

***L. ANGUSTIFOLIUS* VS *L. ALBUS*: A COMBINED CHROMATOGRAPHIC AND ELECTROPHORETIC ANALYSIS TO HIGHLIGHT THE DIFFERENCES IN PROTEIN PROFILE**

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ABSTRACT

Lupin proteins are gaining attention from industry because of their possible role in the prevention of cardiovascular disease. With Australian *Lupinus angustifolius* now available in the European market, it is important to determine whether the characteristics of its main globulin proteins differ from those of *Lupinus albus*, more commonly used in Europe.

In order to characterise the proteins of both *L. albus* and *L. angustifolius*, three varieties of each lupin species were analysed via anion-exchange chromatography and bidimensional electrophoresis, with HPLC-Chip-MS/MS also being used as a tool to confirm the identity of gel spots. New and interesting results have helped to improve understanding on the complexity of the protein families present in lupin seeds. 2D screening revealed noteworthy differences in all the globulin proteins. The anion-exchange chromatography pointed out the differences in the ratio of vicilin/legumin peak area, with *L. angustifolius* having an approximate peak area ratio of 1:2, and *L. albus* 1:1.

In conclusion, highlighting the differences in protein composition between these two lupin species could help to understand differences in their bioactivity and allergenic response.

KEYWORDS

Lupinus albus, *Lupinus angustifolius*, protein heterogeneity, 2D-E, anion exchange chromatography

INTRODUCTION

Recently there is much attention to the use of lupin as a food or food ingredient because of its nutritional properties, the lack of antinutritional factors and the presence of specific bioactive components. Initially its flour, rich in proteins, was being used as a supplement in bread, replacing as much as 10% of the wheat flour [Kanny *et al.* 2000]. Later on, with concerns over genetically-modified organisms (GMO) lupin was heralded as a GMO-free replacement for soybean [Leduc *et al.* 2002]. There is rising evidence showing that sweet lupin proteins may have a hypocholesterolemic effect [Martins *et al.* 2005; Sirtori

et al. 2004; Rahaman *et al.* 1996] similar to that of soybean and other legumes. Moreover, its content of anti-nutritional components is lower than that of other legumes [Peterson *et al.* 1997], thus making it a favourable replacement for soybean in nutraceutical applications.

However, lupin could provoke severe allergic reactions and in 2007 was declared as a new food allergen, and was officially added to the EU list of known allergens, obliging food producers to label its presence, even in traces (Commission Directive 2006/142/EC).

It is interesting to note that in the literature most of the studies on lupin bioactivity and allergenicity used the species *Lupinus albus* [Moneret-Vautrin *et al.* 1999; Moreno-Ancillo *et al.* 2005; Peeters *et al.* 2005; Crespo *et al.* 2001], that is traditionally found in Mediterranean areas. However, because blue lupins from Australia (*L. angustifolius*) are now arriving on the European market it should be important to determine if their proteins possess the same properties as those of *L. albus*. Results presented in this paper provide a first step in that direction.

MATERIALS AND METHODS

TOTAL PROTEIN EXTRACT (TPE)

The defatted lupin flour was extracted with extraction buffer (100 mM Tris-HCl, pH 8.2, 0.5 M NaCl) for 2 hrs at room temperature (RT), with gentle stirring. The slurry was centrifuged at 10,000 rpm, 4°C for 20 min and the extracted proteins present in the supernatant were dialysed to eliminate the salt. The protein content was determined by the colorimetric method of Bradford [Bradford, 1976].

PROTEINS PURIFICATION VIA ANION EXCHANGE CHROMATOGRAPHY

The α -, β - and γ -conglutins were purified according to the method described by Dooper *et al.* 2007. In brief, 4 mL of the TPE was filtered through sterile 0.2 μ m membrane filters, (Econofilter, Agilent Technologies) and loaded onto a DEAE Sepharose Fast Flow column (1.6 cm, 5 mL volume; GE Healthcare, Sweden). The proteins were eluted with a linear salt gradient (0-1 M NaCl) in 100 mM Tris-HCl, pH 8.2. The fractions were

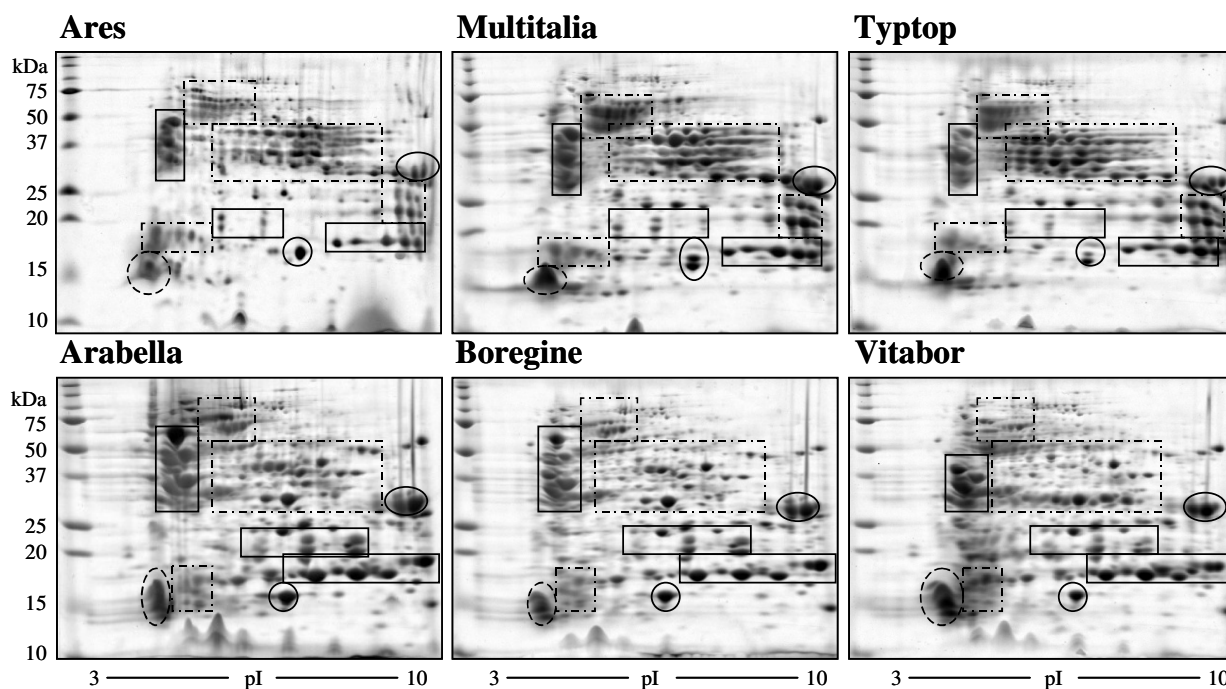


Figure 1. 2D-gels of TPE from different cultivar of *L. albus* (upper gels) and *L. angustifolius* (lower gels). Standard markers are indicated (in kDa) on the left. Solid line rectangles (—) indicate the acidic and basic subunits of α -conglutinin. Dash-dotted rectangles (---) indicate β -conglutinin. The dotted oval (---) highlights the heavy chain of δ -conglutinin. The solid line ovals enclose the large and small subunits of γ -conglutinin.

collected and stored at -20°C . The identity of the column fractions were confirmed by MS/MS analysis (as explained below).

2D-E electrophoresis. One hundred μg of TPE and 40 μg of purified proteins fractions were diluted in 150 μL of IEF solubilisation buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM Tris-HCl pH 8.8, 0.1 mM EDTA); the proteins were reduced with 10 mM DTT for 1 hr at RT and alkylated with 20 mM IAM for 1 hr at RT. Isoelectric focusing was performed on 7 cm, pH 3-10 non linear IPG strips*. Strips were focused for 20,000 V/hrs using the Protean IEF Cell*. Prior to the second dimension strips were rehydrated in equilibration buffer (6 M urea, 30% glycerol, 1% SDS, 0.1 mM EDTA, 50 mM Tris-HCl pH 8.8) for 25 min at RT. The separation was performed on 13% SDS-PAGE gels using the Mini Protean 3 Dodeca Cell*. The gels were stained using Bio-Safe Coomassie*. Gels were scanned in a VersaDoc 3000 Imaging System*. The software used to compare the different maps was PDQuest software*.

* All items were supplied by Biorad Laboratories Inc., USA.

TRYPTIC DIGESTION OF TPE AND COLUMN FRACTIONS

The TPE and column fractions were denatured with 6 M urea, reduced with 1 M DTT (in the ratio 50 mol of DTT vs 1 mol of Cys), and alkylated with 1 M IAM (in the ratio 200 mol of IAM vs 1 mol of Cys); each step was performed for 1 h at RT. The proteins were then digested with sequencing-grade trypsin (0.5 $\mu\text{g}/\mu\text{L}$

(Promega, USA) in the ratio 1:50 (w/w) enzyme/protein for 18 h at 37°C .

RESULTS AND DISCUSSION

As a first step, the TPE from six cultivar (*L. albus* cv. Ares, Multitalia and Typtop; *L. angustifolius* cv. Boregine, Arabella, Vitabor) were extracted and analysed via 2D-E. Focusing conditions to obtain 2D map with high spot resolution, were optimised in our laboratory. From this preliminary screening a lot of differences appeared in all the globulins between the two species, and also between different cultivars of the same specie. Figure 1 shows exemplary 2D-map of each cultivar tested.

In order to confirm these differences, the samples were then analysed via anion exchange chromatography, and then with HPLC-Chip-MS/MS to verify some spots/bands identity.

Figure 2 shows an exemplary chromatogram that points out the differences in the ratio of vicilin vs legumin peak areas. The first eluting peak (~ 7.5 min, 0.15-0.2 M NaCl) is β -conglutinin (or vicilin-like protein) and the second (~ 10 min, 0.3-0.35 M NaCl) is α -conglutinin (or legumin-like protein). In *L. angustifolius* the vicilin:legumin ratio was on average 1:2, while in *L. albus* it was approximately 1:0.9.

In order to identify and characterise the storage proteins of both species, one cultivar for both species (*L. albus* cv. Ares; *L. angustifolius* cv. Boregine) was selected for further experiments.

Coupling the techniques of column chromatography with 2D-E enabled important differences in the two species to be seen. Exemplary 2D-gels of purified proteins fractions of both species are shown in figure 3.

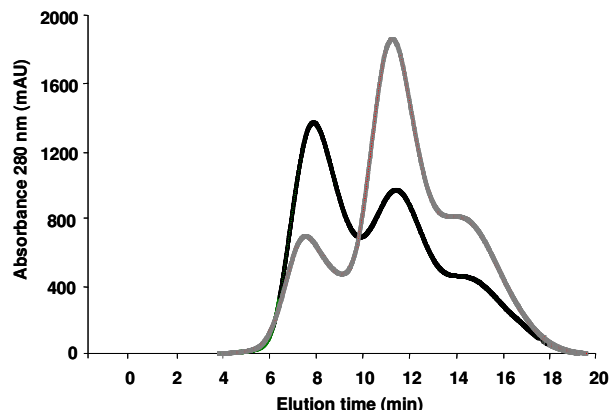


Figure 2. Exemplary elution profiles from the DEAE-FF column under a linear salt gradient. The protein isolates were obtained from *L. albus* cv. Multitalia (dark line) and *L. angustifolius* cv. Arabella (grey line).

Protein profiles of the β -conglutin on 2D gels showed that the dissociated fractions of *L. albus* focus over a large pH range, which reflects the wide eluting peak shown in figure 2. Another observation is the lack of vicilin proteins in *L. angustifolius*. The α -conglutin acidic and basic subunits were seen to have different MWs and the spot at 60 kDa was distinct to the *L. angustifolius* map. Other spots that differ notably between the two species are those of δ -conglutin, with 4

distinct spots being visible in *L. angustifolius*, and only 1 in *L. albus*. To our knowledge this is the first time in *L. angustifolius* that the heavy chain of this protein has been revealed to contain so many isoforms.

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LITERATURE CITED

- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* 72: 248.
- Crespo, J.F., J. Rodríguez, R. Vives and J.M. James *et al.* 2001. Occupational IgE-mediated allergy after exposure to lupine seed flour. *J. Allergy Clin Immunol* 108: 295.
- Dooper, M.M., L. Holden, C.K. Faeste, K.M. Thompson and E. Egaas. 2006. Monoclonal antibodies against the candidate lupin allergens alpha-conglutin and beta-conglutin. *Int Arch Allergy Immunol* 143: 49.
- Kanny, G., L. Guerin and D.A. Moneret-Vautrin. 2000. Le risqué d'asthme aigu grave à la farine de lupin associé à l'allergie à l'arachide. *Rev Med Interne* 21: 191.
- Leduc, V., D.A. Moneret-Vautrin and L. Guerin. 2002. Allergénicité de la farine de lupin. *Allerg Immunol* 34: 213.
- Martins, J.M., M. Riottot, M.C. de Abreu and A.M. Viegas-Crespo *et al.* 2005. Cholesterol-lowering effects of dietary blue lupin (*Lupinus angustifolius*) in intact and ileorectal anastomosed pigs. *J Lipid Res* 4: 1539.

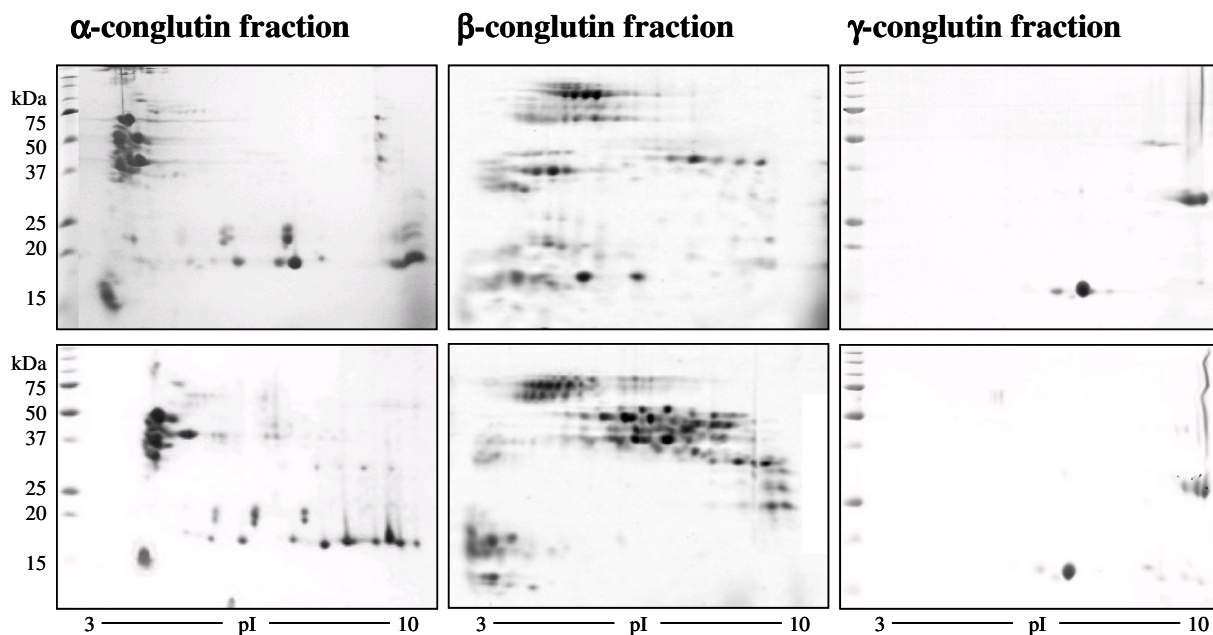


Figure 3. 2D-gels of α -, β - and γ -conglutin purified fractions, eluted from DEAE-FF column. Standard markers are indicated (in kDa) on the left. *L. angustifolius* cv. Boregine – upper gel; *L. albus* cv. Ares – lower gel.

- Moneret-Vautrin, D.A., L. Guérin, G. Kanny and J. Flabbee *et al.* 1999. Cross-allergenicity of peanut and lupine: the risk of lupine allergy in patients allergic to peanuts. *J Allergy Clin Immunol* 104: 883.
- Moreno-Ancillo, A., A.C. Gil-Adrados, C. Dominguez-Noche and P.M. Cosmes. 2005. Lupine inhalation induced asthma in a child. *Pediatr Allergy Immunol* 16: 542.
- Peeters, K.A.B.M., J.A. Nordlee, A.H. Penninks and L. Chen *et al.* 2007. Lupine allergy: not simply cross-reactivity with peanut or soy. *J Allergy Clin Immunol* 120: 647.
- Peterson, D.S., S. Sipsas and J.B. Mackintosh. The chemical composition and nutritive value of Australian grain legumes, 2nd Edn., Grains Research and Development Corporation, Canberra Australia Publications 1997.
- Rahman, M.H., A. Hossain, A. Siddiqua and I. Hossain. 1996. Hematobiochemical parameters in rats fed *Lupinus angustifolius* L. (sweet lupin) seed protein and fibre fraction. *J Clin Biochem Nutr* 20: 99.
- Sirtori, C.R., M.R. Lovati, C. Manzoni and S. Castiglioni *et al.* 2004. Proteins of white lupin seed, a naturally isoflavone-poor legume, reduce cholesterolemia in rats and increase LDL receptor activity in HepG2 cells. *J Nutr* 134: 18.