PARTIAL PURIFICATION AND CHARACTERIZATION OF LECTIN-LIKE ACTIVITIES FROM *Lupinus albus* SEEDS

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ABSTRACT. In our previous work, the haemagglutinating activity present on three *Lupinus* species was studied; from those results it was decided to purify lectin-like activities present in *Lupinus albus* seeds, taking into account the highest specificity previously found in the extracts. In this study, three appropriate affinity chromatography columns were prepared, in order to purify the haemagglutinating activity from *L. albus*. Pellet collected by centrifugation after saturating the extract with 80 % of ammonium sulphate was applied to Mucin-Sepharose, Fetuin-Sepharose and Galactose-Agarose columns respectively. Although lectin-like activity equivalent to about 5 % of the total protein in the extract was purified using the Mucin or Fetuin columns, the major haemagglutinating activity ran through and did not bind to any column tested. Proteins collected in the eluting peaks upon affinity chromatography were partially characterized by SDS electrophoresis and gel filtration in a Superose 12 column, in order to know about monomeric or oligomeric composition as well as their molecular weight. Circular dichroism spectra were also recorded to analyze the secondary structure of the protein.

Key words: *Lupinus albus*, seed, lectins, peptides

RESUMEN. En un trabajo anterior los autores estudiaron la actividad hemaglutinante presente en semillas de tres especies de Lupinus; a partir de esos resultados se decidió purificar las actividades lectínicas presentes en semillas de *Lupinus albus*, tomando en cuenta las mayores especificidades encontradas previamente en los extractos. En este trabajo se prepararon tres columnas apropiadas de afinidad, con el fin de purificar la actividad hemaglutinante de *L. albus*. El pellet colectado por centrifugación a partir del extracto previamente saturado con 80 % de sulfato de amonio, fue aplicado respectivamante en tres columnas de afinidad: Mucina-Sepharosa, Fetuina-Sepharosa y Galactosa-Agarosa. Aunque fue purificada, a través de las columnas de Fetuina y Mucina, una actividad lectínica equivalente al 5 % aproximado del total de proteína encontrado en el extracto, la actividad hemaglutinante pasó a través de la columna sin aparentemente interactuar con las proteínas fijadas en cada una de las columnas probadas. Las proteínas colectadas en los picos de elusión de las corridas cromatográficas fueron parcialmente caracterizadas mediante electroforesis en poliacrilamida y filtración en gel a través de una columna de Superosa 12, con el fin de conocer acerca de su composición monomérica y oligomérica y sus pesos moleculares. Se analizó, además, la posible estructura secundaria de la proteína a partir de un espectro de dicroismo circular realizado a la muestra.

Palabras clave: *Lupinus albus*, semilla, lectinas, péptidos

INTRODUCTION

Many wild and cultivated plants accumulate the so-called lectins or agglutinins in their seeds or vegetatives tissues. These (glyco)proteins are capable of binding reversibly and specifically to mono and oligosaccharides without altering the structure of the bound ligand (1). Lectins are highly represented in plant kingdom occurring in many plant families where they accumulate specially in seeds though they can be found in all vegetal parts (1).

Among the physiological roles attributed to lectins, they have been considered as antinutritional factors against predators (2), as antimicrobial molecules, because of their specificity to bind glycoconjugates present on the surface of microorganism (3) and conversely because of similar reasons: they are, probably, involved in the symbiosis between nodulating bacteria and leguminous plants (4, 5) or as an evolutionary adaptation to storage nitrogen in repellent proteins (3).

Legume lectins have been deeply studied. For instance, from Concanavalin A (the main lectin in the species *Canavalia ensiformes*), great advances have been done to figure out its tertiary and quaternary structure (6) and even in applied studies (7).

Lectins from Lupin species have been motive of research during the last decade, since this legume group offers some advantages against other legumes, such as its high content of protein, less antinutritional factors, adaptability to colder regions, symbiosis and less growing requirements. Despite lectin activity was found and partially studied in *Lupinus albus* (8, 9, 10), still haemagglutinating

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activity has not been purified and characterized. In the former work, the specificity of the haemagglutinin activity that occurred in three Lupinus species with respect to carbohydrates and glycoproteins was studied. In the present paper, it was tried to partially purify and characterize the haemagglutinin activity present in mature seeds of *Lupinus albus.*

MATERIALS AND METHODS

Procedure of protein extraction from Lupin species. Seeds from *Lupinus albus var.* Lublanc were crushed to a fine powder with a grinder. The seed meal was defatted with N-hexane at room temperature (10 mL/g of meal) while stirring. The process was repeated three times during two hours in order to remove most lipids. After the solid material had settled to the bottom of the recipient, the hexane was decanted and the solid was spread on filter paper and let to dry in the fumehood, at least, overnight.

Dried meal was extracted overnight at 4°C using saline (0.9 % w/v NaCl) containing 0.02 % sodium azide and 20 mM of calcium and manganese chloride. Afterwards, the mixture was filtered through cheesecloth and the remaining debris was removed by centrifugation at 12 000 g for 15 minutes at 4°C.

Protein fractionation with ammonium sulphate. The proteins contained in the supernatant of the crude extract were precipitated with ammonium sulphate by adding the salt amount required to achieve 80 % saturation and it was calculated according to the table (11). Once the 80 % saturation was reached in the crude, the solution was let stirring 1 and a half hours at 4°C and to repose for 30 minutes more before centrifuging it at 27 000 g at 4°C for 20 minutes. The pellet was recovered and redissolved in a minimum amount of extraction buffer and dialyzed at least for two hours (or even overnight) against the same buffer. The supernatant from 80 % precipitation was also kept and analyzed for haemagglutinating activity.

Preparation of three different affinity chromatography columns for the purification of lectin-like activities from *Lupinus albus*

Activation of Sepharose-4B with Cyanogen bromide (CNBr) and immobilization of glycoproteins. For the activation a procedure described by Beeckmans (12) was followed. Activated gel was washed very quickly with 0.1M $NaHCO_3$ pH 8.2 on a sintered glass funnel and NaOH pellets were added into the vacuum flask in order to destroy any remaining CNBr.

The washed gel was added into the respective glycoprotein solution (Mucin or Fetuin), which had previously been dissolved in the same cold buffer (0.1M $NaHCO_3$, pH 8.2) at a concentration of 5 mg/mL, giving a final coupling solution of 2 mL/g wet weight of activated Sepharose. The mixture was shaken overnight at room temperature to allow coupling and afterwards, the gel was washed first with a small volume (2 x 5 mL) of 0.1M $NaHCO_3$ pH 8.2, keeping these two washing for further determination of the amount of protein not bound to the gel. Additional washing with about two litres of the same buffer was done and finally the gel was packed into a column of 13 x 1.5 cm.

Coupling of D(+) Galactose to Agarose gel activated with divinyl sulfone. Divinyl sulfone activated agarose (KEM-EN-TEC Company, Denmark) was used as the matrix to bind the galactose. The activated matrix (Mini-Leakä High) was twice washed with distilled water following the instruction manual. Then 10 g (wet weight) of gel were resuspended in 20 mL of Na_2CO_3 containing 2g of D(+)-galactose and shaken overnight at room temperature. The day after, the gel was washed with 8 mL Na_2CO_3 of solution and added into the blocking reagent (0.1M ethanolamine, pH 11) to block all the remaining still active agarose sites, which can provoke nonspecific interactions in the purification process. The mixture was shaken two hours at room temperature and then the beads were extensively washed with saline to wash out weakly or non-covalently bound material before being packed in a column of 12 x 1.5 cm.

Purification of lectin-like activities from *Lupinus albus*

Purification by affinity chromatography on Mucin, Fetuin and Galactose columns. In each case the sample to be applied onto any affinity chromatography column was kept in a solution of ammonium sulphate at 80 % saturation in the cold room till the day before the run; then it was centrifuged 12 000 g for 20 min at 4°C and the pellet obtained dissolved and dialyzed overnight in the equilibrium buffer (Saline: NaCl 0.9 % + $MnCl_2$ 20 mM + $CaCl_2$ 20 mM + NaN_3 0.02 %). The sample was applied onto the column in the equilibrium buffer and allowed to pass through the gel at the lowest speed the pump permits. After the sample was inside, the column was washed with at least three times the volume of the column with equilibrium buffer at a higher speed or until a flat base line was seen in the recorder signal. For the elution of the lectins bound to the column, it was used first 0.25M of D(+) Galactose dissolved in equilibrium buffer (50 mL) and afterwards 0.1M acetic acid (50 mL) in all columns. After each run, each column was regenerated with equilibrium buffer.

Only in the cases of Mucin and Fetuin columns, runs were additionally done under the same conditions, but using D(+) Mannose (0.25M, 50 mL) as first eluent buffer instead of D(+) Galactose and similarly followed by 0.1M of acetic acid. Moreover, in the same columns several unbound peaks from former runs were re-chromatographed under the same conditions. In each case, all protein peaks collected in the run process were dialyzed against equilibrium buffer or saline-azide (without ions), in order to determine lectin activity as haemagglutination of trypsinized RBC and to know the protein concentration, respectively. Finally, all peaks collected containing proteins were dialyzed extensively against bidistilled water and lyophilized.

Protein determinations. Protein concentrations were determined by the Bicinchoninic acid (BCA) method (13) in flat microtiter plates (Nunc Company).

When it was required, as for instance, before run in the FPLC lyophilized samples which were previously dissolved in running buffer, the protein concentration of the sample was determined by measuring the absorbance in a spectrophotometer UVIKON 933 double beam uv/vis (Kontron), in a scanning process between 350-240 nm.

Determination of lectin-like activities. Lectin-like activities in the extracts and all protein samples were determined as the haemaglutinating activity of trypsinized rabbit red blood cells in U-shaped microtiter multi-well plates (Limbro) according to a methodology described by Falcón (14).

Protein characterization

Determination of the protein molecular weight. The native size of the protein in each eluted peak from the affinity chromatography columns (Mucin or Fetuin) was determined by gel filtration in FPLC (Fast protein liquid chromatography) on a Superose 12 column supplied by Pharmacia.

Lyophilized samples from galactose and acetic acid eluted peaks were dissolved in PBS running buffer (50 mM K-phosphate + 15 mM NaCl + 0.02 % NaN_3) pH 7.2 and both sample and buffer were filtered using a 0.22mm filter (from Millipore). With no column in the system, pump A was primed with PBS and the Superose column was connected to the system and equilibrated with PBS for 90 minutes at a flow rate of 0.5 mL/min.

After washing the loading loop with PBS 100 uL of the protein sample, 0.12 mg/mL in Galactose peak and 0.16 mg/mL in the acetic acid peak were applied onto the column across the loading loop and the column was washed with the PBS running buffer while elution was monitored by measuring the absorbance 214 nm and peaks collected using a Superrac collector. Subsequent runs using the following protein standards were carried out to determine the exact molecular mass of the protein peaks obtained: Thyroglobulin (669 kDa), Apoferritin (443 kDa), β-amylase (200 kDa), IgG (150 kDa), BSA (68 kDa) and Myoglobin (17 kDa).

The molecular mass of subunits was determined by SDS-PAGE on 12.5 % polyacrylamide gels on a Mini Protean II apparatus (BioRad) under denaturing conditions (15) using sample solution containing β-mercaptoethanol (reducing sample). The same SDS-PAGE was carried out using nonreducing sample solutions (without β-mercaptoethanol) to know the molecular weight of disulfure bridge containing proteins. Polypeptide bands were stained with Coomasie blue R250. Protein standards used in SDS were: Phosphorylase b (92 kDa), Catalase (60 kDa), Aldolase (40 kDa), Carboxyanhydrase (30 kDa), Trypsin (20 kDa) and Lysozyme (14.3 kDa).

All samples were extensively dialyzed against bidistilled water and lyophilized. Afterwards, each one was redissolved in water or PBS before being used in SDS or gel filtration experiments respectively, and clarified by centrifugation.

In both methods, SDS-PAGE and gel filtration in FPLC, the molecular weight of bands and protein peaks respectively, was calculated by plotting in a logarithmic paper the distance between the buffer front and each band (in SDS) or the top of the peak (in gel filtration) in samples and markers.

Secondary structure by Circular dichroism (CD). CD spectrum was measured using a Jasco J-715 spectropolarimeter. A far UV CD spectrum (260-180 nm) was taken in a thin cell (pathlength 0.1 cm) and the protein was dissolved in bidistilled water. Protein concentration was reduced in order to decrease the noise. The far UV CD spectrum was analyzed for secondary structures, using a computer programme (16). In this programme the structure is calculated by comparing the CD spectrum of the unknown proteins with the spectra of 33 proteins from which the exact 3-dimensional structure is known from X-ray crystallography studies.

RESULTS

Glycoproteins immobilization in activated gels. During the coupling procedure of Mucin and Fetuin to activated Sepharose respectively, the protein concentration was measured in each protein solution before being added to the Sepharose and after the coupling process using the washing buffers kept for this purpose.

In these cases, protein determinations were made by measuring absorbance in the spectrophotometer through a scanning process explained in Materials and Methods. According to the results, there were 3.2 and 2.35 mg/mL of Sepharose of Mucin and Fetuin, respectively. Taking into account that the size of the columns was 13 x 1.5 cm, there was a volume of about 23 mL of gel in each one. Thus, 73 mg of Mucin and 54 mg of Fetuin were bound to the gel of the respective columns.

During the immobilization of D(+) Galactose to activated Agarose, the procedure recommended by the company was followed, but the amount of Galactose bound to the gel was not determined.

Affinity chromatography results in Mucin, Fetuin and Galactose columns. Running either in Fetuin and Mucin columns the results were very similar; an unbound peak running through, which accounts for about 93-95 % of the protein content present in the extract and two elution peaks representing between 5-6 %. Results are shown in Table I. However, 98-99 % of the haemagglutinating activity ran through the column as part of the unbound peak and just a minimal fraction could be eluted as part of the acetic acid peak, whereas no haemagglutinin activity was detected in peaks eluted with D(+) Galactose.

Table I. Protein purification results by affinity chromatography in a Fetuin-Sepharose column. A representative example from several runs

Protein samples	Volume (mL)	Protein		Agglutinating activity	
		Mg/mL	Total (mg)	Titre	Units
Extract added	17	12.4	210.8	256	4352 u♣
Unbound peak	70	2.8	196	64	4480 u
Galactose peak	25	0.143	3.575	—♥	—
Acetic acid peak	24	0.325	7.8	4	96 u

The different fractions obtained were analyzed by SDS-PAGE (Figure 1, lanes c-f). In the extract, lane c, it was possible to appreciate two major and darker bands corresponding to a MM of 31-34 kDa (heavy band) and 17-19 kDa (light band). These two bands were not present in the lane d corresponding to the application of the unbound peak. However, lanes c and f representing the applications of Galactose and acetic acid eluted peaks have justly the bands with the MM aforementioned for the extract case. Moreover, lines g and h corresponding to the peaks eluted with Galactose and acetic acid but dissolved in nonreducing sample solution (without β-mercaptoethanol) containing the same simple and intense band of a MM of about 52 kDa.

Lane a and b: Protein markers applied in high and low amounts respectively. The molecular mass of the markers is as follows: 1-Phosphorylase b (92 kDa), 2-Catalase (60 kDa), 3-Aldolase (50 kDa) 4-Carboxyanhydrase (30 kDa), 5-Trypsin (20 kDa) and 6-Lysozyme (14.3 kDa)
Lane c: Extract applied onto the column
Lane d: Unbound protein peak
Lane e: Protein peak eluted with galactose
Lane f: Protein peak eluted with acetic acid
Lane g: Protein peak eluted with galactose in nonreducing sample solution
Lane h: Protein peak eluted with acetic acid in nonreducing sample solution
Lane i: Protein contained in the unbound peaks after re-precipitation with 80 % ammonium sulphate, centrifugation and dialysis against water

Figure 1. SDS-PAGE results of an affinity chromatography run in the Fetuin-Sepharose column. Each sample was processed as described in Materials and Methods. Unless the contrary is specified, all samples were run in SDS using a reducing sample solution

Several unbound peaks free of any contamination with the fractions eluted with D(+)-galactose and acetic acid (as it was proved by SDS-PAGE beforehand) were re-chromatographed under the same running conditions (results not shown) in order to determine whether any competition occurred that prevented the binding of the haemagglutinin and, instead, allowed the binding of the apparently different lectin protein. However, again the sample applied ran through and no proteins were eluted using neither Galactose nor acetic acid.

From these results, it was decided to run a normal sample under the same conditions and make the elution using D(+) Mannose instead of Galactose dissolved in the equilibrium buffer at the same concentration (0.25 M). The results shown in Table II are, actually, quite similar to the other one obtained before using Galactose as eluent; again haemaglutinating activity was not observed in the mannose peak and it was almost absent in the acetic acid peak. SDS-PAGE analysis (Figure 2) again showed the same two intense bands in the extract (lane c) similar to the bands obtained in mannose and acetic acid lanes (e and f) corresponding to a MM of about 32 (heavy) and 17 (light) kDa, and 52 kDa in the lines where it was run using the nonreducing sample solution.

Table II. Protein purification results by affinity chromatography in a Mucin-Sepharose column. A representative example from several runs

Protein samples	Volume (mL)	Protein		Agglutinating activity	
		Mg/mL	Total (mg)	Titre	Units
Extract added	9.5	8.3	78.85	512	4096 u
Un-bound peak	55	1.26	69.30	64	3520 u
Mannose peak	30	0.18	5.40	—♥	—
Acetic acid peak	25	0.315	7.80	2	50 u

On the other hand, all the runs made in the Galactose-Agarose column were unfruitful, since no protein was bound or eluted from the column under the same conditions described before (Table III).

Finally, proteins belonging to unbound peaks were reprecipitated with ammonium sulphate at 80 % saturation, centrifuged and the pellet dissolved in bidistilled water, dialyzed and lyophilized before being redissolved in water to run in SDS-PAGE (Figure 1, line i and Figure 2, line i and j), showing several bands without any specific intensity at the level of MM of 32 and 17 kDa.

Lane a and b: Same protein markers and conditions described in Figure 1
Lane c: Extract applied onto the column
Lane d: Unbound protein peak
Lane e: Protein peak eluted with mannose
Lane f: Protein peak eluted with acetic acid
Lane g: Protein peak eluted with mannose in nonreducing conditions
Lane h: Protein peak eluted with acetic acid in nonreducing conditions
Lane i and j: Protein contained in the unbound peaks after re-precipitation (same conditions as Figure 1).

Figure 2. SDS-PAGE results of an affinity chromatography run in the Mucin-Sepharose column. Sample processing and conditions are similar to Figure 1

Table III. Protein purification results by affinity chromatography in a Galactose-Agarose column. A representative example from several runs

Protein samples	Volume	Protein		Agglutinating activity	
		Mg/mL	Total (mg)	Titre	Units
Extract added	8.3	14.0	116.2	128	1062.4 u
Un-bound peak	32	4.0	128.0	32	1024.0 u
Galactose peak	17	—♠	—	—♥	—
Acetic acid peak	7	—♠	—	—♥	—

♠: It means that protein was neither detected nor present in a nondetectable concentration using our standard protein curve
♥: No haemagglutinating activity was detected using the current method described in chapter 2

Gel filtration in FPLC. Protein peaks eluted from Mucin or Fetuin-Sepharose columns were extensively dialyzed against bidistilled water and lyophilized before being redissolved in PBS to be used in FPLC experiments. A clear supernatant after centrifugation was applied onto the Superose 12 column following the conditions described in Materials and Methods.

Both samples had the same behavior in the gel filtration runs. Two peaks of fairly equal height were observed running both Galactose and acetic acid eluted samples (Figure 3). According to the molecular mass (MM) of the markers also run under the same conditions, both sample peaks correspond to a MM of about 130 kDa and 52 kDa respectively. No other protein peaks were observed in any repetition.

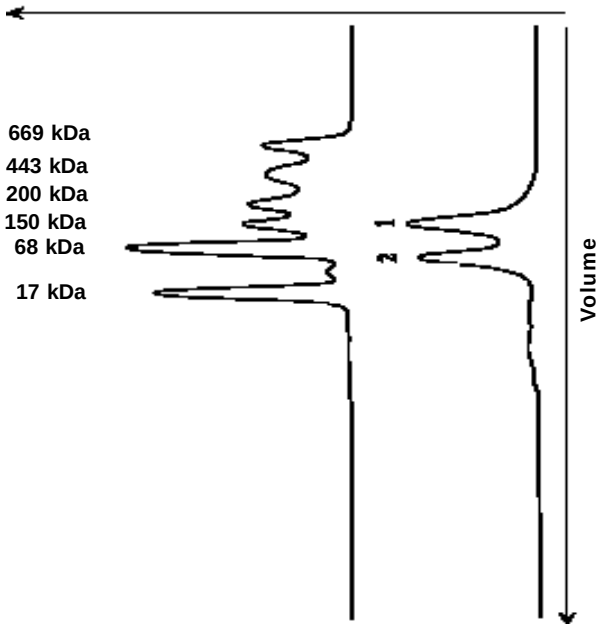

Figure 3. Gel filtration profile in FPLC using a Superose 12 column (1 x 30 cm). Results of the run with the protein markers and the protein sample eluted with acetic acid from the Mucin-Sepharose column are shown

Circular dichroism results. A lyophilized protein sample from the elution with acetic acid in a Mucin-Sepharose run was redissolved in PBS and centrifuged, in order to obtain a clear solution suitable to perform a circular dichroism spectrum following the procedure described in Materials and Methods. Spectrum of the acetic acid peak from the run in the Mucin-column shown in Figure 4 was analyzed according to Deléage and Geourjon's method (16) (see Materials and Methods), in order to estimate the secondary structure of our first lectin:

Acetic acid protein peak

Secondary structure	% present according to CD results
α- helix	6 %
Anti-parallel β-sheet	33 %
Parallel β-sheet	2 %
β-Turn	19 %
Random coil	38 %

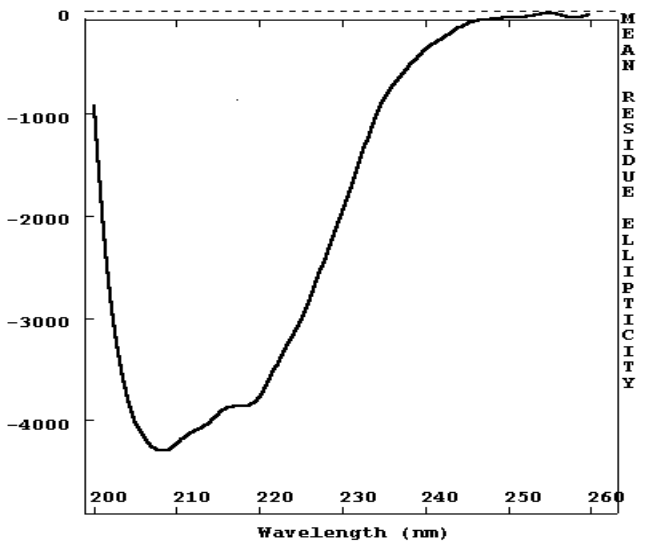

Figure 4. CD spectrum of the protein peak eluted with acetic acid from the Mucin-Sepharose column

DISCUSSION

Legume seeds usually contain high concentrations of haemagglutinins and lectin-like proteins in general, which along with trypsin inhibitors and other components such as alkaloids in a lesser extent, can undoubtedly reduce the efficiency of food conversion by the gut, due to their direct antinutritional effects (17). Particularly, lectins according to some suggestions, may act as extraneous metabolic signal molecules which induce wasteful and harmful changes in metabolism leading to inefficient digestion and to substantial losses of essential body components (17, 18).

Among the advantages that some authors claim for lupin over other legume species, such as soybean, is their lower content of antinutritional factors. Lupin seeds or meal would not need to be heat-treated since trypsin inhibitors and haemagglutinins are practically absent (19).

Affinity chromatography results. Regarding the results shown in Tables I, II and III, it can be concluded that the haemagglutinin activity did not bind to any of the affinity chromatography columns prepared for this purpose.

Nevertheless, at least with the Mucin and Fetuin columns, it was possible to purify another protein which is dominantly present in the extract (i.e. it represents 5-6 % of the total amount of protein in the extract). Since this protein can be eluted by sugars, it can also be considered to be a lectin; however, it does not show haemagglutinating activity (at least not when assaying with rabbit erythrocytes).

In order to know if the lack of adsorption of the haemagglutinin activity might be due to the presence of the lectin purified, unbound peaks containing the haemagglutinins but free from the other lectin (according to SDS-PAGE results) were re-chromatographed using the same conditions and eluents. In the result, as in former runs on Mucin and Fetuin-Sepharose, it was found that haemagglutinin was running through in the unbound peak and no protein was detectable in the elution peaks.

By SDS-PAGE using reducing and nonreducing conditions, the same bands or band were obtained respectively in the peaks eluted with either Galactose and acetic acid. Thus, they belong to the same protein. This was further confirmed by gel filtration in FPLC, where exactly the same elution pattern was observed for both fractions. Apparently, proteins can not be completely eluted with Galactose (0.25 M); thus, a stronger eluent was required such as acetic acid (0.1 M) to release the lectin.

In order to find out whether Galactose is the specific monosaccharide for this lectin, a sample was run under the same conditions but changing the Galactose eluent for Mannose at the same concentration. The results shown in Table II confirm the same behavior obtained in former runs using Galactose as eluent; the lectin was eluted with Mannose but again incompletely, as it was necessary to use acetic acid to elute the rest of the protein. Thus, this lectin shows about the same specificity for Galactose as for Mannose. It may be concluded that the position of the -OH group at the carbon atoms C_2 and C_4, which makes the difference between Galactose and Mannose, does not appear to be important for binding the sugar to the protein.

In mature lupin (*Lupinus albus*, L.) seeds, a globulin protein, which accounts for 5 % of the total protein, was purified (20). The lectin activity of this protein, named conglutin γ, has also been confirmed (9) as well as the molecular mass of its monomer and subunits (21). According to the results of these authors conglutin γ consists of oligomeric units of 51 kDa, which are formed by a glycosylated 30 kDa polypeptide chain linked to a 17 kDa one by a disulphide-bridge.

From our SDS-PAGE results using the elution fractions obtained with either Mannose or Galactose, about the same molecular masses were observed for both monomer (52 kDa) and subunits (32 and 17 kDa). Moreover, the amount of protein eluted with Galactose and acetic acid from Mucin and Fetuin-Sepharose columns was always between 5 and 6 % of the total soluble protein present in the extract. Taking into account the results aforementioned and those obtained from the literature, it might be assumed that the lectin protein purified in our affinity chromatography columns corresponds to the conglutin γ described before (9). Additionally, we can also say that conglutin γ accounts for the major lectin activity contained in *Lupinus albus* seeds, but it is not responsible for the haemagglutinating activity found, which in fact runs through the column in the unbound peak.

Why the haemagglutinins do not adsorb onto any of the affinity chromatography columns tested is not clear. One possibility might be that due to the immobilization of the glycoproteins onto the Sepharose matrix, conformational changes occur in the covalently linked carbohydrate chains, so that the haemagglutinin does not

recognize them anymore. Another explanation could be that the affinity shown for the glycoproteins to inhibit the agglutination of RBC is not strong enough to keep the haemagglutinins bound to the column during the affinity chromatography process. Alternatively, the lack of binding may be due to steric hindrance.

Gel filtration in FPLC. The apparent molecular masses of both peaks obtained from affinity chromatography on Mucin-Sepharose was determined by FPLC gel filtration using a Superose 12 column. It seems that this protein elutes as a mixture of monomers and trimers, at least, under the conditions used (PBS buffer pH 7.2). This result is not in agreement with others found in former reports: a hexameric structure was proposed in *L. angustifolius* (22) and the coexistence of monomers and tetramers, as a function of pH, in *L. albus* (21) as the common quaternary structure for conglutin γ. A further research to confirm this result is required, taking into account the molecular weight of the oligomeric form which did not fully correspond to the theoretical molecular weight of the trimer.

Secondary structure characterization. The secondary structure of our purified lectin is characterized by a high content of anti-parallel β-sheet conformation (33 %) and a very low content of α-helix (6 %) as it is commonly reported for lectins in general. In addition, our CD spectrum is quite similar in shape to a former spectrum reported for conglutin γ (8).

The results obtained from this study about the identification and characterization of the haemagglutinin activity in lupin species drew some valuable conclusions, despite the followings can be considered as preliminary in some aspects:

- Using affinity chromatography in Mucin- and Fetuin-Sepharose columns, the major lectin present in the *Lupinus albus* extract was purified. However, it was also found that this lectin is not responsible for the haemagglutinating activity detected in the same extract. Consequently, there are, at least, two different lectins present in *L. albus*.
- According to our SDS-PAGE, gel filtration and CD spectrum results, the major lectin purified by affinity chromatography might be conglutin γ, a lectin-like protein purified previously by different methods.
- Finally, in order to purify the haemagglutinin still contained in the unbound peaks of the affinity chromatography runs, other chromatographic methods should be used; for instance, gel filtration and ion exchange chromatography have been used with success in other lectin purifications from Leguminous plants (23).

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