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Isolation of a Hemagglutinating γ-conglutin from Seeds of the Andean Legume *Lupinus mutabilis* Sweet (Tarwi)

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ABSTRACT

Background: *Lupinus mutabilis* Sweet is a species of Andean legume with a high content of proteins, which present hemagglutinating lectins. Here, we demonstrated that the γ-conglutin lectin isolated from *L. mutabilis* seeds is responsible for the hemagglutinating activity by evaluating this activity at each step of the isolation process.

Methods: Seeds of three ecotypes of *L. mutabilis* were used. Saline protein extraction size exclusion and ionic exchange chromatography were performed to isolate the lectin. Carbohydrate specificity, thermostability and resistance to chelating and reducing agents of the lectin were tested. SDS-page and mass spectrometry were performed to characterize the isolated hemagglutinating lectin. Rabbit erythrocyte hemagglutination test was performed at each step.

Result: Patón Grande ecotype had higher hemagglutinating titers and therefore was selected for further purification steps. Hemagglutinating activity of the purified lectin, which was identified as a γ-conglutin, was cation-independent and optimal between 15-20°C. Besides, it resisted temperatures up to 70°C, its activity was lost in basic pH and remained active under reducing and chelating conditions.

Key words: Andean legume, Hemagglutinating ϒ-conglutin, Lectin purification, *Lupinus mutabilis*, Tarwi.

INTRODUCTION

Lupinus mutabilis, known as tarwi, is one of the main legumes cultivated by the Andean populations and the only lupin species domesticated in the Andes (Atchison *et al*., 2016). *Lupinus mutabilis* has a wide weather adaptability and its seeds have superior grain quality and a high protein content (44%) in comparison to the other three domesticated lupin species (*L. albus*, *L. angustifolius* and *L. luteus*), which have a protein content between 34-42% (Gulisano *et al*., 2019; Gulisano *et al*., 2022). This legume is a key genetic resource that has many traditional applications such as antiparasitic, analgesic, anti-inflammatory and the treatment of diabetes (Jacobsen and Mujica, 2006). Unfortunately, *L. mutabilis* breeding is decreasing in South American countries due to replacement by other crops and the inconvenient need to treat *L. mutabilis* seeds before human consumption in order to reduce alkaloid content (Jacobsen and Mujica 2006).

Lectins, or lectin-like proteins, are generally found in legume seeds, these proteins have multiple applications in biochemistry, molecular biology and medicine and their presence in *L. mutabilis* seeds could promote the use of this historically relegated plant. Lectins usually have hemagglutinating activity (HA) because they bind carbohydrates in the membrane of erythrocytes (Sharon and Lis, 2007). This activity has been demonstrated in the extract of mature seeds of *L. albus*, *L. angustifolius* and *L. mutabilis* (Falcon *et al.,* 2000a). However, after the purification of the lectin from the extract of *L. albus* by affinity chromatography using galactose, the HA was loss. This lectin was reported to be a non-hemagglutinating protein with a circular dichroism spectrum similar to ϒ-conglutin (Falcon *et al.,* 2000b).

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The ϒ-conglutin is a basic globulin conserved between lupin species and it usually represents 4-5% of the seed protein content (Duranti *et al.,* 2008). Duranti *et al.* (1995) reported a *L. albus* γ-conglutin with lectin-like activity and the ability to bind glycoproteins. Recently, an hemagglutinating γ-conglutin from *L. albus* was isolated (Grácio *et al.*, 2021), but its activity was not constant in different purified batches, indicating that the methodology was not ideal. The hemagglutinating activity (Schoeneberger *et al.,* 1982; Djabayan-Djibeyan *et al.,* 2022) and the affinity for galactose of seed extracts from *Lupinus mutabilis* was reported (Falcon, 2000b), but the purification protocols were not suitable and caused the loss of the hemagglutinating capacity (Falcón *et al*., 2000b).

Thus, the objectives of the study were to purify and characterize a lectin (ϒ-conglutin) from an extract of *Lupinus mutabilis* seeds and to show that ϒ-conglutin is responsible of the HA, evaluating this activity at each step of the process.

MATERIALS AND METHODS

Lupinus mutabilis Sweet seeds were provided by the Germplasm Bank of the Grain and Oilseed Legumes Program of the Universidad Nacional Agraria La Molina. Seeds of three ecotypes were characterized by its composition and agronomic characteristics according to parameters stablished by Camarena *et al.* (2013). The ecotypes were cultivated at multiple locations of Peru: Cholo Fuerte seeds (AC TLM 14) were collected in the Oncoycancha locality (10°24′00″S77°23′46″W), Vicos seeds (AC TLM 47) in Vicos peasant community (9°19′39.5″ S 77°33′8.1″W) and Patón Grande (AC TLM 12) in Otuzco (7°53′54″S 78°33′45″W).

Dry *Lupinus mutabilis* seeds were grinded and sifted. The obtained seed powder was defatted with n-Hexane by stirring for 2h, this step was performed three times. The fat-free powder was dried and incubated overnight in saline extraction solution (0.15 M NaCl, 20 mM MnCl2, 20 mM CaCl2) at 10°C. The aqueous phase was separated by centrifugation (12000 × g, 4°C, 15 min) and proteins were precipitated with ammonium sulphate at 80% saturation. The suspension was centrifuged (21150 × g, 4°C, 15 min) and the pellet resuspended in saline extraction solution and dialyzed against the same solution overnight at 4°C (Falcón *et al.,* 2000a, De Amat, 2016). Extracts were frozen (-20°C) and stored until its further use. This and the following procedures were performed in the Faculty of Biological Sciences of Universidad Nacional Mayor de San Marcos between 2015 and 2019.

Whole rabbit blood was washed with saline physiological solution and the erythrocytes pellet was incubated with 0.01% trypsin for 1 h at 37°C (Falcón *et al*., 2000a, De Amat, 2016). Trypsinized erythrocytes were washed three times with saline and resuspended in N-(2-Hydroxyethyl) piperazine N'-(2-ethanesulfonic Acid) (HEPES) 10 mM (pH 6.8, NaCl 0.15 M) buffer. For hemagglutination, 50 µL of samples of the extracts from the three ecotypes and 50 µL of erythrocytes were mixed in round bottom microplates and incubated at room temperature for 40 min.

Size exclusion chromatography was performed using HEPES 10 mM pH 6.8 (NaCl 0.15 M), which was the optimal buffer for purification. Initial tests were performed with Tris-HCl pH 8. Hemagglutinating fractions were submitted to anion exchange chromatography in DEAE-Sephadex (Life Sciences) in HEPES 10 mM pH 6.8 (and pH 7.2 in preliminary tests). Size exclusion peak I fractions were mixed and subjected to anion exchange chromatography in HEPES 10 mM pH 6.8. Size exclusion and anion exchange fractions were quantified by UV_{280} nm spectrophotometry and Bradford assay (Bradford 1976). HA was assessed following the methodology above described.

Sodium dodecyl sulphate-polyacrylamide electro phoresis (SDS-PAGE) (Laemmli, 1970) was performed under reducing and non-reducing conditions in 10% polyacrylamide gels at 100 V for 2 h. Gels were stained with Coomassie G-250 and the molecular weight was calculated by relative migration analysed in ImageJ (Schneider *et al.,* 2012).

SDS-page was performed in non-reducing conditions as above described. Gels were stained with Coomassie blue G-250 and the band of the isolated hemagglutinating protein was excised from the gel and sent to the Pasteur Institute of Montevideo (Uruguay) for the identification by Peptide Mass Fingerprinting (PMF). Excised band was digested with trypsin and subjected to Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (4800 MALDI TOF/TOF) (Pappin *et al.,* 1993). Obtained mass spectre was analysed in Mascot Software with the Swissprot database.

To perform the hemagglutination inhibition test the following carbohydrates were used: glucose, lactose, galactose, mannose and melibiose were diluted in HEPES 10 mM pH 6.8 buffer. 25 µL of carbohydrates were incubated with 25 µL of 4 hemagglutinating units of the isolated protein for 30 min at room temperature. Following, 50 µL of rabbit erythrocytes were added to the wells. Carbohydrates final concentrations in the well were 50, 10, 2, 0.4 and 0.08 mM.

Determination of the optimal temperature for hemagglutination tests was performed using 10 mM HEPES, pH 6.8 as previously mentioned. The hemagglutination plates containing the purified protein and the rabbit erythrocytes were incubated in temperatures between 4 and 60°C (4, 10, 14, 20, 23, 30, 37, 50 and 60°C) for 1 h. After incubation, the hemagglutinating titers were compared.

To assess the thermostability, the isolated protein obtained by anion exchange was incubated at temperatures between -20 and 90°C (-20, 0, 10, 23, 30, 50, 70 and 90°C) for 30 min. The samples were incubated at room temperature for 20 min and their HA was assessed as previously mentioned.

The demonstration of the effect of a reducing and chelating agent on the hemagglutinating protein was carried up by incubating it with final concentrations of 1, 5, 10 and 20 mM of β-mercaptoethanol and ethylenediamine tetraacetic acid (EDTA) in HEPES 10 mM pH 6.8 for 1 h. After this step, hemagglutination was assessed as previously detailed.

RESULTS AND DISCUSSION

Saline extraction and hemagglutinating protein isolation from seeds

Collected *Lupinus mutabilis* seeds ecotypes presented the phenotype described in literature (Camarena *et al.,* 2013) (Fig 1). Saline extraction from the seeds of the three ecotypes yielded yellow-coloured extracts with a pH between 5-6 and similar amounts of protein quantified by Bradford (100-200 mg protein per 100 g of seed). Extracts from the three ecotypes showed HA on rabbit erythrocytes, with Patón Grande ecotype (PG) presenting the highest hemagglutinating titer (rank of 16-32) in comparison to Cholo Fuerte and Vicos ecotypes (rank of 8-16). Then, PG ecotype was chosen for

the subsequent procedures. Falcon *et al*. (2000a) reported a hemagglutinating titer of 512 for *L. mutabilis* crude, which was higher than the overall titer from our ecotypes. However, not Falcon *et al*. (2000a) or other studies have compared HA between *L. mutabilis* ecotypes.

The size exclusion profiles for PG showed four peaks (Fig 2A). Proteins from peak I retained their HA when 10 mM HEPES buffer pH 6.8 (0.15 M NaCl) was used, while proteins from peaks II, III and IV did not show this activity (Fig 2A). Villamarin (2016) also found 4 peaks in the chromatographic profile using 2.5 mM phosphate buffer pH 7.6. In this study, the peak IV presented the highest hemagglutinating titer with human group O erythrocytes and the activity was probably caused by other lectins. Again, *L. mutabilis* ecotypes were not considered in this study. In our study, the first peak of the anion exchange chromatography showed HA (titer of 8), while no activity was found in the peaks with lower absorbance (Fig 2B).

Chromatographic profiles were similar when size exclusion was initially performed using Tris-HCl buffer pH 8 in the initial tests; however, the HA was completely lost (data not shown). When anion exchange was carried out at pH 7.2, the activity was reduced to 50%, with hemagglutinating titers of 4. Therefore, considering the two chromatographic methods applied, it is shown that the isolated lectin has better HA at lower pH (pH 6.8). The pH sensitivity contrasted with the high pH stability observed in lectin-like proteins or lectins from other legumes. For example, lectin from *Parkia biglobosa* showed optimal HA on rabbit erythrocytes between pH 6-8 and decreased at pH 9-10 (Silva *et al.,* 2012). Similarly, the *Dioclea lasiophylla* mannose-binding lectin has optimal agglutinating activity on rabbit erythrocyte at pH 8 (Pinto-Júnior *et al.,* 2013).

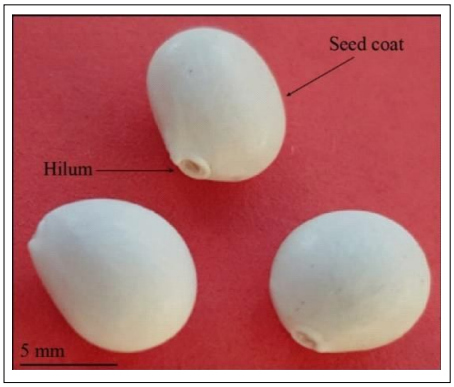

Fig 1: Characterization of *Lupinus mutabilis* Sweet Patón Grande ecotype seeds. This ecotype has oval, white and semi-glossy seeds. The weight of 100 dry seeds is 26.48 g; the average length and width is 9.0 and 6.0 mm, respectively. Figure designed and modified on Illustrator 2019.

Electrophoretic analysis

The extract contained multiple proteins (Fig 3A) that were lost during the purification procedure. The lectin isolated in HEPES pH 6.8 buffer had a molecular weight of 46 kDa in non-reducing conditions (Fig 3D). Under reducing condition, it was separated into two bands of 31 and 16 kDa (disulphide-bond subunits) (Fig 3E). Similar results were obtained by Santos *et al*. (1997) when analysing the γ-conglutin of *L. mutabilis* (Potosí and Inti cultivars), who identified a 42-43 kDa monomer under non-reducing conditions and two 30 and 18 kDa subunits under reducing conditions. Nadal *et al.,* (2011) found that the γ-conglutin from *L. albus* is formed by a 43 kDa monomer under non-reducing conditions and two subunits of 30 and 17 kDa under reducing conditions. It is important to mention that these authors did not perform hemagglutination tests. Thus, our purified lectin had the expected molecular weight of lupins γ-conglutin and differed from the small lectin (17 kDa) isolated from *L. mutabilis* extracts under different purification conditions (Villamarin, 2016).

γ-conglutin identification

The γ-conglutin isolated in the first peak of the anion exchange chromatography at pH 6.8 presented hemagglutinating titers of 8 (50 μg/mL) quantified by Bradford, equivalent to 0.31 μg of protein per hemagglutinating unit (HU) (2.5 μg

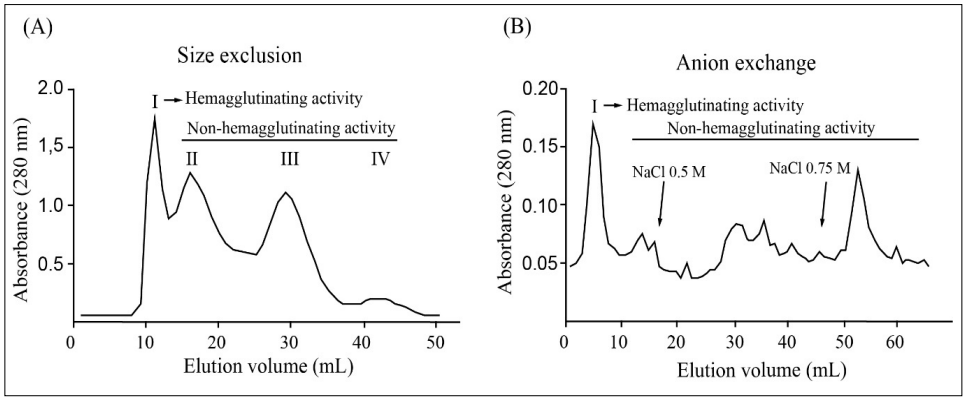

Fig 2: Chromatographic profiles for purification of *Lupinus mutabilis* Sweet lectin γ-conglutin (LMC). (A) Size exclusion chromatography profile. (B) Anion exchange chromatography profile. For both steps of purification peak I contained the LMC. Data was plotted in Prism vers. 7.

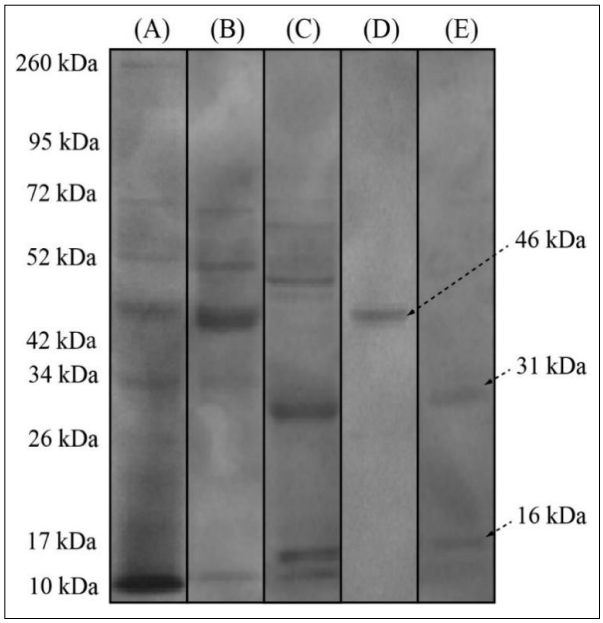

Fig 3: Electrophoresis SDS-PAGE of saline extract and the isolated *Lupinus mutabilis* Sweet lectin γ-conglutin (LMC). (A) Saline extract at non-reducing conditions. (B, C) Size exclusion hemagglutinating fractions (peak I) at non-reducing (B) and reducing conditions (C). (D, E) Purified LMC (46 kDa) at non-reducing (D) and reducing conditions (E) (31 kDa and 16 kDa subunits are observed). Migration analysed on ImageJ (Schneider *et al.* 2012). Figure designed on Illustrator 2019.

in 50 μg/L per well/titer of 8), while Grácio *et al*. (2021) found lower required amounts of protein per HU (0.14 μg) for *L. albus*.

Mascot analysis of the mass spectrometry result (MS/MS spectra) for the purified hemagglutinating protein identified the γ-conglutin from *Lupinus angustifolius* (Gene ID: 109345795) ($p<0.05$) by the peptide RQLEENLV VFDLAKS. The number of hits is limited by the lack of genomic information for *L. mutabilis*, but it was another evidence that, in conjunction with the results obtained in the electrophoretic analysis, showed that the purified lectin from *L. mutabilis* PG ecotype was a γ-conglutin lectin (LMC). The methodology used for its purification allowed to preserve its HA, unlike the isolation of γ-conglutin from *L. albus*, which lost its HA while maintaining its affinity for galactose (Falcon *et al.,* 2000b) and glycosylated polypeptides (Duranti *et al*., 1995). Grácio *et al*. (2021) isolated γ-conglutin from *L. albus* at pH 7.0 preserving its HA; although, it was not constant in different purification batches. These findings indicate that pH is an important factor in maintaining the hemagglutinating capacity of lupins γ-conglutin.

Inhibition of hemagglutination with carbohydrates

The HA of LMC was inhibited by 2 mM galactose and 50 mM melibiose (disaccharide containing galactose and glucose) (Fig 4A). None of the other assessed carbohydrates (glucose, mannose, lactose) inhibited hemagglutination. Rabbit erythrocytes have a high abundance of galactose terminal residues (Yamakawa, 2005), which explain the hemagglutination by LMC. Therefore, the isolated protein

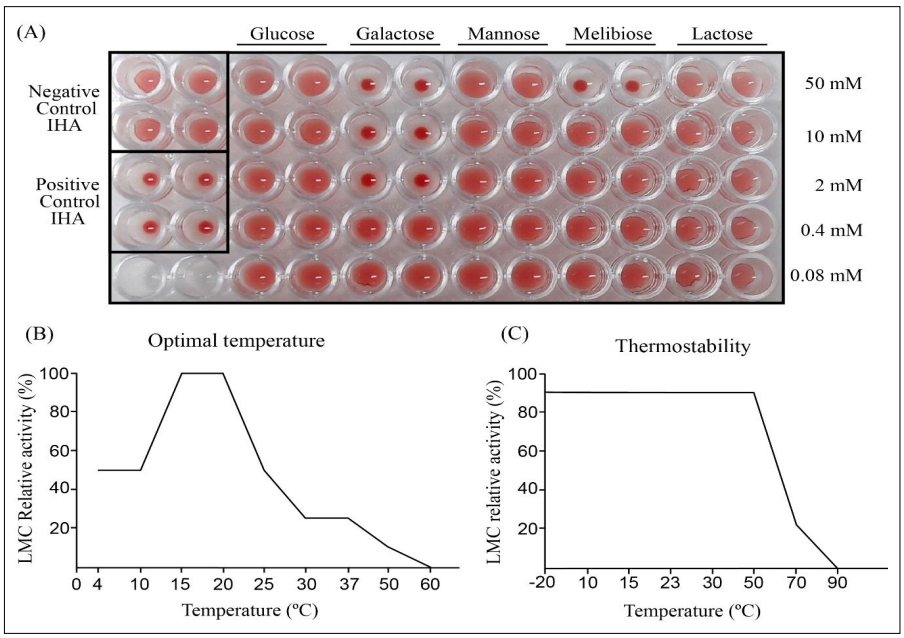

Fig 4: Characterization of the hemagglutinating activity of *Lupinus mutabilis* Sweet lectin γ-conglutin (LMC). (A) Inhibition of hemagglutination (IHA) with monosaccharides (Glu, Gal) and disaccharides (Man, Mel, Lac). Negative control: LMC + rabbit erythrocytes. Positive control: rabbit erythrocytes + HEPES buffer. (B) Optimal hemagglutinating temperature of the isolated LMC (15-20°C). (C) Thermostability of the isolated LMC. Data was plotted in Prism vers. 7.

has specificity for galactose, as previously found for *L. mutabilis* extracts (Falcón *et al.,* 2000a). Villamarin (2016) reported the inhibition of hemagglutinating activity (IHA) of human erythrocytes for mannose and glucose; besides, the IHA was negative for galactose, differing from our results and possibly due to the extraction method used by the author, that yielded other hemagglutinating lectin.

Optimal hemagglutination temperature and thermostability

LMC showed optimal HA at temperatures between 15 and 20°C (Fig 4B). At 60°C, HA was completely depleted while lower temperatures did not inhibit hemagglutination. In the case of legume lectins, hemagglutinating activities are diverse. For example, the optimal HA of the *Sophora japonica* lectin is near 0°C, while the *Canavalia ensiformis* lectin (concanavalin A) has optimal activity at 35°C (Gilboa-Garber and Sudakevitz 1999).

HA was totally preserved after short term incubation (30 min) at temperatures between -20°C and 50°C, was dramatically reduced after incubation at 70ºC and lost after incubation at 90°C (Fig 4C). A similar thermostability was found for the 17 kDa lectin isolated from *L. mutabilis* (Villamarin, 2016), although temperatures below 37°C were not assessed. Other legume lectins, such as the *P. biglobosa* lectin, retain their HA up to 50°C (Silva *et al.,* 2013) while the *D. lasiophylla* and black turtle bean lectins retain their HA up to 70°C (Pinto-Junior *et al.,* 2013; He *et al.,* 2014) and those of *P. vulgaris* up to 80°C (Sharma *et al.,* 2009).

Hemagglutinating activity of LMC is cation-independent and resistant to reducing conditions

LMC was not inhibited by β-mercaptoethanol or EDTA at the concentrations tested (1-20 mM). Legume lectins HA can be cation dependent or independent, as it has been seen in the lectins from *C. ensiformis* and *Vataiera guianensis*, respectively (Sharon and Lis 2007; Silva *et al.,* 2012). In cation-dependent lectins, such as those from *P. vulgaris* var. Jade, the addition of EDTA at concentrations as low as 10 mM can almost completely deplete the HA (Cheung *et al.,* 2013). Unlike our findings, Villamarin (2016) reported that their *L. mutabilis* lectin lost its HA on human erythrocytes (A and AB type) after incubation with EDTA (25 mM). To our knowledge, there are no reports of HA under reducing conditions; however, the fact that LMC hemagglutinates under these conditions suggests that only one of the γ-conglutin subunits exerts the HA.

CONCLUSION

In this study, we successfully isolated and characterized a γ-conglutin from the seeds of *Lupinus mutabilis* Sweet ecotype Patón Grande. The isolated γ-conglutin has a molecular weight of 46 kDa, is formed by two subunits and has affinity for galactose. The used methodology allowed it to preserve its hemagglutinating activity, verified at each step of the isolation process. We consider that this methodology is an important contribution for the purification of γ-conglutin from *L. mutabilis* and can be scaled up to a semi-industrial level.

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Conflict of interest: None.

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