INVESTIGATION ON THE HAEMAGLUTINATING ACTIVITY OCCURRING IN THREE SPECIES OF *LUPINS*

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ABSTRACT. Lupin is a leguminous plant greatly used as human food and animal feed all around the world. It has several agronomic advantages related to other species of the family. In this paper, the haemagglutinating activity from seeds of three species of lupins: two Mediterraneans (*L. albus* and *L. angustifolius*) and one from South America (*L. mutabilis*) was studied. An important haemaglutinating activity was found in extracts from mature seeds, in each case, against rabbit erythrocytes, specially when these cells had been previously treated with a trypsine solution to partially hydrolyze their membranes. Assays were not influenced by different temperatures and similar results were observed at 25 and 4 ºC. Inhibition of haemagglutination with carbohydrates and glycoproteins was performed to determine haemagglutinin specificity; the three species tested followed a highly specific behavior per galactose, as well as per disaccharide (Melibiose) and trisaccharide (Raffinose) containing galactose monomer. Among the glycoproteins tested, it was observed a high specificity towards Porcine Mucin, Fetuin, Asialofetuin, Thyroglobulin and in a lesser extent to Ovalbumin. There was not inhibition of haemaglutination by other carbohydrates and glycoproteins tested or they did it in higher concentrations.

Key words: lupin, leguminous, haemaglutinins, lectins

RESUMEN. El lupino es una leguminosa de gran uso como alimento animal y humano en todo el mundo, que posee ventajas agronómicas en relación con otros representantes de la familia. En este trabajo se estudió la presencia de actividad hemaglutinante en semillas de tres especies de lupino: dos mediterráneas (*L. albus* y *L.angustifolius*) y una sudamericana (*L. mutabilis*). Se encontró una importante actividad hemaglutinante en extracto de semillas maduras contra eritrocitos de conejo, especialmente cuando estas células habían sido previamente tratadas con una solución de tripsina para hidrolizar parcialmente sus membranas. La temperatura del ensayo de hemaglutinación no constituyó un parámetro que influyera en sus resultados, ya que a 25 y 4ºC se obtuvieron resultados similares. Mediante ensayos de inhibición de la hemaglutinación usando carbohidratos y glicoproteínas, se determinó la especificidad de las hemaglutininas, siendo las tres especies altamente específicas por galactosa, así como por disacárido (Melibiosa) y trisacárido (Rafinosa) que la contienen. Entre las glicoproteínas probadas, se observó una alta especificidad hacia la Mucina porcina, Fetuina, Asialofetuina, Tiroglobulina y en menor medida por Ovalbumina. Otros carbohidratos y glicoproteínas no inhibieron la hemaglutinación o lo hicieron a concentraciones mucho mayores.

Palabras clave: lupino, leguminosas, hemaglutininas, lectinas

INTRODUCTION

Lupins are legumes used as human food and animal feed since the early Roman times. Nowadays, over 200 lupin species are grown worldwide and their yield has been increased especially in Australia, Chile and the United States, as a result of breeding programs to produce «sweet» varieties with low concentrations of alkaloids, its main disadvantage for human consumption (Culvenor and Petterson, 1986).

The agronomical benefits of growing lupin include its ability to fix nitrogen and to grow in areas where, for instance, soybean cannot be grown, since lupin can tolerate frost, drought, and poor soils (Rahman and Gladstones, 1987). Other important features which point them as good candidates for food source are lower concentrations of antinutritional factors and haemagglutinins than soybean.

Lupine is one of the legumes with higher protein content in seeds; the actual amount depends on the species and ranges from 36 to 52 % of the dry weight of the whole seed (Gross *et al*. 1988; Petterson and Mackintosh, 1994). Studies carried out at the National Institute of Agricultural Sciences in breeding *Lupinus albus*, as an alternative of green manure to agriculture, have yield harvests ranging between 0.92 and 2.00 $t.ha^{-1}$ from September to January (García, 1997; Alvarez, García and Treto, 1998).

In the latest years, lectin activity has been found and studied in the seed of *Lupinus albus*. Interaction has been demonstrated between its lectin activity with dextrin matrix (Sephadex G-75) and with glycoside peptides including Ovalbumin, human transferrin, glycoside leguminin of lupin

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and peroxidase (Duranti, Gius and Scarafoni, 1995). However, so far the haemaglutinating activity before mentioned has not been duly studied in regards to the kind of blood cells agglutinated and the carbohydrate specificity of these proteins.

Therefore, the aim of this study was to detect and characterize the haemagglutinating activity in three species of *Lupinus*: *L.albus*, *L.angustifolius* and *L.mutabilis*. To that purpose, the behavior of each species was firstly investigated on its agglutinating properties of rabbit red blood cells after different levels of saturation of the extract with ammonium sulfate, using normal or trypsinized red blood cells, and at two different temperatures. Then, the carbohydrate specificity of the haemagglutinins present was determined using agglutination-inhibition studies.

MATERIALS AND METHODS

Protein extraction procedure from Lupin species

Seeds from three lupin species (*Lupinus albus* var. Lublanc, *Lupinus angustifolius* var. Kubeza and *Lupinus mutabilis*) were crushed to a fine powder with a grinder. The seed meal was defatted in each case with N-hexane at room temperature (10 $mL.g^{-1}$ of meal) while stirring. The process was repeated three times during two hours each, in order to remove most of the 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 % (w/v) sodium azide and 20 mM of calcium and manganese chloride. Afterwards, the mixture was filtered through cheese cloth and the remaining debris was removed by centrifugation at 12 000 g for 15 minutes at 4°C.

Ammonium sulfate fractionation

The proteins contained in the supernatant of the crude extract were precipitated with ammonium sulfate at 20, 40, 60 and 80 % saturation and the amount of ammonium sulfate required in each case was calculated according to the table by Green and Hughes (1955).

Once the ammonium sulfate amount necessary to achieve 20 % saturation in the crude was reached, the solution was left stirring one 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 resuspended in a minimum amount of extraction buffer and dialyzed at least for two hours (or even overnight) against the same buffer, whereas the supernatants underwent the same procedure described before to reach 40, 60 and 80 % saturation. The supernatant from 80 % precipitation was also kept and analyzed for haemagglutinating activity.

As a different experiment, lupin seeds from the three species were allowed to stay in distilled water overnight and the day after a sample of water was kept, calling it the inbibition water, for haemagglutinin assays.

Haemagglutination tests

Blood cells. Lupin lectins were assayed for haemagglutination with rabbit blood erythrocytes. To prevent clotting, the blood was collected in an equal volume of Alsever solution (in 100 mL solution: 2.05 g glucose, 0.8 g Na-citrate, 0.42 g NaCl and 10 % w/v citric acid) pH 7.2 containing heparin (1 drop/5 mL). From this stock suspension, 2 mL were taken and washed three times in 0.9 % NaCl + NaN_3 0.02 %, pH 6.5 (Saline) and the cells collected by centrifugation at 500 g for 10 minutes. Afterwards, the cells were resuspended in saline + ions 20 mM (Mn^{2+} and Ca^{2+}) + azide to give a 4 % v/v suspension and then cells were ready for testing directly the haemagglutination or to be trypsinized.

Trypsinization of red blood cells. A suspension was made consisting of 2 mL red blood cells (RBC) from the stock, 8 mL 0.9 % saline/azide pH 6.5 and 1 mL saline/azide containing 0.1 % w/v trypsin. The suspension was incubated at 37°C for one hour. Following the trypsinization procedure, RBC were washed three times with saline and the cells collected by centrifugation at 500 g for 10 minutes. Afterwards, the cell suspension was adjusted with saline/aside+Mn^{2+} and Ca^{2+} to give a 4 % v/v suspension. Trypsinized RBC were used the same day as they are more fragile than the native cells.

The assay. Haemagglutination was carried out in U-shaped microtiter multi-well plates (Limbro). 25 µL of buffer (saline/azide+Mn^{2+} and Ca^{2+}) were added to all the wells of the first row except the second well. 25 µL of the lectin sample was added to the second and third wells of each row. Serial dilutions were made with an equal volume of saline from the third well to the last one of each row to give a final volume of 25 µL in each well. The first well acted as the negative blank since no lectin was added to it. The second well was the undiluted lectin sample. To all the wells of each row, 50 µL of the 4 % erythrocyte suspension was added except to the last one; if necessary, further dilutions could be started from this well. The plates were incubated for one hour at room temperature or 4°C depending on the experiment.

All the samples were tested, at least, in duplicate. The end point of titration was estimated visually as the lowest dilution still showing agglutination and corroborated if required, by observation in microscopy to see the agglutinated erythrocytes. This figure was then called the agglutination titer, and it was given as the inverse of the dilution.

Inhibition of agglutination. The haemagglutination activity of lectin samples was adjusted in such a way that it gave four agglutinating units (That is three wells in which agglutination was seen). This lectin concentration was used fix during the inhibition assay.

The following sugars and glycoproteins (from Sigma Chemical Company) were used in this assay:

Sugars: D(+)-Galactose, D(+)-Galactosamine, N-acetyl-D(+)-Galactosamine, Methyl-α-D-Galactopyranoside; D-Glucose, D-Glucosamine, N-acetyl-D-Glucosamine, Methyl-α-D-Glucopyranoside; D-Mannose, D-Mannosa-mine, Methyl-α-D-Mannopyranoside; α-Lactose, β-Lactose, Raffinose and Melibiose.

Glycoproteins: Ovalbumin (Chick egg), Thyroglobulin, Porcine stomach mucin, Bovine salivary gland mucin, Asialomucin (bovine) from salivary glands, Fetuin and Asialofetuin from fetal calf serum and Peroxidase.

Each sugar was dissolved in saline to give a concentration of 0.4 M, whereas for glycoproteins a stock solution of 15 $mg.mL^{-1}$ was made. In this experiment, both sugars and glycoproteins were diluted in columns of the agglutination plates instead of rows (contrary to the haemagglutination assay). The first column contained only 25 μL of saline buffer and constituted the negative control; the second column had just 25 μL of lectin (4 agglutinating units) being the positive blank. Starting from the third column, it was possible to test one sugar (or glycoprotein) per column and the dilutions were made in a vertical way, as it was already mentioned, in such a way that the first well of the column contained the sugar and lectin (25 μL of each) and from the second well started serial dilutions of sugar with 25 μL of saline buffer before adding the 25 μL of the lectin solution. An incubation at room temperature for 15 minutes was done to allow the possible interaction between sugars (or glycoproteins) and lectin before adding 50 μL of the 4 % (v/v) solution of trypsinized RBC. The plate was incubated for one hour at room temperature or 4°C.'The inhibition of agglutination was visually estimated and expressed as the lowest concentration of sugar/glycoprotein still inhibiting agglutination.

Both haemagglutination and inhibition of haemagglutination were carried out for the three species of Lupinus: *Lupinus albus*, *Lupinus angustifolius* and *Lupinus mutabilis*; using trypsinized RBC.

RESULTS

Haemagglutination results after ammonium sulfate fractionation

The stepwise precipitation (20, 40, 60, 80 % saturation) with ammonium sulfate was carried out on the three species, in order to determine the appropriate degree of saturation with this salt that allows the total precipitation of the haemagglutinating activity. The results are shown in Table I, expressed as haemagglutination titer, which is the reciprocal of the highest protein fraction dilution employed still giving a visible agglutination.

Table I. Haemagglutination results after fractionated precipitation of proteins from the crude extract of each species with ammonium sulfate

Species	% of saturation with ammonium sulfate 0-20	20-40	40-60	60-80
Lupinus albus	32	16	64	64
L. angustifolius	4	16	32	16
L. mutabilis	32	16	32	64

In all cases, it was necessary to precipitate the extract with 80 % (saturation) ammonium sulfate to precipitate all the haemagglutinin activity; only then, no activity was found in any of the supernatant fractions. Thus, it was determined to continue working in the following experiments using the pellet after 80 % precipitation with ammonium sulfate.

Moreover, in all three lupin species, the imbibition water of seeds was found to be devoid of agglutinating activity (data not shown).

Influence of the partial digestion of blood cell membranes and assay temperature on the haemagglutinating activity of Lupinus species

Seeds of the three Lupinus species were analyzed to compare their haemagglutinating response towards trypsinized or non-trypsinized rabbit blood cells according to the process described above. An extract was precipitated with 80 % (saturation) ammonium sulfate and the pellet was redissolved and re-equilibrated by dialysis in saline containing Mn^{2+}, Ca^{2+} and Na-azide. The influence of temperature on haemagglutination was also tested, taking into account the results with some other leguminous lectins where temperature has a role in the lectin-binding process. These results are shown in Table II.

Table II. The influence of partial digestion of blood cell membranes and the assay temperature on the haemagglutination by lectins

Plant species	Trypsinized rabbit blood cells: Room temperature (*Titre)	Trypsinized rabbit blood cells: Cold room (4°C) (*Titre)	Non-Trypsinized rabbit blood cells: Room temperature (Titre)	Non-Trypsinized rabbit blood cells: Cold room (4°C) (Titre)
Lupinus albus	256	512	NAO**	2
L. mutabilis	512	256	8	8
L. angustifolius	16	16	2	2

*Titre: the highest dilution of sample (lectin) where haemagglutination may still be seen both visually and under the microscope
** No agglutination observed (NAO)

Inhibition of haemagglutination by haptens

In order to determine the carbohydrate-binding specificities of the haemagglutinins in each Lupinus species as well as their quantification, inhibition of the haemagglutination tests were carried out using different sugars and glycoproteins according to the procedure described in Materials and Methods. The results of this hapten survey are summarized in Tables III and IV.

Tables III. Sugar specificities in Lupinus species

Carbohydrates	*L. angustifolius* (mM)	*L. mutabilis* (mM)	*L. albus* (mM)
D(+) Galactose	0.195*	0.781	0.097
D(+) Galactosamine	50	-	25
N-Ac. Galactosamine	-	-	-
M-Galac. pyranoside**	12.5	25	12.5
D(+) Glucose	12.5	-	25
D(+) Glucosamine	50	-	25
N-Ac. Glucosamine	-	-	-
M-Glucopyranoside**	6.25	-	-
D(+) Mannose	3.125	-	-
D(+) Mannosamine	25	50	12.5
M-Mannopyranoside**	1.562	-	-
α-Lactose	-	-	-
β-Lactose	-	-	100
Melibiose	1.562	12.5	1.562
Raffinose	12.5	100	12.5

Table IV. Glycoprotein specificities in Lupinus species

Glycoprotein haptens	*L. antustifolius* ($\mu g.mL^{-1}$)	*L. mutabilis* ($\mu g.mL^{-1}$)	*L. albus* ($\mu g.mL^{-1}$)
Mucin (porcine stomach)	2.4*	2.4	0.6
Mucin (bovine salivary glands)	-	-	-
Asialomucin (BSG)	-	-	-
Fetuin (fetal claf serum)	2.4	1.2	0.6
Asialofetuin (fetal calf serum)	2.4	1.2	0.6
Ovalbumin (chick egg)	156	1250	19
Peroxidase	-	-	2500
Thyroglobulin	9.7	4.9	1.2

*: Sugar and glycoprotein specificities are shown as the minimal concentration needed for the inhibition of haemagglutination
-: Negative results (no inhibition of the haemaglutination):The maximal concentrations used were 100 mM and 2.5 $mg.mL^{-1}$ respectively for sugars and glycoproteins
**: methylated derivative

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. 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 (Peumans and Van Damme, 1996).

Haemagglutination results after ammonium sulfate protein fractionation

From our results, it is evident that haemagglutinin activity against rabbit erythrocytes was present in the three species tested and, in all cases, it was needed to add ammonium sulfate until 80 % saturation, in order to precipitate all the haemagglutinins as a whole despite they started to precipitate beginning at 20 % saturation.

However, in contrast to many other legumes, very low or no activity was found when the seeds were imbibed in water overnight and afterwards, for haemagglutinating activity a water sample was tested (imbibition water). Some authors suggest that the presence of lectins in the imbibition water could be related with embryo defense at the moment of germination when the seed takes up water for this purpose (Van Driessche, 1988).

Influence of the partial digestion of blood cell membranes and assay temperature on the haemagglutinating activity

At least in these three lupin species it is necessary, according to the results shown in Table II, to partially trypsinize the membranes of the rabbit blood cells to reach the highest titers of haemagglutinating activity; and, in fact, in the case of *Lupinus albus,* the difference between trypsinized and non-trypsinized blood seemed to be dramatic for the activity. When using trypsinized blood cells, it seems to be the species with the highest concentration of haemagglutinins, whereas *L.angustifolius* has the poorest haemagglutinin content.

Anyway, in all cases, blood trypsinization affected the haemagglutination titer. Apparently, some cryptic sugar groups appear at the membrane surface after a partial digestion with trypsin.

Results on the influence of the assay temperature on the haemagglutination titer are less convincing if we take into account that the range of experimental error in the haemagglutinin assay is about 20 %. Thus, differences of one well in the assay could be interpreted as not significant. We conclude that the temperature of the assay did not influence the haemagglutin titer observed.

Inhibition of the haemagglutination by haptens

The inhibition of haemagglutination by sugar and glycoprotein haptens showed, as it was expected, a carbohydrate specificity relation among three species of the same genus. According to the results shown in the Table III we can conclude that this genus belongs to the legume lectin group specific for galactose. Other members of this group are *Phaseolus* (*P.lunatus*), *Dolichos*, *Glycine* (Brewin and Kardailsky, 1997) and *Vigna* (*Vigna radiata*) (Suseelan, Bhatia and Mitra, 1997a).

Lectins belonging to these genera also have high specificity for N-acetyl galactosamine; however, in these experiments specificity for this sugar was not observed in any of the species tested. A similar behavior has been described in literature for the species *Vigna mungo,* which showed a high specificity for D-Galactose (0.1 mM) in contrast with a low specificity for N-acetyl-D-Galactosamine (100 mM) (Suseelan, Bhetia and Mitra, 1997b).

Results are in agreement with previous works demonstrating the high specificity of *L.albus* and *L.angustifolius* haemagglutinins for galactose (Kim and Madhusudhan (1988) and Van Nevel *et al.* (1998). So far, this is the first time that haemagglutinating activity and its specificity is reported for *L.mutabilis* haemagglutinin.

It is also worth to mention that, in all cases, the specificity for galactose was demonstrated not only with the monosaccharide but also for di and trisaccharides containing galactose in the structure, since the haemagglutination could be inhibited by Lactose, Melibiose and Raffinose. Similar results have been reported in other members of the leguminous family (Sharon and Lis, 1990).

Lupinus mutabilis, an Andean lupin species, showed a high specificity only for galactose. Other sugars do not inhibit agglutination or show inhibition just at high concentrations. *L. albus* and *L. angustifolius*, both European species but broadly extended all around the world (recently adapted to winter conditions in Cuba; Alvarez, García and Treto, 1998), seem to have a broader specificity spectrum and, in fact, they were inhibited at lower concentrations than *L. mutabilis* in the case where the three species share the sugar specificity.

Regarding inhibition by glycoproteins, the three species showed about the same behavior. *Lupinus albus* in some cases showed the highest specificity. Interestingly, inhibition of the haemagglutination was very remarkable with the three species against porcine stomach mucin whereas there was not inhibition while using salivary gland mucin, demonstrating differences in the carbohydrate decoration in equal proteins from different origins.

These results allow us to prepare suitable columns for the ulterior purification and characterization of haemagglutinins present in Lupin species.

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