IDENTIFICATION OF CHEMOPREVENTIVE PEPTIDE LUNASIN IN SOME LUPINUS SPECIES

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ABSTRACT

Lupinus seeds are characterized by elevated contents of high quality protein, so they find uses in both human and animal nutrition. However, little is known about possible bioactive compounds that could be beneficial to health. Lunasin is a 43 amino acid residue peptide isolated by the group of Prof. de Lumen (University of California, Berkeley) from the albumin protein fraction of soybean. Its cancer chemopreventive properties have been demonstrated by in vitro and in vivo assays; currently other plants are being investigated as possible sources of this peptide. This study aimed to detect lunasin in two cultivated and two wild species of Lupinus. Lunasin detection using lunasin monoclonal antibodies was carried out on total protein extracts as well as soluble fractions of defatted flour from seeds with and without testa. Soy (Glycine max) seed extracts served as positive control. Results showed the presence of lunasin in G. max protein fractions of albumin, globulin, and glutelin. Lunasin was found in prolamins fraction of L. albus seeds with testa; whereas it was found in albumin and glutelin fractions of seeds without testa of L. montanus and L. campestris, respectively. Lunasin was not detected in protein extracts of seeds without testa of L. albus and seed with and without testa of L. mutabilis. Peptide bands of molecular weights greater than 25 kDa were detected in the fractions of albumin, globulin and glutelin of all evaluated Lupinus species, suggesting presence of lunasin-related peptides.

KEY WORDS

lupin proteins, monoclonal antibodies

INTRODUCTION

Lunasin is a 43 amino acid residue peptide initially identified in the soybean cotyledon when a cDNA encoding a posttranslationally processed 2S albumin (Gm2S-i) was cloned (Galvez et al. 1997). Gm2S-i codes for a signal peptide, a large subunit methionine-rich protein, a small subunit (called lunasin), and a linker peptide. Its cancer chemopreventive properties have been demonstrated by in vitro and in vivo assays (De Lumen, 2005). Lunasin exerts cancer preventive properties in mammalian cells induced by chemical carcinogens and viral oncogenes. Moreover, lunasin is effective preventing skin cancer in a mouse model induced by a chemical carcinogen (Galvez et al., 2001). Recently, this peptide has been reported to reduce mammary tumour incidence, generation and weight in a xenograft breast cancer mouse model (Hsieh et al., 2010a). Lunasin also prevents breast cancer induced by chemical carcinogens (Hsieh et al., 2010b).

The presence of lunasin has been reported in grains of barley (Jeong et al., 2002), wheat (Jeong et al., 2007a), rye (Jeong et al., 2009) and amaranth (Silva-Sánchez et al., 2008), as well as in plants of the Solanaceae family (Jeong et al., 2007b). With these results, presence of lunasin or lunasin-like peptides in other grains and plants has been suggested.

Lupinus belong to the Fabaceae family and have a worldwide distribution. There are between 200 and 300 species reported, 12 of them from the Mediterranean region, and the rest in the American continent. Some of the Lupinus species have been used since antiquity as protein source for humans and animals. Currently, some species are cultivated for food and feed in several countries: L. angustifolius in Australia and Europe, L. albus and L. luteus in Europe, and L. mutabilis in the Andean Region. Several studies have been performed to characterize storage proteins of these species (Blagrove and Gillespie, 1975; Cerletti et al., 1978, Duranti et al.,...
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However, few data are available about the release of bioactive peptides from lupins proteins (Duranti, 2006; Duranti, 2008, Arnoldi, 2008; Lucari et al., 2002), and no data have been reported about the presence of the chemopreventive peptide lunasin or lunasin-like peptides in Lupinus species.

The aim of present work was to identify by immuno-detection the presence of lunasin or lunasin-like peptides in protein extracts from two cultivated (L. albus, and L. mutabilis) and two Mexican wild species of Lupinus (L. montanus and L. campestris).

MATERIAL AND METHODS

Seeds of the cultivated species L. albus and L. mutabilis were donated by Dra. Martinez Ayala of Centro de Investigación en Biotecnología Aplicada (CIBA-IPN, Tlaxcala, Mexico). G. max seeds were bought in the central market of Yautepec, Morelos. Seeds of wild Mexican species (L. montanus and L. campestris), were collected in La Joya, Popocatepetl-Iztaccihuatl National Park (Estado de Mexico, Mexico) at altitude of 3800 m above sea level (masl) and 2600 masl, respectively.

Protein extraction and fractionation

Seeds were dehulled, handmilling and milled in a seed grinder (Tekmar A-10, Tekmar Ohio, USA). Flour was sieved through a screen (100-mesh) and defatted by exhaustive extraction with petroleum ether.

Defatted flour (5 g) was mixed with water, (1:10 w/v) stirred for 2 h at 4°C, and centrifuged at 17000 g for 30 min at 4°C. The supernatant with the albumins fraction was collected and kept at 4°C until use. The pellet was used for the extraction of globulins. It was suspended in NaCl 10% (w/v), pH 7.0, stirred for 1 h, and centrifuged as above. The supernatant was fractioned as described by Gillespie and Blagrove (1975). Briefly, supernatant was precipitated with 85% saturated (NH4)2SO4, stirred during 2 h and centrifuged at 17000 g for 30 min at 4°C. The pellet was suspended in 0.15 M phosphate buffer (pH 7.0), stirred for 2 h and dialyzed against 0.02 M acetate buffer (pH 4.8) for 18 h. Dialyzed solution was centrifuged under above conditions. α-Conglutin (11S) was obtained in the pellet, while the β-conglutin fraction was separated from the supernatant. Supernatant was dialyzed against water for 24 h and centrifuged. β-Conglutin (7S) was obtained in the pellet. This fraction was suspended in 0.15 M phosphate buffer (pH 7.0) and concentrated by precipitation with 70 and 90% saturated ammonium sulphate. All fractions were obtained by triplicate, and after addition of 0.02% (w/v) sodium azide were kept at 4°C until use. Protein was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

Detection of lunasin by Western-blotting

Lunasin was detected by the method described by Silva-Sánchez et al. (2008) with some modifications. Samples of total protein extracts and protein fractions of lupins were prepared at a concentration of 2 mg/mL, diluted 1:1 with Tricine sample buffer, and boiled for 5 min. Aliquots of 20 μL of each sample were loaded onto the same gels. Standard lunasin was synthesized by Chengdu KaiJie Bio-Pharmaceutical Co. (Chengdu, PR China). An amount of 208 ng of synthetic lunasin was loaded onto the same gels. The gels were run in a Mini Protean-3 Cells (Bio-Rad) with Tris-Tricine-SDS buffer, at 100 V constant for 100 min. Immun-Blot PVDF membranes (Bio-Rad) were prepared for transfer with a soak of 100% methanol and rinsing with distilled water. The proteins on SDS-PAGE gel were transblotted to the membrane for 60 min at 100 V constant for 100 min. Upon completion of transfer, the nonspecific binding sites were blocked by immersing the membrane for 1 h in Odyssey

Table 1. Polypeptides showing affinity with soy lunasin monoclonal antibodies.

<table>
<thead>
<tr>
<th>Species</th>
<th>Albumins PM (kDa)</th>
<th>Globulins PM (kDa)</th>
<th>7S PM (kDa)</th>
<th>11S PM (kDa)</th>
<th>Prolamins PM (kDa)</th>
<th>Glutelins PM (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. albus dehulled</td>
<td>28, 31, 33, 60</td>
<td>25, 31, 58, 60</td>
<td>25, 31, 58, 60</td>
<td>25, 31, 58, 60</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. albus</td>
<td>28, 31, 33, 60</td>
<td>25, 31, 58, 60</td>
<td>25, 31, 58, 60</td>
<td>25, 31, 58, 60</td>
<td>5</td>
<td>25, 60</td>
</tr>
<tr>
<td>L. mutabilis dehulled</td>
<td>28, 31, 33, 60</td>
<td>25, 31, 58, 60</td>
<td>25, 31, 58, 60</td>
<td>25, 31, 58, 60</td>
<td>-</td>
<td>25, 27</td>
</tr>
<tr>
<td>L. mutabilis</td>
<td>28, 31, 33, 60</td>
<td>25, 31, 58, 60</td>
<td>25, 31, 58, 60</td>
<td>25, 31, 58, 60</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. montanus</td>
<td>31, 40, 57</td>
<td>25, 31, 40, 57, 61</td>
<td>25, 31, 40, 57, 61</td>
<td>25, 31, 40, 57, 61</td>
<td>-</td>
<td>5, 57</td>
</tr>
</tbody>
</table>

PM: Molecular weight; 7S: Globulins 7S, 11S: Globulins 11S
Blocking Buffer (Li-Cor Biosciences, Lincoln, NE, USA). The membranes were washed three times and incubated with monoclonal primary antibody of lunasin (diluted 1:5000 in Odyssey Blocking Buffer-0.2% Tween 20) for 1 h at room temperature. After washing four times with PBS-0.1% Tween 20, the membranes were incubated for 1 h with an anti-mouse fluorescence-labeled secondary antibody (Li-Cor Biosciences) at 1:15,000 dilution in Odyssey Blocking Buffer-0.2% Tween 20-0.02% SDS. The membranes were washed four times with PBS-0.1% Tween 20 and once with PBS. Finally, each membrane was scanned and analyzed using an Odyssey Infrared Imaging System (Li-Cor Biosciences). Lunasin content in samples was determined comparing their band intensities with that corresponding to standard lunasin run under the same conditions. Results were expressed as the mean of the three values ± standard deviation.

RESULTS AND DISCUSSION

In all the Lupinus species, protein bands with molecular weight equal and greater than 25 kDa were recognized by lunasin monoclonal antibody in the fractions of albumin, globulin, and glutelin (table 1). Silva et al. (2008) reported lunasin-like peptides with molecular weight larger than that of lunasin for Amaranthus hypochondriacus, these peptides showed the typical lunasin biological activity as chemopreventive peptide. Lupinus peptides with affinity to lunasin could represent lunasin like-peptides; however, a further work has to be done to

**Figure 1.** A Western-Blot of protein fractions of *L. albus* of dehulled (A) and non dehulled (B) seeds. M: molecular marker, L: synthetic lunasin, Alb: albumin, Glo: total globulin, 7S: globulin 7S, 11S: globulin 11S, Pro: prolamin and Glu: glutelin.

**Figure 2.** A Western-Blot of protein fractions of *L. mutabilis* of dehulled (A) and none dehulled (B) seeds. M: molecular marker, L: synthetic lunasin, Alb: albumin, Glo: total globulin, 7S: globulin 7S, 11S: globulin 11S, Pro: prolamin and Glu: glutelin.
understand characteristics of bands identified as lunasin-like peptides.

In the protein fractions (PFs) of dehulled and non dehulled seeds from *L. albus* bands greater than 25 kDa were detected by the monoclonal lunasin antibody (fig. 1). In PFs of dehulled seeds, antibody had specially affinity for the albumin and globulin fractions; while in PFs of non dehulled seeds, affinity to lunasin antibody was found in the albumin, globulin, prolamin, and glutelin fractions.

For the south-American species (*L. mutabilis*), the lunasin antibody had affinity for the PFs of albumin, globulin and glutelin in dehulled seeds. For non dehulled seeds, affinity by lunasin antibody was found in albumin and globulin fractions (figure 2).

Western blot of PFs of non dehulled seeds from *L. montanus* showed that all PFs present affinity for lunasin monoclonal antibody. Specially, a protein band of 5 kDa was observed in the glutelin fraction. This molecular size is similar to that reported to lunasin, suggesting the presence of lunasin in the glutelin fraction of *L. montanus*.

Affinity for the lunasin antibody was found also in the fractions of albumin and globulin of non dehulled *L. campestris* seeds. In the albumin fraction a protein band of approximately 7 kDa was detected.

In total globulin and subfractions of all evaluated species, bands with affinity to the lunasin antibody were observed at same molecular weight: 28, 31, 33 and 60 kDa. While, for the albumin fraction were observed differences between cultivated and wild species. For the cultivated species, *L. albus* and *L. mutabilis*, results show an affinity for lunasin of proteins with molecular weights of 25, 31, 58 and 60 kDa; while, for the proteins of the two wild species there were affinity differences: *L. montanus* showed bands of 5, 31, 40 and 57 kDa; whereas, *L. campestris* 7, 31, and 100 kDa (table 1).

This diversity of proteins with affinity to lunasin antibody found in the lupin species shows that there is an expression of the polymorphism of the storage proteins in seeds. This polymorphism was reported for several cultivated and wild varieties of various crops, such as bean (*Phaseolus vulgaris*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), pea (*Pisum sativum*) and corn (*Zea mays*) (Becerra and Paredes, 2000), and used for identification between cultivated and wild species. Our results show that wild species present higher polymorphism as cultivated species, these differences could be the result of the domestication process, which allows the selection not only of varieties with higher grain yield and quality, but also of biochemical phenotypes.

Lunasin play a role in the development of seeds, as it is working directly on the suppression of mitosis during endoreplication, and presence of this peptide can be expected in the second stage of maturity, before drying, so that we can think that this peptide is common to all angiosperms. This might explain the absence of lunasin in evaluated *Lupinus* seeds, we don’t know in which maturity stage they were.

On the other side, there are several studies showing that lunasin is not present in all studied seeds. Several reasons could explain this fact: 1) lunasin may be lost during processing of samples; 2) monoclonal antibodies are soy lunasin specific; 3) lunasin is present in seeds at a specific maturity state; 4) lunasin-like peptides represent a form of lunasin, and after processing they have the same activity; 5) other peptides/substances play similar role as lunasin.

The presence of lunasin-like peptides in flour of dehulled and non dehulled seeds of four *Lupinus* species was demonstrated in this work. However, is necessary to perform research to characterize these bands, such as sequencing and determination of biological activities.

![Figure 3](image3.png)

**Figure 3.** Western-Blot of protein fractions of *L. montanus* none dehulled seeds. M: molecular marker, L: synthetic lunasin, Alb: albumin, Glo: total globulin, 7S: globulin 7S, 11S: globulin 11S, Pro: prolamin and Glu: glutelin.

![Figure 4](image4.png)

**Figure 4.** Western-Blot of protein fractions of *L. campestris* none dehulled seeds. M: molecular marker, L: synthetic lunasin, Alb: albumin, Glo: total globulin, 7S: globulin 7S, 11S: globulin 11S, Pro: prolamin and Glu: glutelin.
Furthermore, this work demonstrates that lunasin-like peptides are not associated only with globulin fraction of soy. We found affinity of lunasin antibody also for the soy fractions of albumin, glutelin and prolamin.

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