

# ISOLATION AND PURITY ASSESSMENT OF LUPIN ALBUS CONGLUTINS

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## ABSTRACT

An increasing demand of *Lupinus* proteins for feeding as well as the need of isolating large quantities of pure conglutin- $\gamma$  for nutraceutical purposes prompted us to search for adequate scale preparative and analytical procedures. *L. albus* seeds were dehulled, finely ground and defatted with hexane. Being careful of performing all steps of the procedure at 4°C and centrifuging always at 5000 rpm we performed water extraction of the defatted flour, pooled all water supernatants for vacuum drying and labelled this powder as *albumins*. Pellets were dissolved in 10% NaCl solution (3X). Their pooled supernatants were mixed with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to reach 85% saturation. After centrifugation *globulins* were dissolved in 0.15 M Na<sub>2</sub>HPO<sub>4</sub>/0.15 M NaH<sub>2</sub>PO<sub>4</sub> buffer, pH 6.8 and dialyzed against 0.2M acetate buffer, pH 4.8 to isolate  $\alpha$ -conglutin. The dialyzed supernatant against water during 48 hours was centrifuged to obtain  $\beta$ -conglutin, and the crude conglutins- $\gamma$  in the supernatant. The latter was suspended in 0.5 M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> buffer, pH 7 and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 50% saturation to obtain conglutin- $\gamma$  in the precipitate. All freeze dried protein fractions were SDS-PAGE and PAGE separated using 17 to 250 Kda reference proteins as: total proteins, globulins,  $\alpha$ ,  $\beta$ , and conglutin- $\gamma$ . Portions of each sample were analysed for their aminoacid content. Solubilised proteins either in phosphate buffer or acetonitrile:TFA:water mixture were separated through a 300SB-C3, 9.4 mm x 25 cm Zorbax column for a reverse phase HPLC while performing TFA water and acetonitrile gradient in order to obtain each protein fraction molecular weight and ascertain  $\gamma$ -conglutin presence by recording the resulting fractions in a Matrix-assisted laser-desorption ionisation mass spectrometer (MALDITOF-MS) to ascertain fractional molecular weights. The  $\alpha$ , and  $\beta$  fractions contained conglutin- $\gamma$  (46 KDa) and the latter still requires further purification.

## KEY WORDS

*Lupinus albus*, protein isolates, Conglutin- $\gamma$  isolation, conglutins, methods

## INTRODUCTION

An increasing demand of *Lupinus* proteins for feeding through products for human consumption as a replacement for soybean proteins as well as the need of isolating large quantities of pure conglutin- $\gamma$  for nutraceutical purposes, requires, adequate analytical and scale preparative procedures (D'Agostina, A., 2006). The reported polypeptide heterogeneity, post-traslational processing, physical size, heterogenous disulphide linking, constitute intrinsic difficulties that might explain the scarce solubility encountered for these conglutins, in particular for conglutin- $\gamma$ . On the other hand, the storage globulins which make up the bulk of the protein of *L. albus* seeds contain almost the recommended level of most of the essential amino acids: met, lys, asn, asp, gln, glu, whose molar concentrations were found present either equal or over levels in human serum albumin and those of FAO reported requirements, particularly in protein fractions precipitated at pH 5.5 where the conglutin- $\gamma$  is more likely to be isolated than other pH values (Garzon-de la Mora *et al.* 2008). Therefore, the aim of this work was to analyze the purity of attained fractions of  $\alpha$ ,  $\beta$  and conglutins- $\gamma$  following salt extraction and precipitation, applying two additional proteomic procedures. An additional purpose was to compare the proteomic rapid methods of protein separations and molecular mass ascertainment of the conglutins isolated through salt extraction and dialysis method with the classic techniques in order to investigate the quality of isolated conglutin- $\gamma$ .

## MATERIALS AND METHODS

### REAGENTS AND CHEMICALS

NaCl, NaOH, Na<sub>2</sub>HPO<sub>4</sub>, NaH<sub>2</sub>PO<sub>4</sub>, 2H<sub>2</sub>O, sodium azide, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, N-hexane, HPLC grade acetonitrile, sodium acetate and potassium acetate were analytical grade reagents obtained from Sigma (Sigma-Aldrich St Louis MI.). Reagents for derivatisation, OPA-3-MPA, FMOc, borate buffer and amino acid standards (10, 25, 100, 250 pmol) were supplied by Hewlett-Packard (AA reagent kit high sensitivity No. 5061-3347), and diluted with 0.1 M hydrochloric acid by a factor of 1:10. THF (silylation grade) was obtained from Pierce (Rodgau, F.R.G.). All the reagents and samples were stored at 4°C.

## PLANT MATERIAL

*Lupinus albus* seeds were donated by E. van Santen (Auburn University, USA). After dehulling, *L. albus* seeds were ground into a fine flour using a homemade grinder (JNF\_2006) such that the flour passed through a 0.5 mm mesh sieve. The fat was removed from the flour for 9 h in hexane using a Soxhlet distillation equipment at 40°C until less than 5% of the fat remained. This flour was then stored within a drying chamber at 4°C until use.

## GLOBULIN RECOVERY

The globulin fraction was recovered, from the albumin isolate, by solubilisation in NaCl and precipitation with  $(\text{NH}_4)_2\text{SO}_4$ . The albumin fraction was washed and centrifuged 10 min at 5000 rpm and 4°C three times with 10% NaCl salt. After each extraction, the supernatant was decanted and saved. Enough  $(\text{NH}_4)_2\text{SO}_4$  was added to the pooled supernatants until an 85% saturated solution was reached and the slurry centrifuged for 10 min at 5000 rpm. The supernatant was decanted and the precipitate, containing the globulin fraction, recovered.

## CONGLUTIN- $\alpha$ RECOVERY

Conglutin- $\alpha$  fraction was recovered from globulins using 85% saturation  $(\text{NH}_4)_2\text{SO}_4$ . Then, after centrifugation, pellets were dissolved in 0.02% sodium azide, 0.15 M  $\text{Na}_2\text{HPO}_4/0.15$  M  $\text{NaH}_2\text{PO}_4$  buffer, pH 6.8 and dialyzed three times against 0.2M acetate buffer, pH 4.8. After centrifugation, pooled supernatants were mixed with to isolate other conglutinins from  $\alpha$ -conglutinins devoid globulins.

## CONGLUTIN- $\beta$ AND CONGLUTIN $\Gamma$ RECOVERY

The above supernatant was dialysed against water for 48 hours, and centrifuged to obtain conglutin- $\beta$ , and the crude conglutin- $\gamma$  in the supernatant. The latter was suspended in 0.5 M  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  buffer, pH 7 and  $(\text{NH}_4)_2\text{SO}_4$  was added to 50% saturation to obtain conglutin- $\gamma$  in the precipitate. Each precipitate was freeze-dried at  $-50^\circ\text{C}$  and  $13 \times 10^{-3}$  mbar, weighed and stored for further proteins studies (Blagrove, R.J. *et al.* 1980).

## AMINO ACID ANALYSIS

Each protein sample (5 mg) was hydrolysed under a vacuum for 24 h at  $108^\circ\text{C}$  with 6 M HCl and then freeze-dried before the amino acid content was analysed on a Beckman 120C amino acid analyser. The analysis of primary and secondary amino acids was carried out by the automated pre-column derivatisation technique in which the amino acids were derivatised in a two-step reaction using OPA for the primary amino acids and FMOC for the secondary amino acids. The derivatisation is performed within the injection cycle. A Hewlett-Packard HP 1090 Series M liquid chromatograph fitted with a DR5 solvent delivery

system, a variable volume autoinjector, a cooled autosampler (4°C), a temperature controlled column compartment, a solvent-preheating device and a 5  $\mu\text{m}$  Hypersil ODS (20 cm x 2.1 mm I.D.) were used. The amino acid analysis of the protein hydrolysate was performed with a diode array detector at a wavelength 338 nm for primary and 266 nm for secondary amino acids. For trace levels in the low picomole range, an HP 1046A programmable fluorescence detector was used with excitation and emission wavelengths of 230 and 455 nm for the primary amino acids. This detector was switched to 266 and 310 nm for secondary amino acids (Schuster, R., 1988).

## REVERSE PHASE HPLC

Ten mg of *L. albus* globulins, albumins, conglutinins  $\alpha$ ,  $\beta$ , and  $\gamma$  samples were solubilised in 0.25 M  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  buffer at pH 8.5, sonicated for 5 min. Turbid samples were centrifuged at 3,000 rpm to remove the sediment and the supernatant was collected for protein measurement (Bradford) and HPLC separation of the proteins. The latter was carried out using a 9.4 mm x 25 cm Zorbax analytical column, equipped with a 0.5 mL extension loop attached to a Beckman System Gold with a 126 programmable solvent module and a 166 programmable detector module, as well as a uv detector set at 280 nm. The column was eluted with 10 mM TFA in HPLC water (A) and 10 mM TFA in acetonitrile (B) gradients at a flow rate of 3.5 mL/min starting with a gradient from 10 to 60 or 70% acetonitrile in water (with 10 mM TFA in both solvents) over about 30 minutes followed by ramping to 95% to remove more hydrophobic components from the column. After finding where the proteins of interest eluted, the gradient was modified so that the acetonitrile concentration increased at a rate of about 1% per minute, and the concentration at the start and at the end of the gradient was adjusted to optimise separation of the proteins of interest. Protein fractions were solubilised in a solvent mixture containing 300  $\mu\text{L}$  of HPLC water, 100  $\mu\text{L}$  acetonitrile, 5  $\mu\text{L}$  trifluoroacetic acid and 30% acetonitrile in 10 mM trifluoroacetic acid (TFA).

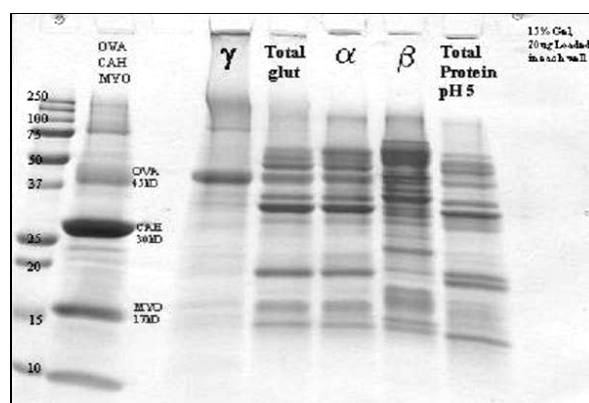


Fig. 1. Denatured gel (conglutinins).

Although the 45 Kda enolase does not identify conglutin- $\gamma$ , as occurred in size exclusion chromatography, we used it as a molecule that might migrate close to conglutin- $\gamma$ . Once the protein profiles were recorded and the fractions collected, an aliquot was prepared for mass spectrometry analysis (Szepesi, 1992).

#### SDS-PAGE AND PAGE

SDS-PAGE and PAGE was used to determine the molecular weight and to separate proteins present in the total protein, globulins, and *conglutins* ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) fractions. SDS-PAGE and PAGE were carried out on 12 and 15% polyacrylamide gels in Tris-HCl buffer, respectively. A standard kit (Biorad prestained standard Catalog #161-0318) containing proteins with molecular weights ranging from 17 KDa to 250 Kda,) was used for the calibration. Electrophoretic separations were performed using a Minigel Protean II system (BioRad). Enough lyophilised protein sample to achieve a protein concentration of 0.9 to 1.2  $\mu\text{g}/\mu\text{L}$ , was re-suspended in 5 mL 1X PBS, pH 7.7 buffer and vortexed vigorously until fully dissolved. Solubilisation of conglutin- $\gamma$  sample required the addition of 1.0 mM NaOH and extensive vortexing and sonication in order to achieve fully soluble stock of 1  $\mu\text{g}/\mu\text{L}$ .

After the samples were solubilised, they were loaded in the gel as follows: 5  $\mu\text{L}$  of a commercial Molecular Weight Standard was loaded in lane 1. Three purified protein standards, ovalbumin 45 KDa, Carbonic anhydrase 30 KDa, Myoglobin 17KDa, were also prepared with native sample buffer and run on lane 2; an equal protein concentration (10  $\mu\text{g}/\mu\text{L}$ ) of the conglutin- $\gamma$ , total globulins,  $\alpha$ -conglutins,  $\beta$ -conglutins fractions and the total protein pH 5.0 precipitate was loaded on lanes 3 to 7, respectively. PAGE electrophoresis was carried out using 20  $\mu\text{g}$  of each sample dissolved in 2X native sample buffer containing no SDS, neither a denaturant nor boiling, and loaded onto 15% Tris-glycine gel. Samples were run under constant voltage until dye front migrated off of the gel using tris-glycine buffer without SDS (30 mM Tris/192 mM glycine). Coomassie stained gel overnight (Biorad biosafe gel stain, Cat # 161-0786). Gels were washed three times with 50% acetonitrile/50 mM Tris-HCl, pH 8.0, for 15 min to remove the Coomassie blue dye, then soaked in 100% acetonitrile for 5 min. Gels were digitalised with a Studio Scan II scanner (Agfa) and quantitatively processed with Cream (Kem- En-Tec, Copenhagen, Denmark) software (Costa, J. *et al.* 2005).

#### MASS SPECTROSCOPY

Positive ion mass spectra were obtained by matrix-assisted laser desorption/ionisation delayed extraction time-of-flight (MALDI-TOF) mass spectrometry using a Voyager DE STR Biospectrometry Work Station (Foster City, CA). The spectrometer was operating in reflector mode using the manufacturer's default settings. The nitrogen laser excitation frequency was set at 3 Hz

and the laser power was optimised to obtain a good signal-to-noise ratio after averaging 200–400 single-shot spectra. Mass calibrations were performed over several m/z ranges, using a ProteoMass TM Peptide Protein MALDIMS Calibration Kit (Sigma-Aldrich, USA). Each sample obtained from each peak, or two globulins isolated from *L. albus* at different pHs, were separated in the reverse phase HPLC or size exclusion HPLC after mixing with 100  $\mu\text{L}$  of a solvent mixture of 30% acetonitrile containing 0.1% trifluoroacetic acid (TFA).

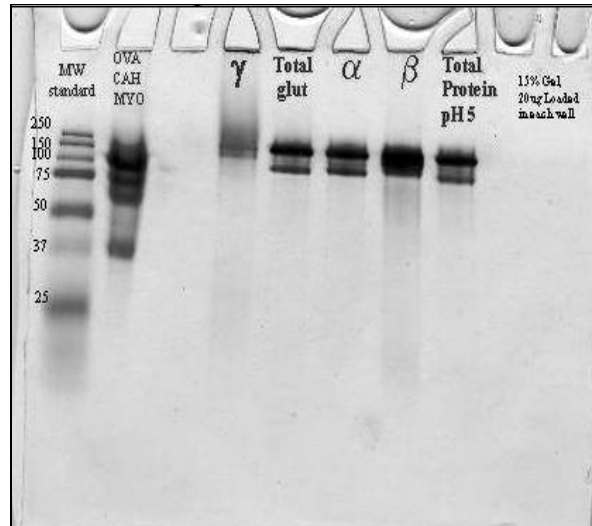


Fig. 2. Native PAGE.

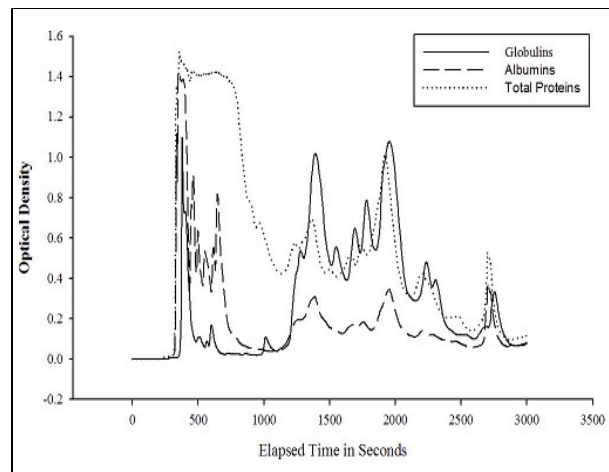


Fig. 3. *Lupinus albus* proteins, RPHPLC.

The sample solution (1  $\mu\text{L}$ ) was mixed with 1  $\mu\text{L}$  of a 10  $\mu\text{g}/\mu\text{L}$  solution of 2,5-dihydroxy benzoic acid (DHB) in TFA, and this preparation (1.4  $\mu\text{L}$ ) was placed onto a MALDI-sample plate. The enolase migrating peak of each fraction was dried under  $\text{N}_2$  and layered to record the molecular weight of each fraction by Matrix-assisted laser-desorption ionisation (MALDI-TOF-MS) analysis of the recovered 46 KDa migrating enolase fraction (Lin, D. *et al.* 2003).

## RESULTS AND DISCUSSION

Table 1 shows the amino acid profile of *L. albus* native protein, albumins, globulins and conglutins. These results confirm the poor methionine content of the lupin seed major proteins, while the discovery of conglutin- $\gamma$  displays a favorable imbalance that resembles the increase found in globulins isolated at pH values of 5.0, 5.5 and 6.0 (Garzon-de la Mora *et al.* 2008) where this conglutin demonstrated to be less soluble than remaining globulins and globulin fractions. Differences in essential amino acid content among conglutin and globulin fractions are observed. In fact, the over control values of essential amino acids in conglutin- $\gamma$ , allow us to think of genetic modifications to improve the amount of this fraction to equilibrate total sulfur and essential amino acids in this crop. Ovoalbumin 45 KDa, Carbonic anhidrase 30 KDa, Myoglobin 17 KDa standards allowed us to distinguish the 46 KDa conglutin- $\gamma$  run on lane that should not be present in  $\alpha$  and  $\beta$ -conglutins, as expected in TP and Globulins. Therefore, the reported separation sequence following an important conglutins separation method (Blagrove, R.J. 1980) still preserve the total globulins pattern in the  $\alpha$  and  $\beta$ -conglutins fractions; however conglutin- $\gamma$  was almost purely separated at the pH and Ip that correspond to the one unit left of the plateau where the phosphate buffer display a pK of 6.8 and the maximal acetate buffer pK value whose right deviation from pK correspond to 5.8.

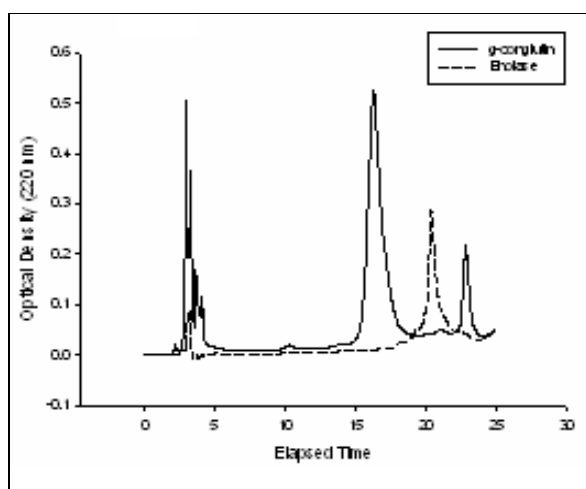


Fig. 4. Gama conglutin RPHPLC.

The 17 Kda subunit but not the 30 Kda subunit is distinguishable in all protein fractions except in conglutin- $\gamma$  despite of denaturing conditions where only the 46 Kda appear (Fig.1). In fact, although conglutin- $\gamma$  required alkalisation to enhance solubility, only the 46 KDa band and a normal resolution of the major globulin components were observed. Finally, the native PAGE did not reveal differences among loaded proteins (Fig. 2). Although the globulins are large and usually move very slowly in polyacrylamide gels, it was not necessary to increase ionic strength (I) neither gel

porosity nor the running time to control the state of subunits association and dissociation as reported elsewhere (Marcote, M.F. 1999). In the 7S group of seed storage globulins, the glycoprotein of 46 KDa named conglutin- $\gamma$  accounts for about 5-6% of the total globulins, and is composed of two disulphide bridged subunits of 29 and 17 kDa (Blagrove R.J. *et al.* 1980). Although, solubilisation difficulties were observed when conglutin- $\gamma$  fraction was dissolved in a mixture of water, trifluoroacetic acid and acetonitrile before reverse phase HPLC gradient partitioning using a 10 mM TFA in HPLC water and 10 mM TFA in acetonitrile at a flow rate of 3.5 mL/min.,  $\alpha$  and  $\beta$  conglutins were more soluble (Fig.3).

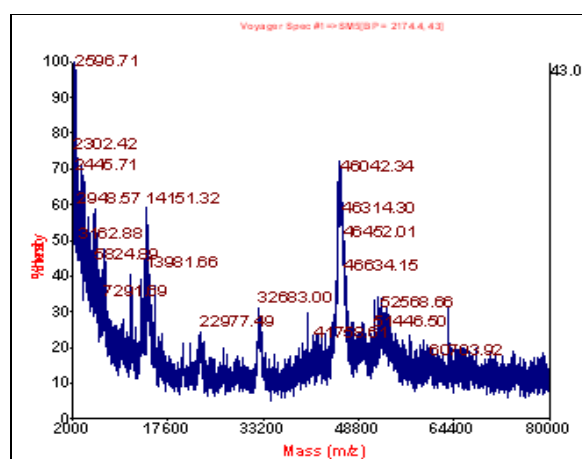


Fig. 5. RPHPLC of *L. albus* conglutin- $\gamma$ .

Therefore, we also dissolved conglutins in 3% NaCl in 0.25 M  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  buffer pH 9.0 and run them through the reverse phase column using a 0.25 M  $\text{PO}_4$  buffer, pH in 0.25 M  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  buffer at pH 8.5 at similar flow rate. It is possible that TFA might have induced a conglutin- $\gamma$  protonation state affecting water:TFA: acetonitrile solubilisation. On the other hand, because the separation of conglutin- $\gamma$  is achieved at high acetonitrile concentration while running the gradient low polar interactions between the hydrophobic support and the proteins might have occurred. An increase of ionic strength and pH was necessary to solubilise conglutin- $\gamma$  until a 0.25 M  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  buffer at pH 8.5 at a similar flow rate was enough to clearly separate this protein that we recommend for quality control during conglutin- $\gamma$  isolation.

Each protein fraction molecular weight from the conglutin- $\gamma$  RPHPLC separated peaks (Fig. 4) was recorded in a Matrix-assisted laser-desorption ionisation mass spectrometer (MALDI TOF-MS) in order to ascertain fractional molecular weights (Fig. 5). The  $\alpha$ , and  $\beta$  fractions contained  $\gamma$ -conglutin (46 KDa) and the latter did not require further purification. The reported amino acid content of essential and sulfur containing

amino acids of conglutin- $\gamma$  is similar to the composition of the isolated globulins at pH 5.5 and 6.0 (Table 1), suggesting that an amino amino acid improvement in lupin seed could be achieved through genetical means.

**Table 1.** Aminogram of *L. albus* proteins.

Amino acid	Albumins	Globulins	Conglutin-		
	mole%	mole%	$\alpha$ mole%	$\beta$ mole%	$\gamma$ mole%
Asx	11	9.9	10.2	9.6	9.5
Glx	24.8	27.6	12.6	26.5	20.9
Ser	7.4	7.1	6.9	5.1	7.4
His	2.6	3.5	2.9		1
Gly	9	6.8	9.6	7.5	9.5
Thr	6.1	4	5.9		4.5
Ala	6.9	4.4	8.5	4.2	5.7
Arg	4.9	7.5	5.7	7.7	5.8
Tyr	3.1	2.5	2.3		1.8
Val	4.6	3.5	6.6	4.1	4.9
Met	0.3		1		0.5
Phe	2.5	2.6	3.9	5.9	3.9
Ile	4.2	4.5	5.4	10.1	5.9
Leu	6.8	7.4	8.8	7.5	7.9
Lys	2.4	4.7	5.4		5
Pro	3.6	3.9	4.2	11.8	5.7

This particular composition, together with a different amino acid sequence when compared to other legume proteins have elicited research on divalent metal ions binding, protein cleavage during and after seed germination, in vitro proteolysis resistance and isolating procedures that follows proteomic strategies and allow us to know better analytical and preparative conditions in order to continue our ongoing pre-clinical and clinical assays with purified globulins and conglutin- $\gamma$  as well.

## LITERATURE CITED

- D'Agostina, A., C. Antonioni, D. Resta, A. Arnoldi, J. Bez, U. Knauf and A. Wäsche. 2006. Optimisation of a pilot-scale process for producing lupin protein isolates with valuable technological properties and minimum thermal damage. *J Agric Food Chem.* 54(1): 92.
- Garzón-de la Mora, P., G. Avalos-Alcantara, J.R. Villafán-Bernal, E.A. Maciel Hernández, C. Gurrola-Díaz, J. López, D.C. Brune, J.S. García-López, P.M. García-López and M. Ruiz-López. 2008. Chemicalphysical Properties Of Globulins And  $\Gamma$ - Conglutins Isolated At Different pH Values From Lupin Albus. 12th Internat. Lupin Conf.; Fremantle, Western Australia, 14-18 Sept.
- Blagrove, R.J., J.M. Gillespie, G.G. Lilley and E.F. Woods. 1980. Physicochemical studies on conglutin- $\gamma$ , a storage globulin from seeds of *Lupinus angustifolius*. *Aust. J. Plant Physiol.* 7:1.
- Schuster, R. 1988. Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatisation and high-performance liquid chromatography. *J. Chromatogr.* 431(2): 271.
- Szepesi, G. 1992. How to use Reverse-Phase HPLC, VCH Publishers, N.Y.
- Barth, H.G., B.E. Boyes and C. Jackson. 1994. Size exclusion chromatography. *Anal. Chem.* 66: 595R.
- Costa, J., D.A. Ashford and C.P. Ricardo. 2005. One- and two-dimensional electrophoretic identification of IgE-binding polypeptides of *Lupinus albus* and other legume seeds. *J. Agric. Food Chem.* 53: 4567.
- Lin, D., D.L. Tabb and J.R. Yates III. 2003. Large-scale protein identification using mass spectrometry. *Biochimica et Biophysica Acta.* 1646: 1.
- Joubert, F.J. 1956. Lupin seed proteins. 111. A physicochemical study of the proteins from white lupin seed (*Lupinus albus*). *Biochim. Biophys. Acta.* 19: 172.
- Marcote, M.F. 1999. Biochemical and biophysical properties of plant storage proteins: a current understanding with emphasis on 11S seed globulins. *Food Research International* 32: 79.