

Maize food allergy: lipid-transfer proteins, endochitinases, and alpha-zein precursor are relevant maize allergens in double-blind placebo-controlled maize-challenge-positive patients

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Abstract Italian patients with maize anaphylaxis have been shown to have IgE toward two major maize allergens: an alpha-amylase inhibitor and a 9-kDa LTP. A complete study on maize food allergens in patients with positive maize double-blind, placebo-controlled food challenge (DBPCFC) is lacking. The objective was to utilize the three maize protein fractions to identify and characterize the most relevant IgE-binding proteins recognized by the sera of Italian and Swiss patients with either a positive maize-DBPCFC or a history of maize-induced anaphylaxis. Osborne's protein fractions of maize were extracted to obtain water-soluble, total zein,

and total protein fractions. Protein IgE-binding capacity was investigated by SDS-PAGE immunoblotting using the sera from DBPCFC-positive patients and from patients with maize-induced anaphylaxis. Purified maize LTP was used to inhibit the IgE immunoblotting of the three protein fractions. IgE immunoblotting demonstrated that the 9-kDa LTP was recognized by all the Italian patients and by none of the Swiss patients. Other allergens were: 14-kDa α -amylase inhibitor, 30-kDa endochitinases A and -B, 19 kDa zein- β precursor, and 26kDa zein- α precursor; a newly described allergen, the globulin-2 precursor, identified in the total protein fraction. It is noteworthy that maize LTP and endochitinase were cross-reactive with grape LTP and one grape endochitinase. LTP was found to be the only major allergen in Italian patients with either positive maize challenge or a history of maize-induced anaphylaxis. We have identified other maize allergens in subjects with maize food allergy, as grape cross-reactive endochitinase, however, the clinical significance of these proteins needs to be investigated in larger groups of patients with allergy to these food items.

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Introduction

Allergy to maize can cause different clinical manifestations according to the kind of environmental exposure, dietary

habits, and pre-existing sensitizations. Thus maize allergy can be represented by maize-pollen-induced respiratory symptoms [1], by occupational asthma [2] or dermatitis [3] due to inhaled or handled maize flour, or by food allergy due to maize ingestion [4]. The different tissues of this crop are all allergenic constituents and present several IgE-binding proteins that have been related in some cases to different clinical manifestations. For example, Zea m1, a β -expansin of 28–35 kDa [5, 6] is a major maize-pollen allergen responsible for seasonal symptoms; Zea m13 [6, 7], which is a poly-galacturonases, and Zea m12 which is a profilin [6] are also pollen allergens. A 9 kDa kernel LTP, named Zea m14 [8], is the unique major food allergen [8]; a 16 kDa α -amylase trypsin inhibitor [8], and a recently identified thioredoxin [9], named Zea m 25, are minor maize food allergens [9, 10]. The latter protein has been shown to be cross-reactive with Tri a25 of wheat [11] and has been reported to be involved in baker's asthma [11]. Another food allergen that has been identified but not characterized is a reduced soluble 50-kDa protein described by Pasini et al. [12]; we recently confirmed the presence of this allergen in a 2D study [10]. It is noteworthy that a recent comprehensive editorial on maize allergy reported that maize food allergy has been described mainly in Italian patients and only isolated cases have been reported in other countries [13]. The demonstration that the most relevant maize food allergen is an LTP that is highly cross-reactive with peach LTP, which is one of the most common food allergen in our country and characterizes a good part of the Italian peach-allergic population, may explain the high frequency of maize allergy in Italy [8]. However, maize LTP recently has been found to be a relevant allergen also in a group of patients from the US, thus suggesting that it is a very important and unique maize food allergen [14]. This may be due to the characteristic resistance to thermal treatment of maize LTP, i.e., it is the only maize allergen that maintains IgE-binding property at 100°C for 161 min [15]. Another possible explanation of the relative high frequency of maize allergy in Italy can be the high exposure to airborne maize dust, due to its high production, in fact production has reached about 10.5 million tons per year (ISTAT official data 2008) with nearly all of the corn originating from Northern Italy, where the majority of the Italian maize-allergic patients come from (www.istat.it).

Few studies have comprehensively addressed the IgE-binding properties of the three maize fractions, subdivided on the basis of the different solubility, in a conspicuous number of patients with proven maize food allergy. For this reason, we took part in a study that identified subjects with DBPCFC proven maize food allergy [4] and employed the sera obtained from challenge-positive patients and maize-anaphylaxis patients to better characterize maize food allergens. The aim of this study was to identify the most

relevant IgE-binding proteins of all the extractable maize proteins (i.e., the albumin/globulin, zeins and glutelins fractions) as well as new cross-reactive allergens in a population of Italian and Swiss patients with maize anaphylaxis or documented positive maize-DBPCFC.

Materials and methods

Patients

Patients admitted to the study were selected in three Allergy Units: Niguarda Cà Granda Hospital (Milan, Italy); Milan University Hospital, Department of Internal Medicine, and Dermatology, IRCCS (Milan, Italy) and Allergy Unit, Department of Dermatology, University hospital (Zurich, Switzerland).

All of the patients included in this study had a clinical history of allergic reaction to maize: 12 were maize-DBPCFC-positive, six had a documented history of life-threatening anaphylactic reaction after eating maize, and one was positive to open challenge with raw maize. The sera of 14 patients with suspected maize allergy but negative maize-DBPCFC were used as controls, as described in the *in vivo* study [4].

Skin test with purified maize LTP

In order to test the biological activity of LTP, patients n.1 and n.8 underwent SPT with purified LTP.

Extraction proteins from corn flour

Maize flour was obtained from Agnesi SpA. Sequential extraction of maize proteins was performed according to the method described by Pasini et al. [12]: 5 g of commercial corn flour were stirred in 25 ml of 0.5 M NaCl for 2 h at 4°C. After centrifugation (12,000×g, 20 min, 4°C) the supernatant containing the salt-extractable proteins (albumin–globulin fraction) was collected and stored at –20°C. The residue was washed with 50 ml of 0.5 M NaCl and centrifuged again; the procedure was repeated and a final wash with distilled water was done. This albumin/globulin-free residue was then extracted with 25 ml of Tris–HCl, pH 8.5 containing 50% (v/v) propan-2-ol, 2.5% (v/v) 2-mercaptoethanol (2-ME) overnight at room temperature with continuous stirring. After centrifugation (12,000×g, 20 min, 4°C) the supernatant containing alcohol-extractable proteins (zein fraction) was collected and stored at –20°C. A total protein extract of maize was performed as follows: 100 mg of flour with 4 ml of 100 mM Tris–HCl buffer, pH 8.3, containing 6 M urea, 2.5% (w/v) sodium dodecyl sulfate (SDS), 2.5% (v/v) 2-ME and 10% (w/v) glycerol and heated for 5 min at 100°C. After

centrifugation (12000 g, 20 min), the supernatant containing the total protein fraction (glutelin), was collected and stored at -20°C .

The protein concentration of three maize fractions was determined by Bradford assay [16]. The protein concentrations in the various albumin/globulin, zein, and glutelin fractions were: 1.15, 0.25, and 0.25 mg/ml respectively.

SDS-PAGE immunoblotting

The separation procedure is based on the method described by Neville [17].

Every fraction was run in a discontinuous buffer system in a SDS-polyacrylamide gradient gel with a 6% stacking gel and a 7.5–20% separation gradient gel at 6 mA for 16 h in a BIO-RAD Protein IIxi vertical electrophoresis Slab Cell (BIO-RAD Laboratories, Richmond, California). Before electrophoresis, albumin/globulin and zein fractions were diluted 1:2 in sample buffer containing Tris (hydroxymethyl-amino methane) at pH 6.1, 2% sodium dodecyl sulfate, 3% β -mercaptoethanol, 5% glycerol, and 0.001% Bromophenol blue. After dilution, the samples were denatured by heating at 100°C for 5 min. The glutenin fraction was loaded into the gel without the procedure described above.

After electrophoresis each fraction was electro-blotted to a nitrocellulose membrane (pore size $0.45\text{ }\mu\text{m}$; Amersham, Bucks, UK) by using a Trans-Blot Cell (BIO-RAD Laboratories, Richmond, California) at $0.45\text{ }\text{\AA}$, 100 V for 4 h at 4°C . The nitrocellulose paper was cut into strips, which were incubated with the sera of each patient and with the sera of control patients. The bound specific antibodies were detected by incubation with ^{125}I -labeled anti-IgE antibodies (Pharmacia) and exposure on auto radiographic film (Hyperfilm Amersham) at -70°C for 4–7 days.

Extraction and purification of the maize 9-kDa allergen by HPLC

The 9-kDa protein was purified from the albumin/globulin fraction by high-performance liquid chromatography (HPLC) as previously described [8] in order to perform skin prick tests and immunoblotting inhibition.

Extraction of proteins from grape

An extract of grape was prepared to investigate the relevant homology that maize has with grape as regards a new maize allergen such as endochitinase. The grape extract (*Vitis vinifera*) was prepared as described by Pastorello et al. [18]. The protein concentration was determined by using the method of Bradford assay. The protein concentration of the grape extract was 3.2 mg/ml.

Immunoblotting inhibition experiments

1. Maize/maize 9 kDa

An immunoblotting inhibition experiment was performed to confirm that the purified maize 9-kDa protein identified in the three different fractions corresponded to an LTP.

Briefly, 500 μl of pooled sera of patients were incubated with 500 μl of 9 kDa maize-purified LTP at different concentration (500 μg , 50 μg , and 5 μg for albumin/globulin and glutelin fractions and 1,000 μg , 500 μg , 50 μg , and 5 μg for zein fraction). The inhibited sera were matched with the nitrocellulose strips with blotted proteins of every maize fractions and IgE binding was detected as described above.

2. Maize/grape–Grape/maize

In consideration of the high homology between maize and grape endochitinase, we performed an immunoblotting inhibition experiment to assess IgE cross-reactivity between grape and maize. Briefly, 500 μl of pooled sera of patients that had both maize and grape symptoms (n. 1, 4, and 6), were preincubated with 500 μl of undiluted maize albumin/globulin fraction. Later, the grape IgE immunoblotting was repeated as above. Afterward, maize immunoblotting was repeated after preincubating 500 μl of the same pool sera with 500 μl of undiluted raw grape extract.

Amino acid sequencing

To determine the N-terminal sequence, separated proteins were excised from the SDS-PAGE gel and microsequenced as already described elsewhere [19, 20].

“In gel” reduction and digestion

Bands in the SDS-PAGE gel that corresponded to the immunoreacting bands obtained from immunoblotting were cut and reduced by the addition of 100 mM DTT (56°C , 1 h) and alkylated by addition of 200 mM iodoacetamide (room temperature in the dark for 45 min). The proteins were then digested with sequencing grade, modified trypsin (Promega, Madison, WI, USA) as already described, the only difference being that the volume of the combined extracts was not reduced by rotary evaporation [20]. A desalting and concentrating step by ZipTip_{C18} devices (Millipore, MA, USA) was introduced and the manufacturer's instructions were followed. The peptide elution step was performed with 50% acetonitrile/1% formic acid.

Mass spectrometry

For matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, 0.5 μl of each

Table 1 Demographic data, clinical history, skin test with maize and pollen, maize-DBPCFC results, provocation dose (PD)

	Sex	Age	History	Maize SPT	Maize CAP (ku/l)	Pollen	Pollens with symptoms	Results maize-DBPCFC	PD (g)
1	F	50	U, OAS, AE	+++	68.3	G, B, P, R	G, B, P, R	+	2.5
2	F	35	AE	+++	7.51	G, B, R	G, B, R	+	0.5
3	F	12	V, GI	+	4.34	M	M	+	0.1
4	F	24	A, AD	++	63.4	G, B, P, M, R	G, B, P, M, R	+	1,6
5	M	32	U	+++	2.8	G, P, M	G	+	10
6	F	25	OAS, E	–	5.59	G, B, P	G, B, P	+	0.1
7	F	24	OAS, U	++	1.36	M		+	25
8	F	24	OAS, U	+++	2.54	G, B	G, B	+	25
9	M	37	U, AE	–	0.42	R		+	25
10	F	38	AS	N.D.	3.2	G, P, M		N.D.	N.D.
11	F	22	AS	++	8.2	G, B	G, B, P, M, R	N.D.	N.D.
12	F	65	AS	+	1.5	G, B, P		N.D.	N.D.
13	M	30	U, LE, D	++	11.6	G, B, M, R		N.D.	N.D.
14	F	27	U, D, BP	N.D.	2.07	G, B		N.D.	N.D.
15 ^a	F	29	OAS, U, AE, D	++	N.D.	–		+	Not known
16 ^a	M	39	E, OAS, BP	++	1.9	–		N.D.	N.D.
17 ^a	F	47	P, D	+	3.3	–		+	Not known
18 ^a	F	25	OAS	+	N.D.	G, B		+	0.6
19 ^a	F	35	OAS	++	N.D.	G, B		+	1.6

GI abdominal colic/diarrhea, V nausea/vomit, AD atopic dermatitis, OAS oral allergy syndrome, P pruritus, E erythema, U urticaria, AE angioedema, D dyspnea, A asthma, AS anaphylactic shock, LE larynx edema, BP drop of blood pressure, G grass, B birch, P parietaria, M mugwort, R ragweed, N.D. not done, PD provocation dose

^a Swiss patients

peptide mixture from the “in gel” digestion was applied to a target disk before the desalting step and allowed to air dry. Subsequently, 0.5 μ L of matrix solution (1% w/v α -cyano-4-hydroxycinnamic acid in 33% acetonitrile, 0.1% TFA) were applied to the dried sample and again allowed to dry. Spectra were obtained using a Bruker ReflexIII MALDI-TOF (Bremen, Germany). To interpret the mass spectrometry (MS) spectra of protein digests, Profound (<http://prowl.rockefeller.edu/cgi-bin/ProFound>), MASCOT (<http://www.matrixscience.com/home.html>), MS-Fit (<http://falcon.ludwig.ucl.ac.uk/MS-Fit.html>) software were used by means of the peptide mass fingerprinting method [21].

For MS/MS sequencing experiments with the ion trap mass spectrometer, 4 μ L of the tryptic peptide mixture eluted from the ZipTipC18 were applied into the borosilicate capillary. The capillary voltage was set at 46 V and spray voltage at 1.6 kV. The most stable signals from the full-scan mass spectrum were trapped and fragmented by low-energy collision-induced decomposition (CID), with normalized collision energy ranging from 22% to 26%. MS/MS spectra were automatically analyzed using SEQUEST software screening OWL database.

Skin prick tests with purified allergens

To determine the biological activity of the 9-kDa purified protein, we performed skin prick tests with the purified

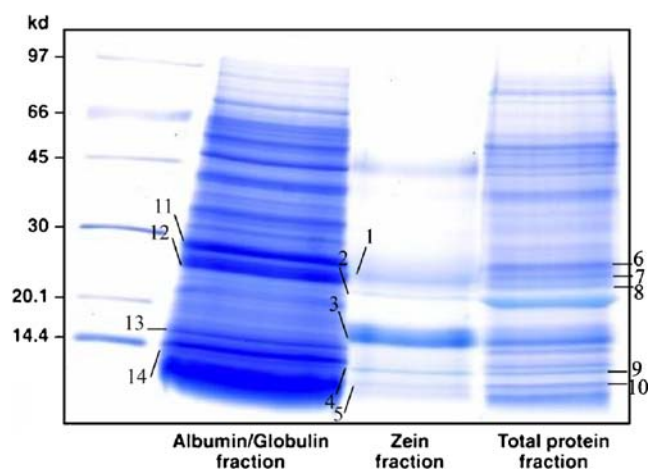
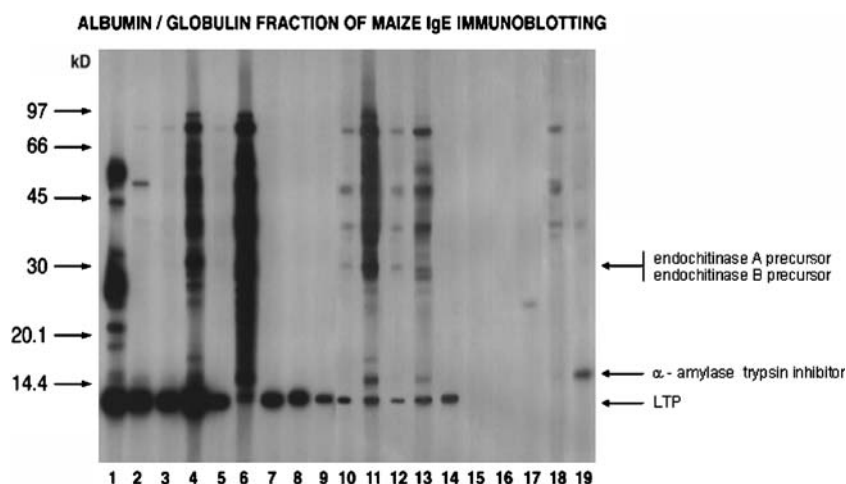


Fig. 1 SDS-PAGE profile of the three protein fractions (albumin/globulin, zein, total protein fractions) of maize. The numbers indicate the bands corresponding to the allergens identified and reported in Table 2 (bands 1–5 correspond to proteins of the zein fraction; bands 6–10 correspond to total proteins fractions; bands 11–14 correspond to proteins of the albumin/globulin fractions)

Fig. 2 IgE immunoblotting of the albumin/globulin fraction of maize with the sera of 19 patients studied. The proteins identified are endochitinase A and B precursor, α -amylase trypsin inhibitor, LTP



protein. Briefly, four samples of 9 kDa purified protein diluted in 0.9% NaCl and 50% glycerol at the protein concentration of 2.5, 5, 10, and 20 mcg/ml respectively quantified by the Bradford microassay [16] were tested by skin prick test. Skin prick tests were considered positive with a weal diameter ≥ 3 mm. Histamine hydrochloride (10 mg/ml) and 0.9% NaCl, and 50% glycerol served as positive and negative controls, respectively.

Results

Patients

Nineteen patients with maize allergy were included in the study (18 adults and one child). Twelve patients were maize-DBPCFC-positive (n. 1–9, 17–19): nine were Italian (eight adults and one child) and three were Swiss adults (n. 17–19). One Swiss patient (n. 15) was positive to open maize challenge. Six patients had a history of maize

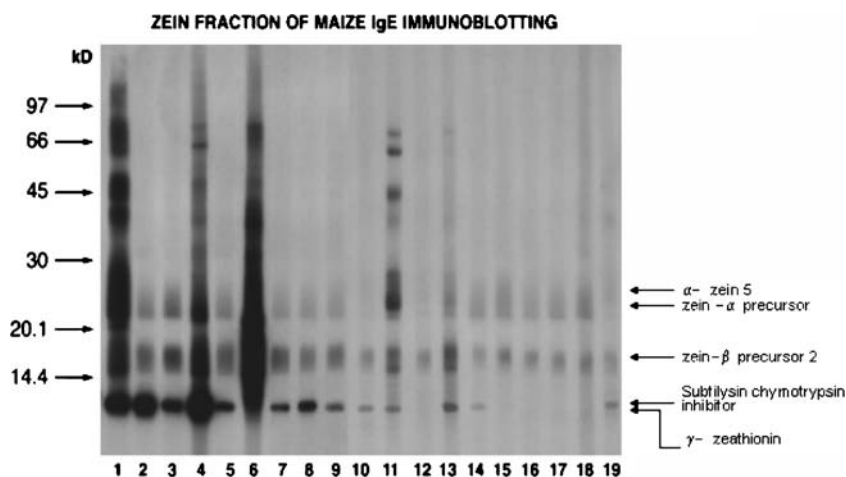
anaphylaxis (n. 10–14 and 16): five patients were from Italy (n. 10–14) and one was from Switzerland (n. 16). The clinical characteristics, the skin prick test, and CAP results of the patients are reported in Table 1. Seven maize-allergic patients also were allergic to grape (patients 1, 4, 6, 10–13); patients 1, 4, and 6 correspond to patients 2, 4, and 5 of the clinical paper [4].

Identification of allergens

SDS-PAGE

Figure 1 shows the SDS-PAGE of the three maize fractions extracted following the method described by Pasini [12]. The albumin/globulin fraction contains several proteins with MWs between 9 and 97 kDa; three areas are of particular interest: 9 kDa, 14–16 kDa, and 27–30 kDa. The zein fraction shows proteins between 9 and 45–50 kDa, with major bands at 18–19 kDa and at 26–28 kDa. The total protein fraction shows proteins with apparent MWs ranging

Fig. 3 IgE immunoblotting of the zein fraction of maize with the sera of 19 patients studied. The proteins identified are α -zein 5, zein- α precursor, zein- β precursor 2, subtilysin chymotrypsin inhibitor, γ -zeathionin



from 9 to 97 kDa with in particular a group of bands at 20–30 kDa and at 9 kDa.

Immunoblotting-IgE

Albumin/globulin fraction

Fourteen of the 19 patients (73%) recognized a 9-kDa protein corresponding to the LTP named Zea m14 in the albumin/globulin fraction (Fig. 2). It is noteworthy that six patients reacted only towards this band (n. 3, 5, 7–8, 9, 14), whereas eight patients also reacted towards bands of higher molecular weight (n. 1, 2, 4, 6, 10–13). Seven of the 19 patients recognized the group of proteins in the 14–16 kDa range (n. 1, 2, 4, 6, 11, 13, 19), previously identified as alpha-amylase trypsin inhibitors [8]. Proteins in the 27–30 kDa range, which correspond to the endochitinase A and B precursors (access N.P29022 and P29023), also were recognized by the sera of seven of 19 (36%) patients (n. 1, 4, 6, 10–13).

Patients 1 and 6 recognized an allergenic band at 17 kDa that was identified as a truncated species of a vicilin-like embryo storage protein with MW of 60 kDa (Q03866). Other allergenic proteins were detected in the range of 45–60 kDa (nine of 19 patients, 47%) and of 80–90 kDa (seven of 19 patients, 36%). Specific IgE reactivity was not detected in the negative control sera.

Zein fraction

The sera of 14/19 (73%) patients recognized an allergenic component located at 9 kDa in the zein fraction (n. 1–11, 13, 14, and 19), see Fig. 3. This band corresponded to a subtilisin/chymotrypsin inhibitor of maize that was identified by MS analysis. Moreover, the sera of five of 19 patients (n. 1, 4, 6, 11 and 13) showed clear IgE binding in the range of 18–20 kDa and 16–28 kDa, corresponding to

beta- and alpha-zein precursors, respectively. However, this area showed slight general binding with the majority of the patients' sera as well as with the sera of five negative control (data not shown), thus suggesting a non-specific binding.

Total protein fraction

In the total protein fraction, 11/19 sera (57%) recognized the 9-kDa protein corresponding to LTP (n. 1–5, 7, 8, 10, 11, 13 and 14), see Fig. 4. Two IgE-binding components, at 25 and 28 kDa, corresponding to an alpha-zein precursor and a globulin-2 precursor, respectively, were recognized by pts n. 1, 4, 6, 11, and 13. These patients also recognized a 30-kDa protein corresponding to the same endochitinase A precursor previously identified in the albumin fraction. The IgE immunoblotting of the albumin/globulin, zeins, and total protein fraction with four out of 14 sera of DBPCFC-negative patients (data not shown) showed an allergenic profile that was very similar to that of DBPCFC-positive patients.

Purification and characterization of maize 9 kDa

The 9-kDa component identified on the IgE immunoblotting as the major allergen in the albumin/globulin and in the total protein fraction, was purified with HPLC and was identified by MALDI as a non-specific lipid-transfer protein (LTP). The purity and IgE-binding capacity of the LTP was confirmed by immunoblotting-IgE using the sera of patients n. 1–3.

Characterization of other maize allergens

Table 2 shows the allergens identified either by Edman degradation or by mass spectrometry approaches in the different maize fractions.

Fig. 4 IgE immunoblotting of the total protein fraction of maize with the sera of 19 patients studied. The proteins identified are endochitinase A precursor, globulin 2 precursor, zein- α precursor, LTP, γ -zeathionin

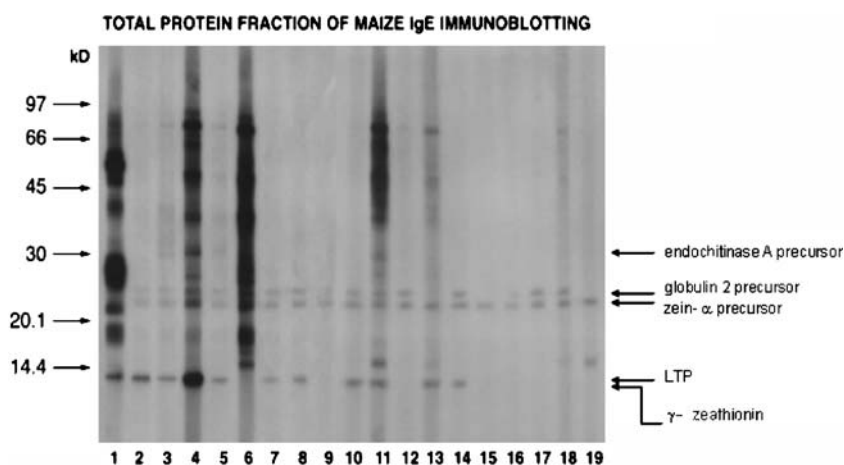


Table 2 Allergens identified in the three maize protein fractions

SDS-PAGE band N.	Allergen	MW kDa	Identification	
			Mass Spectrometry MALDI (%coverage) ESI-MS/MS (peptide sequences)	Edman Degradation N-terminal sequence
1	Azs 22-12 (22kDa alpha-zein 5) (Q9SYT3)	25	–	FIXPQXSLAPQA
2	Zein-alpha [precursor] (P24449)	25	–	ATIFPQXSQAPIA
3	Zein-beta precursor (zein 2) (P06673)	20	–	MQMPXPXAGLQG
4	Subtilisin/chymotrypsin inhibitor-maize (S61830) Q43723	7	MALDI-TOF 72%	–
5	Gamma-zeathionin 2 (P81009)	5	MALDI-TOF 53%	–
6	Endochitinase A precursor (P29022)	30	MALDI 45%	–
7	globulin-2 precursor (S15675)	50	MALDI 26% confirmed by ESI-MS/MS VVMLLSPVVSTSGR (141–154) FTHELLEDAVGNYR (56–69)	–
8	globulin-2 precursor (S15675)	50	MALDI 39% confirmed by MS/MS AFLQPSHYDADEVFMVK (79–95) FTHELLEDAVGNYR (56–69) VVMLLSPVVSTSGR (141–154) VAELEAAPR (70–78)	–
	zein-alpha precursor (P06678)	26	MS/MS FEYPTIQSYR (40–49)	–
9	Non-specific lipid-transfer protein precursor (P19656)	9	MALDI 90%	–
10	Gamma-zeathionin 2 (P81009)	15	MALDI 74%	–
11	Endochitinase B precursor (seed chitinase B) (P29023)	30	–	PGRVARDAVVA
12	Endochitinase A precursor (seed chitinase A) (P29022)	30	–	PNRVAQDAVIAF
13	Trypsin/Factor XIA Inhibitor precursor (P01088)	16	–	LPWPELKRRCCRELA
14	Non-specific lipid-transfer protein precursor (P19656)	9	–	AISXGQVAS

Skin prick test with purified maize LTP

The skin prick test with purified 9-kDa protein performed on the two patients tested resulted positive. Patients n.1 and n.8 reacted to maize LTP at a protein concentration of 2.5 mcg/ml with a weal diameter of and 8 mm. This confirms the high biological activity of the purified 9-kDa maize LTP.

Immunoblotting inhibition experiments

1. Maize/maize 9 kDa

Figure 5 shows that LTP inhibited the IgE binding to the 9 kDa band in all three fractions. It is noteworthy that in the zein fraction the 9 kDa allergen was not identified as an LTP but as a subtilisin/chymotrypsin inhibitor, belonging to the alpha-amylase inhibitor family. However, this allergen was totally inhibited by LTP.

2. Maize/grape–Grape/maize

In Fig. 6a, we can see that preincubation with grape completely inhibited the IgE binding to the maize 9 kDa while inhibited by 90%, the IgE bind to endochitinase A and less significantly that one to endochitinase B; Fig. 6b also shows the high rate of cross-reactivity between the 25-kDa protein that we have previously identified in grape as being an osmotin-like protein.

Discussion

We investigated the maize allergens of the three extractable maize fractions responsible for symptoms in 13 patients with a positive maize-DBPCFC and six patients with

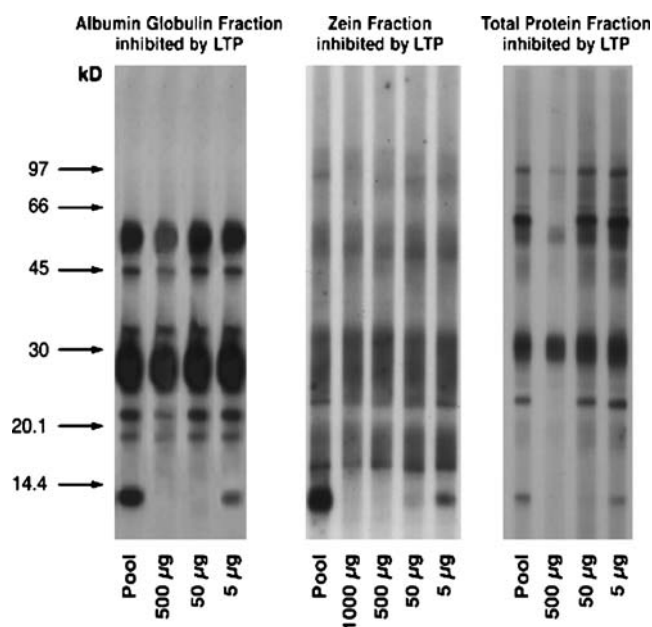


Fig. 5 IgE immunoblotting inhibition of the albumin/globulin and total protein fraction with 500, 50, 5 mcg of the maize-purified 9-kDa protein and zein fraction with 1,000, 500, 50, 5 mcg of maize-purified 9-kDa protein

documented history of maize-induced anaphylaxis. We thus confirmed our previous observation that the LTP designated Zea m14 is the major allergen of maize in Italy inasmuch as specific IgE reactivity towards this allergen was seen in all the Italian patients enrolled in this study (Fig. 2). The 9-kDa protein was demonstrated to be an LTP by the inhibition that was observed with 50 mcg of purified maize LTP.

The lipid-transfer proteins are low-molecular-weight (9 kDa) members of the prolamine superfamily; these proteins are also included in the list of pathogenesis-related proteins (i.e., PRP-14) and are important plant food allergens that are not related to concurrent pollen sensitization [22].

When compared to plant food allergens that are homologous to birch allergens, lipid-transfer proteins are usually considered responsible for systemic food allergic symptoms. In this study, we demonstrated that 11 Italian patients were all LTP positive and that eight manifested systemic symptoms with severe reaction in three of eight patients; only three of 11 patients manifested only OAS; however, these patients reacted to a very low maize-challenge dose, and the challenge was discontinued, thus possible systemic symptoms were not manifested.

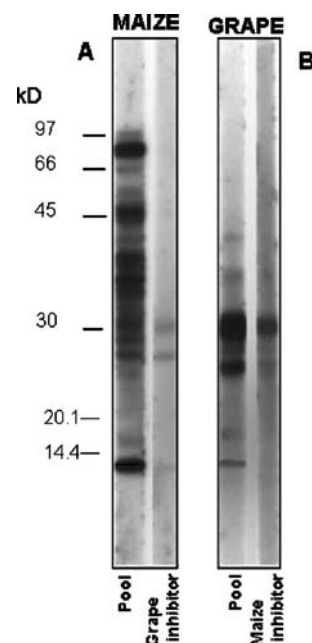
Moreover, we found that the IgE immunoblotting with the sera of patients that were negative to maize-DBPCFC also had IgE that bound the 9-kDa protein. Thus, maize LTP sensitization also can be detected in patients that have no symptoms with maize. The sensitization observed in

asymptomatic patients may be an indication of clinical symptoms that will become manifest at a later time. Although maize challenge was negative, these patients need to undergo follow up so as to detect future development of symptoms. In the meantime, patients have been instructed to be cautious as regards the ingestion of maize.

The prominent allergenic and biological activity of the purified maize LTP was shown by the positive skin prick test results obtained with solutions containing the 9-kDa protein at concentrations of 2.5 and 5 mcg/ml. Of great interest was the observation that five patients showed IgE reactivity to the prolamin of maize called zein. The fact that only five patients showed reactivity to these storage proteins allowed us to identify alpha-zein (22 kDa), alpha-zein precursor (19 kDa), and a beta-zein (16 kDa) as minor allergens of maize. These five patients had evident IgE binding to these proteins; however, it is not clear why slight non-specific binding also was observed in negative controls. Perhaps these proteins possess an allergenic potential that needs to be better investigated especially when one considers that zeins are found in cooked maize. It is noteworthy that similar results were found in a previous study on wheat [19].

A protease inhibitor also was identified in the zein fraction, subtilisin/chymotrypsin inhibitor (7 kDa); this protein belongs to the α -amylase inhibitor family and is an inhibitor of the serin-endopeptidase that is induced by external microorganisms. The binding to this protein was apparently inhibited by preincubation with LTP. This could be related to the presence of a small amount of LTP in this fraction or to a high rate of cross-reactivity between the two

Fig. 6 a IgE immunoblotting inhibition of albumin/globulin fraction of maize extract by undiluted raw grape extract; **b** IgE immunoblotting inhibition of raw grape extract by undiluted albumin/globulin fraction of maize extract



proteins. It is noteworthy that LTP was directly identified in the total protein fraction.

All these results are particularly interesting because they add emphasis to the clinical role of LTP, inasmuch as this protein can be extracted not only in the water-soluble fraction but also in the alcohol-extractable and total protein fractions, clearly demonstrating that it is a major allergen that can be found in all the forms under which maize can be consumed.

Two new allergens, endochitinase A and B precursor, were identified in the water-extractable (albumins and globulins) and in the total protein fractions and were recognized in seven of 19 patients. These proteins belong to the PR3 chitinases, class I group of the “pathogenesis related protein” family, and present significant homology with tomato endochitinase C (homology 58.4%) and with grape endochitinase IV (homology 62%). This protein is responsible for allergic reactions to grape and possibly to wine [18] as we had previously presumed on the basis of the reactivity toward a grape endochitinase IV in subjects allergic to both grape and wine [18]. Sbornik et al. have definitely demonstrated the allergenic role of endochitinase IV in wine by studying a subject with grape and wine anaphylaxis, via specific IgE detection to this allergen in red wine as well as in grape extract [23]. The high rate of homology between grape and maize endochitinase and LTPs (Fig. 6a and b) can explain both the high rate of cross-reactivity between the homologous maize and grape allergens and the clinical reactions to maize and grape in the positive patients. It is noteworthy that the allergenicity of this maize endochitinase was already described in a recent in vitro 2D study [10], and the epidemiological relevance demonstrated by the present study let us conclude that endochitinase A is a newly described maize allergen that belongs to the class of pathogenesis-related proteins. Other minor allergens were a vicilin-like protein, found in the albumin/globulin fraction, and a globulin-2 precursor, found in the total protein fraction; these proteins also had been identified in the previous qualitative 2D study [10]. It is interesting to note that most of these proteins have been reported by Zolla et al. in a study that mapped all wild-type maize proteins on 2D gel [24].

In conclusion, this study has identified LTP, endochitinase, zein, and vicilin precursors as maize allergens with variable importance as regards maize food allergy. The results of our findings lead one to conclude that LTP is the principal maize food allergen in Italian patients; moreover, the cross-reactivity seen in vivo probably depends on clinical allergy to more than one protein, as we have seen for grape, i.e., subjects that are allergic to both maize and grape were positive to both LTP and endochitinase, whereas subjects that were allergic to maize LTP were cross-reactive to grape in vitro but did not have symptoms with grape.

Lastly, considering that maize-allergic patients showed a provocation dose that varied a great deal [4] and that subjects that had undergone maize challenge showed a wide range of symptom severity [4], it may be that co-sensitization to minor allergens modulates the spectrum of symptom severity and provocation dose whereas sensitization to only maize LTP is associated with severe maize allergy.

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