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Original scientific paper

Mitigating the allergenicity of lupin seeds through germination to enhance food safety

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ABSTRACT

The search for novel plant proteins presents a major challenge to the global food industry. Beyond the sustainability and nutritional value of seed proteins, there is a growing focus on exploring their potential health benefits for human consumption. However, the endeavour to evaluate the allergenic risks associated with plant-based proteins will require substantial efforts in the coming years. One key strategy to address this concern is the reduction of allergenicity in seeds through the process of germination, which triggers the strong proteolysis of storage proteins. In this study, *Lupinus luteus* L. seeds were germinated at 25ºC for 0, 3, 6 and 9 days under a constant humidity of 80%. Peptide extracts derived from sprouted lupin seeds were subsequently separated under reducing conditions by SDS-PAGE. The resulting protein bands were digested and subjected to mass spectrometry analysis (SWATH-MS) for accurate identification of allergenic proteins. This study's findings revealed the great impact of lupin seed germination on the protein patterns of hydrolysates, particularly noticeable after a germination period of 6 days. The main protein bands (90, 70, 64, 52, 46 and 37 kDa) exhibited a notable decrease in relative intensity as a result of germination. Furthermore, SWATH-MS analysis successfully identified two distinct isoforms (B0YJF7 and B8Q5G0) of the major allergen, β-conglutin, in all tested bands, both prior to and following germination. This study provides evidence for the crucial role of germination in the degradation of this allergen, leading to the elimination of epitopes and subsequently reducing its allergenicity.

1. Introduction

One of the major challenges for the global food industry is the search for plant proteins. Beyond the sustainability and nutritional value of seed proteins, there is an increasing emphasis on exploring potential benefits on human health (*López-Pedrouso, Lorenzo, Alché, Moreira & Franco, 2023)*novel food is becoming an emerging trend increasingly more demanding in developed countries. Food proteins from vegetables (pulses, legumes, cereals. White lupin (*Lupinus albus* L.), yellow lupin (*L. luteus* L.) and narrow-leafed lupin (*L. angustifolius* L.) are native European species known for their high protein content of up to 44%. These lupin varieties are commonly utilized in the food industry, particularly in the production of bakery and gluten-free products where lupin flour, protein isolates and concentrate are frequently employed (*Lucas et al*., 2015).

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In the case of lupin, three allergens were recognized by the WHO/IUIS Allergen Nomenclature Sub-Committee: Lup a 5 (profilin; 15 kDa), Lup an 1 (β-conglutin, 7S seed storage globulin, vicilin; 55–61 kDa) and Lup an 3 (non-specific lipid transfer protein; 11 kDa). Another potential risk arises from the cross-reactivity between lupin and peanuts, which can potentially trigger allergic reactions *(Mennini, Dahdah, Mazzina & Fiocchi*, 2016)but the identification of the involved individual allergens is still limited. The aim of this review is to describe new allergenic findings, of potential relevance for cross-reactivity among peanut and lupin. Recent Findings: Seventeen allergens of peanut have been included in the official allergen nomenclature database to date. Lupin sensitization has been observed in 15–20% of individuals with known peanut allergy, The majority of lupin seed proteins are comprised of α-conglutins (legumin-like. The immunoreactive properties of seeds can be reduced through germination and other processing conditions. Thus, proteins from cotyledons undergo hydrolysis and the removal of multiple epitopes takes place, with beneficial effects on seed consumption (*Ravindran & Ramaswamy*, 2023). This process has been proven to result in the suppression of epitopes and the reduction of allergenicity through protein degradation (*Sathe, Teuber & Roux*, 2005; *Pi, Sun, Fu, Wu & Cheng*, 2021). Moreover, the germination process of some seeds, including varieties such as beans, chickpeas, lentils and lupins, enhances their nutritional quality by increasing protein, ash and mineral (sodium, magnesium, zinc and iron) contents (*Atudorei, Stroe & Codină*, 2021).

2. Materials and methods

2.1. Germination of lupin seeds (Lupinus luteus L.) and protein extraction

The seeds of *Lupinus luteus* L. were germinated on filter paper at 25ºC, with a constant humidity of 80% maintained throughout the whole germination period. The germination process was conducted under dark conditions. A maximum germination time of 9 days was allowed to preserve the nutritional profile of the germinated seeds. Finally, freeze-drying was employed to store the seeds at –80ºC. Proteins from germinated and non-germinated seeds were prepared by homogenization using a TissueLyser II (Qiagen) in cold RIPA buffer [containing 200 Mmol/L Tris/HCl (pH 7.4), 130 Mmol/L NaCl, 10% (v/v) glycerol, 0.1% (v/v) SDS, 1% (v/v) Triton X-100, 10 Mmol/L $MgCl_2$] with anti-proteases and anti-phosphatases (Sigma-Aldrich). The tissue lysates were centrifuged for 20 minutes at 14,000 g in a microfuge at 4 °C. The protein quantification was assessed for each sample using RC DC™ kit (Bio-Rad) according to the manufacturer's recommendations.

2.2. SDS-Page analysis

Peptide extracts obtained from germinated and non-germinated lupin seeds were subjected to separation under reducing conditions using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Mini-Protean Tetra Cell equipment (Bio-Rad Lab, Hercules, CA, USA) was utilized to separate 15 µg amounts of the extracts onto pre-cast 10% gels. To dissolve and denature the samples, Laemmli buffer (62.5 mM TrisHCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 100 mM DTT) was employed and incubated at 95ºC for 5 minutes. Staining was performed using Coomassie Brilliant Blue G-250 solution, and the Gel Doc XR+ system (Bio-Rad Laboratories) was used to capture the images. The obtained images were subsequently analysed using the Image Lab™ software (Biorad Lab, Hercules, CA, USA).

2.3. Mass spectrometry: Digestion and analysis

The selected bands from 1-DE gels were excised and washed with a solution containing 50 mM ammonium bicarbonate (ambic) and 50% MeOH. Proteins were reduced with 10 mM DTT in 50 mM ambic and alkylated with 55 mM IAA in 50 mM ambic, and subsequently rinsed with 50 mM ambic in 50% MeOH, dehydrated through the addition of acetonitrile (ACN) and dried in a SpeedVac (Thermo Scientific, USA). Modified porcine trypsin was added to the dried gel slices at a final concentration of 20 ng/µL in 20 mM ambic, followed by incubation at 37°C for 16 h. The peptides were extracted three times by incubation in 40 µL of 60% ACN in 0.5% HCOOH for 20 min. The resulting peptide extracts were pooled, concentrated, and dried in a SpeedVac and stored at –20 °C until their analysis through LC-MS/MS.

A TripleTOF 6600 System (SCIEX, Foster City, CA) was employed for the data acquisition using a Data-dependent workflow. After MS/MS analysis, data files were processed using Pro-

teinPilot™ 5.0.1 software from SCIEX, which uses the algorithm Paragon™ for database search and Progroup™ for data grouping. Data were searched using a UniProt database. False discovery rate was calculated using a non-lineal fitting method displaying only those results that reported a 1% global false discovery rate or better.

3. Results and Discussion

3.1. Analysing SDS protein profile of lupin seeds during germination

The germination of lupin seeds had a significant impact on the protein banding patterns of hydrolysates over nine days, as illustrated in Figure 1. Protein analysis through SDS-PAGE revealed the presence of polypeptide bands with molecular weights below 90 kDa. Protein bands at 90, 70, 64, 52, 46 and 37 kDa were predominantly observed in non-germinated seeds. However, with longer germination periods, these storage proteins underwent degradation to supply amino acids for the synthesis of biomolecules, resulting in a remarkable decrease in the intensity and volume of protein bands after six days. Most edible pulse seeds, such as chickpea, fava bean, kidney bean, green lentil and yellow pea, germinate within three or five days. This process enhances *in vitro* digestion in the majority of cases, with the exception of kidney bean, suggesting an important bioprocess (*Ohanenye, Tsopmo, Ejike & Udenigwe*, 2020)major dietary sources in developed countries are of animal origin. However, the association of red meat consumption to the increased risks of some health conditions and its unsustainable pressure on the environment have increased the interest in plant proteins as healthier and sustainable alternatives. Of these, legumes have a great potential, but part of their proteins are indigestible due to interaction with other components such as phytate and polyphenols. As such, the quest to improve protein accessibility has become of interest to many researchers. Germination is proposed to be a bioprocess method to improve protein digestibility and protein biological properties. Scope and approach: This review discusses the importance of plant proteins and the hindrance of protein digestibility. This paper also highlights the role of germination in the deactivation of antinutritional factors, hydrolysis of indigestible proteins, and improvement of properties and content of proteins of different legume seeds. Key findings and conclusions: Protein digestibility is dependent on the nature of antinutritional factors (e.g. trypsin inhibitors and phytate. During ger-

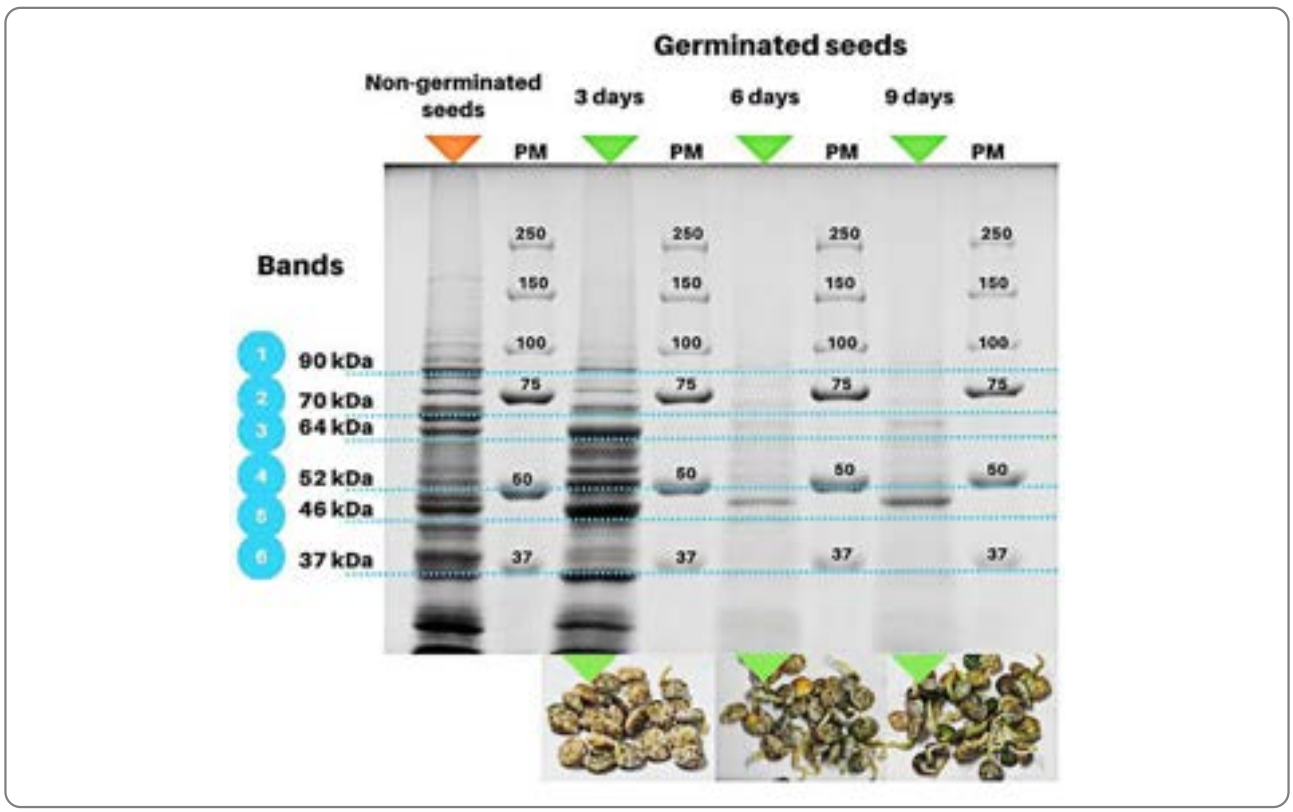

Figure 1. SDS-PAGE analysis of seed proteins from *Lupinus luteus* L seeds on different days of germination

mination, a significant protein transformation occurs through proteolysis increasing the content of free amino acids, which enhances digestibility and reduces the levels of anti-nutrient protease inhibitors. This enzymatic breakdown by protease activity leads to the degradation of storage proteins, including allergens (*Bera, O'Sullivan, Flynn & Shields*, 2023). In short, germination plays a crucial role in modifying the reactivity of seed allergens by effectively eliminating conformational and linear epitopes. This process reduces the allergenicity of lupin seed products. According to this strategy, germination significantly reduced the immunoreactivity of vicilin by 55% and 74% after ten days in black gram (*Vigna mungo*) and mung bean (*Vigna radiata*), respectively (*Gupta, Sathe, Su & Liu*, 2021). Similarly, in soybeans, the three major allergens (Gly m Bd 60K, Gly m Bd 30 K and Gly m Bd 28 K) underwent degradation earlier in the embryonic axes compared to cotyledons during the process of germination and seedling growth (*Wu et al*., 2012).

However, it is expected that each band will contain a complex mixture of polypeptides belonging to different seed proteins. A variety of proteins were present, resulting in albumin (water-soluble), globulin (salt-soluble), glutelin (alkaline-soluble) and prolamin (ethanol-soluble) being the most abundant. These proteins exhibit distinct polypeptide bands due to differences in their molecular weights. Among them, globulin constitutes the highest proportion (approximately 90% of the total protein) and is characterized by bands at 8, 11, 17, 23, 25, 30, 38, 40, 45 and 60 kDa (*Idowu, Alashi, Nwachukwu, Fagbemi & Aluko*, 2021).

3.2. Identification of allergens by mass spectrometry

Mass spectrometry analysis revealed the presence of two distinct isoforms of β-conglutin, B0YJF7 and B8Q5G0, in all tested bands both before and after germination (Table 1). These isoforms consisted of 521 and 611 amino acids, respectively, and exhibit great similarity (86.56% identity according to Uniprot). The major difference between them is the inclusion of an additional terminal sequence of 82 amino acids in the B8Q5G0 isoform. β-conglutin is a protein belonging to the cupin family. The cupin family comprises a diverse range of proteins sharing a common β-barrel structure. The two major storage proteins of lupin seed are α-conglutin (legumin-like or 11S globulin), and ß-conglutin (vicilin-like or 7S globulin). Vicilin proteins exhibit a characteristic structural arrangement comprising

Table 1. Identification of β-conglutin, the major allergen in lupin seeds, in the gel bands of non-germinated seeds (NGS) and geminated seeds at 6 days (GS)

Band	Status seed	Accession No. (Uniprot)	Name allergen	Mr (KDa) expected	Coverage (%)	No. of non-reductant peptides (95%)
1 (90 kDa)	NGS	B0YJF7	Cupin 1	61.47	13.44	3
	GS	B0YJF7	Cupin 1	61.47	17.47	5
2 (70 kDa)	NGS	B0YJF7	Cupin 1	61.47	32.44	23
	GS	B0YJF7	Cupin 1	61.47	70.63	147
		B8Q5G0	Lup an 1	71.9	53.36	117
3 (64 kDa)	NGS	B0YJF7	Cupin 1	61.47	33.40	27
	GS	B0YJF7	Cupin 1	61.47	57.01	21
4 (52 kDa)	NGS	B8Q5G0	Lup an 1	71.9	57.20	22
	GS	B8Q5G0	Lup an 1	71.9	38.95	146
5 (46 kDa)	NGS	B8Q5G0	Lup an 1	71.9	61.47	8
	GS	B0YJF7	Cupin 1	61.47	31.86	9
6 (37 kDa)	NGS	B0YJF7	Cupin 1	61.47	44.15	24
	GS	B8Q5G0	Lup an 1	71.9	41.73	22

two cupin domains, forming barrel-shaped structures composed of α-helices. Thus, β-conglutin, also referred to as vicilin-like globulins, is a 7S globulin with a trimeric structure. It is composed of polypeptides ranging from 16 to 70 kDa. Additionally, a glycosylated precursor of 64 kDa has been identified (*Duranti, Consonni, Magni, Sessa & Scarafoni*, 2008).

One of the most abundant allergen families identified was the cupin family. Among the food allergens within this group are 7S globulins, including soybean (β-conglycinin), peanut (conarachin; Ara h 1), walnut (Jug r 2) and lentil, as well as 11S globulins like peanut (arachin; Ara h 3) and soybean (glycinin) (*Mills et al*., 2002). In the case of lupin-derived foods, the main allergen of concern is β-conglutin, considered the most relevant issue associated with lupin consumption. Therefore, detecting and quantifying β-conglutin in processed products is a suitable strategy *(Lima-Cabello, Alché & Jimenez-Lopez*, 2019)sensitive and accurate ELISA method to detect, identify and quantify the lupin main allergen β-conglutin (Lup an 1, as is reducing their allergenicity, shown in this study. A massive degradation of β-conglutin isoforms (B0YJF7 and B8Q5G0) took place during the germination as shown in Figure 1, providing further evidence of their role as storage proteins. In most cases, germination led to the elimination of conformational and linear epitopes, thereby modifying the immunoreactivity, particularly after day 6 of germination.

4. Conclusions

The food safety of vegetal foods should be enhanced to facilitate these products into the market. It is crucial to develop methods for detection, identification and quantification of allergens, and to decrease allergenicity in fresh and processed foods. The most relevant allergen in seeds from *Lupinus luteus* L is β-conglutin, and particularly, two isoforms (B0YJF7 and B8Q5G0) were detected. This study demonstrates that germination plays a vital role in the degradation of this allergen, thereby indicating the elimination of epitopes and resulting in reduced allergenicity.

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