FPLC Superdex peptide column of 10 mg of pronase-digested 210 kDa protein (A), β-conglutin (B) and goat immunoglobulin G (C). The eluate was continuously monitored for $A_{280}$ (–). 1 ml fractions were collected and assayed for the presence of carbohydrates (▒). Regression analysis of the molecular masses of standards is represented by the dashed line.

explain the calcium-induced self-aggregation of the 210 kDa protein.

Similar experiments to the one depicted in Fig. 2 were performed with increasing concentrations of chitin (0–0.2 mg $ml^{-1}$) and bovine submaxillary mucin (0–0.2 mg $ml^{-1}$) (data not shown). Chitin was selected due to the strong affinity of the 210 kDa protein for this polysaccharide, whereas mucin is a glycoprotein particularly rich in sialic acid. None of these compounds produced any effect or interfered in any way with the self-aggregation of the 210 kDa protein, suggesting that this process may be electrostatic in nature.

As mentioned above, the multisubunit and glycosylated 210 kDa protein binds to immunoglobulins G in a lectin-type manner [10]. On the other hand, if the self-aggregation process is lectin-mediated, i.e. if the protein carbohydrate-binding sites of the 210 kDa protein do recognize and bind to their own oligosaccharidic side chains, then the isolated free oligosaccharidic side chains will inhibit the self-aggregation process. To establish whether the self-aggregation is due to lectin-type interactions, the oligosaccharidic side chains of β-conglutin, 210 kDa protein and goat immunoglobulins G were separated from the apoprotein and fractionated by gel filtration. To this end, 10 mg of each of the above glycoproteins was digested with pronase and the resulting samples evaluated by electrophoretic (Fig. 3A) and glycoprotein detection analysis (Fig. 3B). The results presented in Fig. 3 show

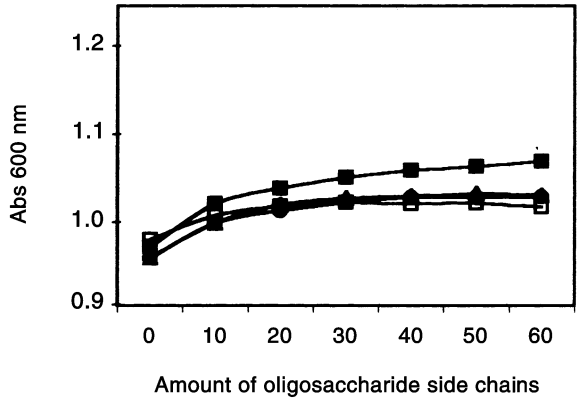


Fig. 5. Effect of the oligosaccharidic side chains of the 210 kDa protein (■), β-conglutin (●) and goat immunoglobulins G (▲) on the Ca+Mg-induced self-aggregation of the 210 kDa protein. A buffered solution (10 mM Tris–HCl, pH 7.5) containing 0.5 mg $ml^{-1}$ of the 210 kDa protein, 5 mM $CaCl_2$ and 5 mM $MgCl_2$ was incubated in the presence of increasing amounts of the proteins' oligosaccharidic side chains and the effects on turbidity determined as described in Section 2. (□) Control (buffer).

that the three glycosylated proteins were readily digested by pronase. The digested samples were subsequently fractionated by gel filtration on a FPLC Superdex peptide column. The fractions collected were assayed for the presence of carbohydrates (Fig. 4) and those containing the oligosaccharidic side chains (fraction numbers 11–15) were pooled and concentrated by liophylization. Increasing amounts of the isolated oligosaccharidic side chains were subsequently added to a buffered solution containing the 210 kDa protein (0.5 mg $ml^{-1}$), 5 mM $CaCl_2$ and 5 mM $MgCl_2$ and the effect on turbidity detected by spectrophotometry (Fig. 5). The result of this experiment supports those of the previous experiments and indicates that the calcium/magnesium self-aggregation of legume seed storage proteins is electrostatic in nature and not lectin-mediated. It seems reasonable to extrapolate the conclusions of these in vitro studies to in vivo conditions, i.e. that calcium and magnesium ions are electrostatically involved in the macromolecular aggregation of legume seed storage proteins, ensuring an efficient packing inside the protein storage vacuoles.

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