Seed Storage Proteins, 2S Albumin And 11S Globulin, Associated to Severe Allergic Reactions after Flaxseed Intake

Short title: Food Allergy to Flaxseed Storage Proteins

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Resumen

Antecedentes: Dada la creciente popularidad de la linaza en las comidas, se han notificado varios casos de alergia a estas semillas. La información acerca de los alérgenos implicados en las reacciones de hipersensibilidad a estas semillas es escasa. El presente trabajo pretende identificar los alérgenos implicados en las reacciones mediadas por IgE en cinco pacientes con una historia clínica de síntomas sistémicos graves tras el consumo de linaza.

Métodos: Las proteínas susceptibles de ser alérgenos con capacidad de unir IgE se purificaron a partir del extracto de linaza mediante técnicas cromatográficas. Su identificación se realizó mediante espectrometría de masas MALDI-TOF. Se realizaron inmunoensayos con los sueros de los cinco pacientes alérgicos, utilizados de forma individual o como mezclas.

Resultados: Cuatro de los cinco pacientes reconocieron una proteína de baja masa molecular (alrededor de 13 kDa) en inmunoensayos con extracto de linaza, mientras que dos pacientes reconocieron una proteína de aproximadamente 55 kDa. Se identificaron por espectrometría de masas como albúmina 2S de linaza, incluida en la nomenclatura de alérgenos de la OMS/UIS como Lin u 1, y globulina 11S, respectivamente. Los ensayos de inhibición in vitro revelaron la existencia de reactividad cruzada de la Lin u 1 con las proteínas del cacahuete y del anacardo, mientras que el reconocimiento por parte de la IgE de la globulina 11S por parte de los sueros de los pacientes fue parcialmente inhibido por varias fuentes vegetales.

Conclusiones: Las proteínas de almacenamiento de las semillas de lino estaban implicadas en el desarrollo de síntomas graves en cinco individuos y mostraron una reactividad cruzada con otras fuentes alergénicas. Además de la gravedad de la alergia a la linaza en los pacientes sensibilizados a la albúmina 2S, es la primera vez que se identifica la globulina 11S como un alérgeno potencial. Consideramos que estos datos deben ser tenidos en cuenta para un diagnóstico más preciso de los pacientes.

Abstract

Background: Given the increased popularity of flaxseed in meals, several cases of allergy to these seeds have been reported. Little is known about allergens implicated in hypersensitivity reactions to these seeds. The present work aimed to identify the allergens involved in IgE-mediated reactions in five patients with a clinical history of severe systemic symptoms after flaxseed consumption.

Methods: Proteins susceptible to be allergens with IgE-binding capacity were purified from flaxseed extract by chromatographic techniques. Their identification was achieved via MALDI-TOF mass spectrometry. Immunoassays were performed using the five allergic patient’s sera either by testing them individually or as a pool.

Results: Four out of five patients recognized a low-molecular-mass protein (around 13kDa) by immunoblotting of the flaxseed extract, while two patients recognized a protein of approximately 55kDa. They were identified by mass spectrometry as flaxseed 2S albumin, included into WHO/IUIS allergen nomenclature as Lin u 1, and 11S globulin, respectively. Inhibition assays revealed in vitro IgE-cross-reactivity of Lin u 1 with peanut and cashew nut proteins, while IgE recognition of 11S globulin by patients’ sera was partially inhibited by several plant-derived sources.

Conclusions: Seed storage proteins from flaxseed were involved in the development of severe symptoms in five individuals and exhibited cross-reactivity with other allergenic sources. Besides the severity of flaxseed allergy in patients sensitized to 2S albumin, it is the first time that the 11S globulin is identified as a potential allergen. We consider that these data should be taken into account for a more accurate diagnosis of patients.

Keywords: Food allergy. Flaxseed. 2S albumin. 11S globulin. Cross-reactivity. Anaphylaxis.
Introduction

In recent years, edible seeds from sesame, sunflower or pumpkin, among others, have been incorporated into regular diet of humans due to their putative beneficial properties[1]. However, this has led to the appearance of hypersensitivity cases against these foods which very often involved severe allergic symptoms. The identification of the proteins that act as elicitors of those reactions is still a big challenge for specialists since these allergens are generally present in small amounts or as hidden components in many food formulas[2].

Among seeds recently incorporated in diet, *Linum usitatissimum* also known as flaxseed or linseed, is considered a “superfood” for its content on protein, dietary fibre, polyunsaturated fatty acids and lignin. Some of these compounds have been described by their helpful nutritional benefits, such as anti-inflammatory, anti-oxidant and cardio-protective properties[3]. Some proteins from flaxseed present emulsification capacities, of a great utility in food technology industry [4] and lately these seeds are highly consumed because of their laxative effect[5, 6]. This frequent use would explain the increase in cases of food allergy after its intake, displaying severe symptoms such as anaphylaxis.

Few cases of hypersensitivity manifestations to flaxseed have been described. In 2002, Leon et al. firstly reported a patient sensitized to a 56 kDa protein from flaxseed, pointing out amalate dehydrogenase (MDH) as an allergen from this seed [7]. Two other case reports of flaxseed hypersensitivity [8,9] involved proteins bands of around 22 and 20 kDa in the sensitization of two patients and a potential LTP has been indirectly involved in a patient with anaphylaxis to linseed [10]. A 2S albumin cDNA and its genomic sequence was identified from flaxseed and also named as conlinin, by Truska et al. in 2003[11]. However, it was not until 2018 when its potential implication in flaxseed allergy was reported[12].

The aim of the present study was to identify and characterize the allergens involved in severe allergic reactions of five patients after consumption of flaxseed, resulting in a 2S albumin and a 11S globulin. Attending to patients’ clinical histories, we also evaluated the implication of these allergens in the cross-reactivity with other seeds and nuts containing homologous proteins.
Materials and methods

Patients’ sera

Five well characterized patients with a clear history of immediate systemic reactions after flaxseed ingestion were recruited, three from Allergology services of Hospital Universitario Fundación Jiménez Díaz, one from Hospital Clínico San Carlos III and one from Hospital de la Princesa, all in Madrid, Spain. All individuals showed positive skin prick test (SPT) and/or specific IgE (ImmunoCAP, ThermoFisher) to flaxseed extract following the diagnostic algorithm of the European Academy of Allergy and Clinical Immunology [13]. The study was approved by the Ethic Committee of the Hospital (PIC28/2014) and written informed consent was obtained from all patients.

Linseed skin tests were carried out by means of prick by prick technique. The size of the reaction was recorded as a mean wheal diameter (D+d/2) with D indicating the larger diameter of the wheal and d indicating the largest diameter orthogonal to D. A prick test with a response of at least 3-mm diameter in wheal more than saline control was considered positive.

The work was performed accomplishing the Ethic Guidelines of Complutense University of Madrid.

Preparation of protein extracts from plant-derived sources and purification of seed storage proteins

Delipidated protein extracts from different vegetable sources, flaxseed included, were obtained as previously described [14]. Protein concentrations were determined by Lowry method.

Seed storage proteins from flaxseed were firstly isolated by a size exclusion chromatography using a Sephadex G-50 medium column equilibrated with 0.15 M ammonium bicarbonate pH 8.0 at a flow rate of 3 mL/min. SDS-PAGE analysis of the resulted fractions was performed, making a pool of the low molecular-mass protein fractions and applied in a second step to a High-Performance Liquid Chromatography (RP-HPLC), using a C-18 reverse phase column, with an acetonitrile gradient from 0% to 60% at a flow rate of 1.5 mL/min. Fractions were analysed again by SDS-PAGE and stained by means of Coomassie Blue R-250 (CBS) (Sigma-Aldrich). Cashew nut protein Ana o 3 was isolated following this methodology, while peanut allergen Ara h 2 was commercially available (Indoor Biotechnologies, USA).
For high molecular-mass proteins isolation, an anionic exchange chromatography was performed using DEAE-Cellulose matrix and a gradient of 0.02 M to 0.5 M ammonium bicarbonate pH 8.0 at a flow rate of 1 mL/min, followed by a second size exclusion chromatography superfine. The obtained fractions were examined by SDS-PAGE and bands visualized by Coomassie Blue R-250 staining. Mustard allergen Sin a 2 was isolated by this method as well.

Identification of 2S albumin and 11S globulin by Mass Spectrometry

The identity of the purified low molecular-mass protein was evaluated by matrix-assisted laser desorption/ionization (MALDI)-Time of flight (TOF) Mass-Spectrometry. For this purpose, SDS-PAGE in “sterile” conditions (buffers prepared in sterile Milli-Q® water) were performed after proteins isolation, staining the gel with colloidal Coomassie BlueG-250. Stained bands were excised and conserved in ultrapure Milli-Q® water to perform peptide mass fingerprinting in the Proteomics facilities at the Complutense University of Madrid (Spain). Samples were analysed in a mass spectrometer 4800 Proteomics Analyzer (AB SCIEX). This spectrometer has a MALDI ionization source and two TOF analysers in tandem, allowing to know the molecular masses of proteins and their fingerprint patterns. For high molecular-mass protein, apart from its fingerprint determination, four peptides were obtained by de novo synthesis.

Analytical procedures for proteins characterization

Purity and apparent molecular masses were monitored by SDS-PAGE in 15% (for high molecular mass proteins) or 17% (for low molecular-mass proteins) polyacrylamide gels. Samples were diluted in loading buffer (50 mM Tris-HCl, pH 6.8; 2% (w/v) SDS, 10% (w/v) glycerol, 2 mM EDTA, 0.01% (w/v) bromophenol blue), in the presence or absence of the reducing agent β-mercaptoethanol (βME, 5% of sample volume) and were denatured for 10 min at 90 °C. Coomassie Blue R-250 was employed for staining. Molecular mass determinations were done with protein markers (ThermoFisher, Massachusetts, USA). 2S albumin concentration was determined by measuring their absorbance at 280 nm using $E^{0.1%} = 1.28 \mu\text{L}/\mu\text{g} \cdot \text{cm}$, while 11S globulin concentration was obtained by using bicinchoninic acid (BCA) method (BCA Pierce, ThermoFisher, Massachusetts, USA).
Western blotting and inhibition assays

Purified proteins (2 μg) and food extracts (20 μg) were blotted onto nitrocellulose membranes (Amersham Biosciences, Barcelona, Spain) after SDS-PAGE in non-reducing conditions (-βME). Immunodetection of potential allergenic proteins were performed as previously described [15], using individual or a pool of sera diluted 1:5 in blocking solution 3% skim milk powder (SMP) in PBS buffer (8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.1% Tween-20 (PBS-T buffer), at a final volume of 300 μL per lane of extract or isolated protein. Non-atopic serum was employed as negative control. For human IgE-binding detection, mouse anti-human IgE monoclonal antibody was used (1:5000 diluted), kindly provided by ALK-Abelló (Madrid, Spain), and followed by horseradish peroxidase-labelled polyclonal rabbit anti-mouse IgG (1:2000 diluted; Pierce, Rockford, Illinois). The chemiluminescent signal was developed by ECL-Western Blotting reagent (Clarity Western ECL Substrate, Bio-Rad, Spain) and detected in a luminescent image analyser LAS3000 (FujiFilm). Quantitation of the signal was performed in triplicate using the computer program Multi gauge V3.0.

For the immunoblotting inhibition assays, equivolumetric pools of patients’ sera diluted 1:5 in blocking buffer were pre-incubated with 20 μg of the corresponding extracts (0.1 mg/ml inhibitor), or 5 μg of purified allergens (0.025 mg/ml inhibitor), using BSA as negative control, at room temperature for 2 h, before the incubation with membranes. Following steps were as described above.

ELISA assays

Indirect ELISA was performed in 96-well polystyrene plates (3950 High-binding plates, Costar) coated with purified proteins (2 μg/mL in PBS, pH 7.5, 100 μL/well) and incubated overnight at 4 °C. Plates were washed with PBS-T and blocked with 3% skim milk powder (SMP) in PBS-T, followed by the incubation with patients’ sera diluted 1:5 for 2 hours at 37 °C. Plates were washed with PBS-T and incubated with mouse anti-human IgE antibody, followed by HRP-labelled goat anti-mouse IgG antibody, both for 1 h at 37 °C, with PBS-T washes after each incubation. IgE binding was detected using o-phenylenediamine and H₂O₂ in 0.1 M citrate buffer and the reaction was stopped with the addition of 3N H₂SO₄. The absorbance in each well was measured at 492 nm in an iMark Microplate Absorbance Reader (Bio-Rad, Alcobendas, Spain) and non-specific
binding of antibodies was eliminated by subtracting the absorbance for control wells containing no antigen from the absorbance for antigen-coated wells. Positive reactions were considered those absorbance measurements at 492 nm greater than three-fold the standard deviations above the mean. A non-atopic serum was employed as negative control.

**Results**

*Immunological detection of potential allergens from flaxseed extract by patients’ sera.*

General characteristics of selected individuals are shown in Table 1. All patients described immediate allergic reactions after flaxseed intake as part of a snack or meal, developing severe and systemic reactions, such as anaphylaxis, showing positive skin prick test (SPT), from patient #2 to #5, to flaxseed extract. All of them exhibited different sIgE levels to flaxseed extract, being the lowest value for patient #1 (0.85 kU/L), whose serum recognized only the high molecular mass protein, and from 3.2 to 10.5 kU/L for the other four patients.

In order to determine the proteins involved in allergic reactions against flaxseed, patients’ sera were tested by immunoblotting with flaxseed extract in the presence and also in the absence of the reducing agent β-mercaptoethanol (βME). Reducing conditions disrupted the disulphide bonds that maintain the heterodimeric structure of many storage protein families present in the seeds, visible in the changes of electrophoretic mobility of the extract. Immunoassays (-βME) revealed two IgE-binding bands with an apparent molecular mass of 13 and 55 kDa. Four out of five patients presented IgE reactivity to the 13 kDa IgE binding band that drops to a band of 10 kDa, when the reducing agent was added. Serum #1 only recognized a ~55 kDa protein (-βME) that seems to split into a 34 kDa and 22 kDa bands (+βME) that could correspond to polypeptide chains of the 55 kDa protein; serum #2 recognized both proteins from flaxseed extract.[Figure 1]

*Isolation, identification and IgE-recognition of flaxseed 2S albumin and 11S globulin*

Attending to IgE-recognition of patients’ sera to flaxseed extract, immune-reactive proteins were isolated by different chromatographic methods as described.[Figure 1Supplementary]. CBS after electrophoretic analysis corroborated that 13 kDa protein split into two subunits of 10 kDa and ~ 3kDa, heavy and light polypeptide chains,
respectively [Figure 2A], while 55 kDa protein showed the 34 kDa and 22 kDa subunits as in the extract [Figure 3A].

In order to identify the nature of these proteins and which families belonged to, mass-spectrometry analyses were carried out with purified proteins. Low-molecular mass protein was identified as flaxseed 2S albumin - previously known as conlinin according to UniProt database- by peptide mapping, obtaining its fingerprint by MALDI-TOF-TOF techniques, with a 66% coverage [Figure 2A]. On the other hand, 55 kDa flaxseed protein required de novo sequencing procedures for its identification, obtaining four peptides that matched with 11S globulins from different vegetable species [Figure 3A]. Flaxseed 2S albumin was recognized by four out of five allergic patients’ sera, as shown in Figure 2B and C, by western blotting and ELISA assays respectively. Under reducing conditions, patient sera reacted exclusively against the 10 kDa heavy chain of the 2S albumin. Sera from patients #1 and #2 recognized flaxseed 11S globulin as well as its two subunits under reducing conditions [Figure 3B].

*In vitro cross-reactivity of flaxseed storage proteins with other plant-derived food sources*

Inhibition assays were conducted to determine the potential role of the identified allergens from flaxseed in cross-reactivity processes with other food sources. Thus, 2S albumin Lin u 1 IgE-recognition by a pool of patients’ sera (#2, #3, #4, #5)was completely blocked by cashew nuts extract (97%) and partially inhibited by peanut (57%). No inhibition was obtained with pine nut, and seeds from both mustard and sesame although patient #2 is sensitized to the last one and developed clinical symptoms after sesame seed intake [Figure 4A].

The IgE binding to the 11S globulin was partially blocked when a mixture of two sera from patients #1 and #2 that recognized the 11S globulin was incubated with extracts from mustard (30%) and sesame seeds (32%), as well as peanut (24%), cashew nut (26%) and pine nut (30%) [Figure 4B]. The cross-reactivity exhibited by this 11S globulin might explain the severe allergy symptoms to yellow mustard seed suffered by patient #1 and to sesame by patient #2, as their medical histories revealed. An equivolumetric mixture of IgEs from these two patients was able to recognize mustard seed 11S globulin (Sin a 2) and the purified flaxseed 11S globulin was able to partially inhibit this reactivity (47%) [Figure 4B]. Those data revealed a possible cross-reactivity
between flaxseed and yellow mustard seed due to their 11S globulins. Inhibition immunoblotting showed that flaxseed and mustard seed inhibited the recognition to each other 11S globulin.

**Discussion**

Seeds are important allergic inducers of systemic reactions including anaphylaxis. Incorporation of novel ingredients, like edible seeds from vegetable foods such as sunflower, pumpkin, flax or sesame seeds, into normal diet and other products has led to the description of new hypersensitivity cases due to those compounds either consumed as a food, in cosmetics applied by contact, or as laxative drugs as in the case of flaxseed [16]. Here we reported five cases of food allergy after flaxseed intake, developing systemic symptoms. Even though hypersensitivity reactions to these seeds has been previously described [17], there was little information related to molecular protein components involved.

Immune profiles resulted from testing patients’ sera with flaxseed extract revealed the presence of two potential allergens. The protein of 13 kDa, recognized by most individuals’s era, was identified as a 2S albumin, whose cDNA was previously sequenced and named as conlinin[11], recently related with the allergy of one patient against flaxseed[12]. Nevertheless, the low number of patients involved in that study and the lack of biochemical data were not enough to consider conlinin as a relevant allergen. The present work provides information about the structure of these allergens and more evidence of the severity of the reactions to flaxseed in patients sensitized to 2S albumins, as it happens with other proteins from the same family [18]. Due to the data provided in this study, conlinin has been included as allergen Lin u 1.01 in the official allergen database maintained by the World Health Organization and International Union of Immunological Societies (WHO/IUIS)Allergen Nomenclature Sub-committee.

Regarding IgE-binding profiles, Lin u 1 displayed similar features to other counterparts from different vegetable sources, as a heterodimer with two polypeptide heavy and light chains linked by disulphide bonds[19]. 2S albumins are characterized by a hypervariable region located in the heavy chain and containing the most relevant epitopes involved in food allergy [20].
The low similarity between the amino acid sequences of 2S albumins has made difficult to predict their potential cross-reactivity [Figure 2 Supplementary], usually being restricted to those from the same phylogenetic family [21, 14]. It is known that, despite the low conservation of amino acid sequences, 2S albumins present a highly preserved 3D structure[22]. Then, possible similarities at 3D level (structural epitopes) may be involved in the cross-reactivity observed between flaxseed, cashew nut and peanut through their 2S albumins. Most patients were polysensitized to several plant derived foods (mustard, tree nuts, sesame seeds, etc). However, here we have reported the in vitro cross-reactivity only between flaxseed and either cashew nut or peanut mediated by their respective 2S albumins. The link between peanut and cashew nut with flaxseed was already reported at clinical level[23], although no data had been described at molecular level until date. It is not the first time we report cross-reactivity between non-related sources mediated by their 2S albumins [24, 19]. These allergens have usually been associated to primary sensitization as described for Sin a 1 from mustard seed [25]. The current study supposes a valuable information for the diagnosis of patients since little was known until date about the cross-reactive potential of these allergens.

On the other hand, patients’ sera #1 and #2 also recognized a protein around 55 kDa, resulting in the 11S globulin of flaxseed according to proteomics. The small amount of patients did not allow to include this protein as an allergen in the IUIS database; however, inhibition assays showed the great potential of this protein in cross-reactivity with several plant-derived sources, as previously observed[26]. A bigger cohort of patients is needed in order to determine the relevance of this protein in allergy to flaxseed.

Summarizing, here we describe two IgE-binding storage proteins, a 2S albumin and an 11S globulin, involved in severe allergic reactions after flaxseed consumption. The 11S globulin showed a broader and partial cross reactivity with other plant-derived sources (seeds and nuts) than the one displayed by flaxseed 2S albumin. Interestingly, 2S albumin from flaxseed was cross-reactive to that from cashew nut, a finding that deserves future evaluation as it has been reported for the first time. The identification of potential allergens from edible seeds supposes a big challenge for specialists since these seeds are part of complex foods or snacks, acting as a hidden allergenic source. However, we should remember that these interesting data have to be corroborated with a higher number of flaxseed allergic patients. Nevertheless, the description of these two
flaxseed IgE-binding proteins and their cross reactivity will help, not only to improve the diagnosis and treatment of the allergic patients, but also to solve the complex world of food allergy cross-reactivity between seeds and nuts. The proper management of allergic reactions to food can have a great impact not only on the healthcare system and economy, but also on the lifestyle of individuals.

Informed consent was obtained from the patients to write the manuscript.

Conflicts of interest
The authors declare that they have no relevant conflicts of interest.

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Statement of Ethics
All authors have no ethical conflicts to disclose.

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References


Figures and Table

Figure 1. IgE-recognition of proteins on flaxseed extract.

SDS-PAGE and Coomassie Blue staining (CBS) of flaxseed extract. Western blotting (WB) of flaxseed extract tested with five patients’ sera, under non-reducing (-βME) and reducing (+βME) conditions. A non-atopic serum was used as negative control (C: control).

Figure 2. Identification and immunodetection of flaxseed 2S albumin.
(A) SDS-PAGE with and without βME of purified flaxseed 2S albumin. Signal peptide determined with SignalP program is shown in italics; fingerprint-matched peptides with conlininare shown in bold. This protein has been included in the WHO-IUIS database as an allergen, named Lin u 1.01. (B) IgE recognition by western blotting of isolated 2S albumin from flaxseed by serum#2, #3, #4 and #5, under non-reducing (-βME) and reducing (+βME) conditions. (C) ELISA test of purified 2S albumin from flaxseed (Lin u 1), cashew nut (Ana o 3) and peanut (Ara h 2) with individual serum from flaxseed-allergic patients. Non-atopic serum was employed as negative control (WB: C; ELISA: OD_{490 nm} ≤ 0.1).

Figure 3. Identification and immunodetection of flaxseed 11S globulin.

(A) SDS-PAGE and identification of flaxseed 11S globulin, by de novo sequencing. Possible variations in amino acid sequences are shown initalics bold. Obtained peptides matched with 11S globulins from several plant sources. (B) Isolated 11S globulin recognition by serum#1 and #2, under non-reducing (-βME) and reducing (+βME) conditions. A non-atopic serum was used as negative control (C: control).
Figure 4. Immunoblotting inhibition assays (-βME).

(A) Inhibition of IgE-recognition to purified flaxseed 2S albumin (2 µg) by different extracts (200 µg; 100 µg/mL) as inhibitors, pooling patients’ sera #2, #3, #4 and #5. (B) Inhibition of IgE-recognition to purified 11S globulins from flaxseed and mustard seed (2 µg) by different extracts (200 µg; 100 µg/mL) and proteins (5 µg; 25 µg/mL) as inhibitors, using a pool of patients’ sera #1 and #2 (FS: flaxseed).
### Table 1. Patients’ clinical information

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AN, anaphylaxis; D, diarrhea; G, gastric symptoms; OAS, oral allergy syndrome; F, female; M, male; ND, no determined; Neg, negative (wheat mean diameter< 7 mm for SPT); Pos, positive (wheat mean diameter> 7 mm for SPT); al, almond; e, egg; h, hazelnut; m, mustard seed; p, pistachio; s, sesame seed; sf, seafood; soy, soya; w, wheat; wa, walnut; wm, watermelon.

*SPT: Skin Prick Test (mean diameter, mm)

†Specific IgE determined by ImmunoCAP (kU/L)