Food Chemistry 244 (2018) 60–70

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Characterization of narrow-leaf lupin (*Lupinus angustifolius* L.) recombinant major allergen IgE-binding proteins and the natural β-conglutin counterparts in sweet lupin seed species

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ARTICLE INFO

Keywords:
Conglutins
Cross-allergenicity
Diagnosis
Food allergy
IgE-binding activity
Immunotherapy
Seed storage proteins
Sweet lupin
Recombinant allergen
Vicilin

ABSTRACT

β-conglutin has been identified as a major allergen for *Lupinus angustifolius* seeds. The aim of this study was to evaluate the binding of IgE to five recombinant β-conglutin isoforms (rβ) that we overexpressed and purified and to their natural counterparts in different lupin species and cultivars.

Western blotting suggested β-conglutins were the main proteins responsible for the IgE reactivity of the lupin species and cultivars. Newly identified polypeptides from "sweet lupin" may constitute a potential new source of primary or cross-reactive sensitization to lupin, particularly to *L. albus* and *L. angustifolius* seed proteins. Several of them exhibited qualitative and quantitative differences in IgE-binding among these species and cultivars, mainly in sera from atopic patients that react to lupin rather than peanut.

IgE-binding was more consistent to recombinant β2 than to any of the other isoforms, making this protein a potential candidate for diagnosis and immunotherapy.

1. Introduction

Lupin is a popular pulse worldwide. From more than 450 species of the *Lupinus* family, only lupins known as "sweet lupins", such as white lupin (*L. albus*), yellow lupin (*L. luteus*) and blue lupin, also known as narrow-leaf lupin (NLL) (*L. angustifolius*) are being used in food production. NLL flour is used in bakery products and other food to improve the nutritional value, with additional health related benefits of a high percentage of protein, low fat and starch content, reasonable levels of dietary fibre and the absence of gluten (Villarino et al., 2016).

As is the case for all edible legume seeds, the major protein fraction of lupin seeds is storage proteins, comprised of α- (legumin like-protein or 11S globulin) and β- (vicilin like-protein or acid 7S globulin) conglutin as the two major globulin fractions, and γ- (basic 7S globulin), and δ- (2S sulphur-rich albumin) conglutin in lower amounts (Foley et al., 2011; Foley et al., 2015).

Sweet lupin seeds seem to be particularly promising as a source of innovative food ingredients due to a protein content similar to soybean and an adequate composition of essential amino acids (Duranti, 2006). Foods based on sweet lupin proteins, including bakery products, pasta formulations and gluten-free products, are gaining more attention from industry and consumers because of a number of health-promoting benefits that have been ascribed to lupin seed components, e.g. prevention of cardiovascular disease, reduction of blood glucose and cholesterol levels (Arnoldi, Boschin, Zanoni, & Lammi, 2015).

On the other hand, with the rapid introduction of novel foods and new ingredients in traditional foods, the number of people allergic to lupin is also rising. Food allergy is a serious and growing problem in many parts of the world. Food allergy is estimated to affect about 1–3% in the general population and 3–8% among children (Prescott et al.,

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http://dx.doi.org/10.1016/j.foodchem.2017.10.015
Received 30 June 2017; Received in revised form 14 September 2017; Accepted 5 October 2017
Available online 06 October 2017
0308-8146/

2013). There is some limited evidence that legume sensitization may be a dynamic process, where symptoms may start with reactions to single legume proteins, with individuals becoming progressively sensitized to other legumes, such as lentil, beans, lupin and pea (Matheu et al., 1999).

In recent years, reports on sensitization or immunoglobulin E (IgE)-mediated allergic reactions to lupin, either as a primary response or as a result of cross-reactivity with other legumes, particularly peanut (Ballabio et al., 2013; Mennini, Dahdah, Mazzina, & Fiocchi, 2016), have increased, and this parallels the increasing number of food applications for this legume. Lupin flour has also been recognized as a cause of occupational airborne allergy (van Kampen et al., 2015), where IgE-mediated allergy was reported after inhalation or contact with lupin flour (Prieto et al., 2010).

The frequency of sensitization and allergic reactions to lupin in the general population is unknown. However, as lupin becomes more prevalent as an alternative protein source for human use, it can be expected that demand for it will increase and more consumers will be exposed to lupin antigens. Serological cross-reactivity of IgE with other legume antigens, particularly those from peanut, has also been reported in both non-occupational and occupational settings (Campbell & Yates, 2010). Allergic reactions to lupin can be triggered via ingestion of lupin in some peanut-allergic individuals (Peeters et al., 2009), although triggering via ingestion, inhalation and occupational exposure in individuals without peanut allergy has also been reported (Peeters, Koppelman et al., 2007; Peeters, Nordlee et al., 2007).

In consideration of the increasing number of clinical cases of lupin allergy reported in the literature, in 2008, lupin was added to the list of foods that must be labelled as an allergen in pre-packaged foods as advised by the European Food Safety Authority (EFSA) (http://www.efsa.europa.eu/).

IgE-binding proteins described for lupin seeds ranged from 13 to 108 kDa (Foley et al., 2011; Goggin, Mir, Smith, Stuckey, et al., 2008; Hefle, Lemanske, & Bush, 1994; Moneret-Vautrin, Guerin, Kanny, Flabbee, et al., 1999; Peeters, Koppelman et al., 2007; Peeters, Nordlee et al., 2007). Different polypeptides belonging to the main lupin protein families α-conglutins (~55 kDa), and β-conglutins (~13–80 kDa), are the most likely involved in the *in vitro* and *in vivo* allergenic responses (Ballabio et al., 2013). Moreover, conglutin β has been identified as a major allergen in *L. angustifolius* (Goggin et al., 2008), as well as in *L. albus* (Guillamon et al., 2010), being designated Lup an 1 and Lup a 1, respectively, by the International Union of Immunological Societies allergen nomenclature subcommittee (http://www.allergen.org/), and included in the allergen database Allergome (http://www.allergome.org/). In addition, three other proteins have been included in this allergen database for both species, corresponding to α-, γ- and δ-conglutins (http://www.allergome.org/). However, the number and the polypeptide composition of these major allergen proteins is yet to be investigated in other lupin species or in the major cultivars used for food production.

In this study, we present molecular data aiming to identify and compare the protein profiles of β-conglutin, a major group of lupin allergen proteins among lupin species including *L. albus* and *L. angustifolius*. We also aimed to compare the IgE reactivity to β-conglutin polypeptides from different lupin species, as well as to recombinant β-conglutin proteins encoded by different β-conglutin genes in NLL.

2. Materials and methods

2.1. Construction of expression plasmids

β1-, β2-, β3-, β4- and β6-conglutins were over-expressed using the pET28b construct (Novogen, www.novogen.com) containing an N-terminal poly-histidine (6xHis) tag and thrombin cleavage site for removal of the tag when necessary. A pUC57 vector containing conglutin beta genes coding for conglutins with GenBank accession numbers HQ670409 (β1), HQ670410 (β2), HQ670411 (β3), HQ670412 (β4) and HQ670414 (β6), were synthesized to include optimal codon usage for bacterial expression (GenScript). The beta genes, β1, β2, β3, β4 and β6 were cloned into the *Nco*I/*Xho*I restriction site of the bacterial expression vector, pET28b and transformed into Rosetta™ 2(DE3) pLysS Singles™ Competent Cells (Novagen).

2.2. β-conglutin protein over-expression

All β-conglutin recombinant proteins were expressed in Rosetta™ 2(DE3) pLysS Singles™ Cells (Novogen). Protein expression was performed using an auto-induction method (Studier, 2005) with small modifications. Briefly, a colony of *E. coli* containing the expression construct was isolated and grown for 20 h in ZY-medium plus kanamycin at 50 μg/ml at 37 °C and continuous shaking (200 rpm). The culture was diluted 1:150 in ZYM-5055 medium (tryptone 1%, yeast extract 0.5%, Na_2HPO_4 25 mM, KH_2PO_4 25 mM, NH_4Cl 50 mM, Na_2SO_4 5 mM, glycerol 0.5%, glucose 0.05%, α-lactose 0.2%, $MgSO_4$ 2 mM) and grown for a further 5 h before subsequently inducing over-expression of the proteins by adjusting the temperature to 19 °C for another 20 h. Cells were collected by centrifugation at 5000 × *g* at 4 °C. The bacterial cell pellet was rinsed twice with phosphate buffered saline (PBS), pH 7.5, the supernatant was removed and the cell pellet was flash frozen using liquid nitrogen. The bacterial cell pellet was stored at −80 °C until further use.

2.3. Purification of recombinant β-conglutin proteins

Protein purification from bacterial pellets was performed following the manufacturers' recommendations for His-tagged proteins (Qiagen).

Briefly, the first step was the lysis of the bacterial cells followed by nickel affinity chromatography using Ni-NTA spin columns (Qiagen) that interact with the histidine (6xHis) tags at the C-terminus of the recombinant β-conglutin proteins, since the interaction between Ni-NTA and the 6xHis tag of the recombinant proteins does not depend on tertiary structure. After elution of 6xHis-tagged proteins from the column with an increasing imidazole concentration gradient (10–300 mM), 2.5 ml fractions were collected.

Fractions containing protein were analyzed using SDS-PAGE and fractions showing a single band corresponding to the expected molecular weight were pooled, and dialyzed 5 times against Tris-HCl 100 mM, pH 7.5, 150 mM NaCl to eliminate the imidazole reagent. The protein was concentrated using a 30 kDa Amicon centrifuge filter (Millipore, www.emdmillipore.com). The aliquots were flash-frozen in liquid nitrogen and kept at −80 °C until further use.

Protein purity was > 95% as determined by densitometry analysis of the SDS-PAGE gel image. An aliquot of each protein was used to measure their concentration using Bradford assays (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as a standard. The β-conglutin purification yields ranged between 2–7 mg/ml.

2.4. Patients

Twenty-seven patients with positive skin prick test (SPT) and three with negative SPT were utilised in this study. Thirteen patients had known clinical reactivity to lupin and exhibit specific serum IgE to lupin (class 2–5; ImmunoCAP, Phadia, Uppsala, Sweden). Of these, nine patients reacted to lupin but not peanut and were recruited from workplaces involved in lupin research or in processing lupin flour or had presented to medical clinics with allergic reactions (Goggin et al., 2008). Five patients were peanut allergic individuals who had a positive food challenge to lupin and their sera were collected at the Allergy Unit at the Royal Prince Alfred Hospital, Sydney, Australia. Another fourteen exhibited specific serum IgE to peanut (class 2–4) but their reactivity to lupin was not known. They were recruited at the Allergy Unit, "San Cecilio" University Hospital, Granada, Spain. ImmunoCAP