

Biochemical, immunological and clinical characterization of a cross-reactive nonspecific lipid transfer protein 1 from mulberry

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Abstract

Background: Mulberry (*Morus* spp.) is a genus comprising several species of deciduous trees whose fruits are commonly eaten in southern Europe. Subjects with severe systemic reaction have been described. The aim of this study was to isolate the allergens of this species.

Methods: A nonspecific lipid transfer protein 1 (ns-LTP1) was purified from black mulberry by ion exchange and reverse phase high-performance liquid chromatography, and the primary structure was elucidated by direct protein sequencing. Its allergenic activity was evaluated *in vivo* by skin prick test and *in vitro* by Western Blot, CD203c basophil activation assay and high throughput multiplex inhibition method on immunosolid-phase allergen chip (ISAC).

Results: Mulberry ns-LTP (Mor n 3) comprises 91 amino acids producing a molecular mass of 9246 Da. This protein shows high sequence identity with several allergenic ns-LTP1. Immunoblot analysis and CD203c activation assay demonstrated its allergenic activity in symptomatic subjects and in ns-LTP allergic patients who are not mulberry consumers. Immunological co-recognition was studied *in vivo* on a selected group of well-characterized ns-LTP allergic patients showing a high percentage of nMor n 3⁺ subjects (88.46%) even in patients who have never eaten mulberry before. IgE inhibition on ISAC micro-array demonstrated an almost complete cross-reactivity to nArt v 3, rCor a 8 and a very high percentage of inhibition to nPru p 3.

Conclusions: Mor n 3 is the first allergen isolated in black mulberry and immunologically characterized. It displayed allergenic activity among symptomatic and nonconsumer patients and a pattern of cross-reactivity to other plant-derived LTPs.

Plant nonspecific lipid transfer proteins (ns-LTPs) are a widely distributed superfamily of related proteins. They are divided into two subfamilies according to their molecular masses: the 9-kDa ns-LTP1 and the 7-kDa ns-LTP2.

Literature available suggests that ns-LTPs might be involved in a wide range of biological functions. Some

reports describe the over-expression induced by a-biotic and biotic stresses. For these reasons, they have been classified as pathogenesis-related group 14 (PR-14) (1). Several ns-LTPs with allergenic activity have been identified in plant foods and in pollen so far. In some cases, a marked degree of similarity among them has been observed, which may explain the frequency of

individuals sensitized to several plant-derived foods in the Mediterranean area (2). The most frequently implicated foods belong to the Rosaceae fruits, but ns-LTPs with allergenic activity have also been detected in tree nuts, peanut, beer, maize, mustard, asparagus, grapes, cabbage, dates, orange, fig, kiwi, lupine, fennel, celery, tomato, eggplant, lettuce, chestnut, and pineapple (2). Because of their stable structure and resistance to proteolytic digestion and heat treatment (3), ns-LTPs are regarded as primary sensitizers responsible for severe allergic reactions leading to the definition of ns-LTPs as true food allergens (4, 5). A relationship between IgE to ns-LTPs and systemic symptoms has been observed in patients allergic to apple (6), cherry (7), peach (8), hazelnut (9), maize (10), and more recently to peanut (11). In particular, an Italian study performed on 1100 food allergic patients showed that ns-LTPs are the most important allergen causing food-induced anaphylaxis, where peach was the most frequently offending food (12).

The clinical picture following ns-LTP sensitization spans from local oral symptoms to anaphylaxis, and either ingestion or inhalation have been postulated to be routes of sensitization (13).

Mulberry (*Morus* spp.) is a genus comprising several species of deciduous trees native to warm temperate and subtropical regions worldwide. The involvement of white mulberry pollen in cases of respiratory allergy (14) and in contact urticaria has been reported (15). The multiple fruit is 2–3 cm long, it is edible and widely used by industry. Black (*Morus nigra*), red (*Morus rubra*), and white (*Morus alba*) mulberries are eaten as fresh food or processed for ice creams in southern Europe. Recently, patients with skin sensitivity to mulberry fruit have also been described (16–19) with severe systemic reaction after its ingestion (16). However, no allergens have been identified and characterized in fruits of the mulberry genus so far.

In this article, we report the identification and immunological characterization of a natural ns-LTP1, purified from black mulberry, with allergenic activity and high cross-reactivity to other fruit ns-LTPs. Official WHO-IUIS allergen nomenclature has been requested, and the Mor n 3 name has been assigned for the identified *M. nigra* ns-LTP1.

Material and methods

Purification of ns-LTP from black mulberry and peach

Black mulberry (*M. nigra*) fruit used for the purification of ns-LTP (nMor n 3) was purchased in a local market. Peach fruit (*Prunus persica* cv Stark Saturn) used for the purification of nPru p 3 was kindly provided by

CRA-FRC, Fruit farming Research Unit, Caserta, Italy. The black mulberry fruit and the peach peel were homogenized in water (1 : 1, w/v) and centrifuged at 10 400 g for 30 min. The supernatant was discarded and the pellet, containing the cell wall fraction, was collected and homogenized again after addition of 0.5 M NaCl. After a 60-min extraction followed by centrifugation, the supernatant was collected, dialyzed against 10 mM Tris-HCl, pH 7.2, and loaded on a DE52 (Whatman, Brentford, UK) column, equilibrated in the same buffer. Nonspecific lipid transfer protein was eluted in the column flow-through. The sample was then adjusted to pH 5.0 and loaded on a SP-Sepharose (Amersham Biosciences, Uppsala, Sweden) column, equilibrated in 10 mM sodium acetate, pH 5.0 (buffer A). Elution was carried out by increasing the concentration of buffer B (50 mM sodium acetate, pH 5.0, containing 0.5 M NaCl). The fractions containing ns-LTP were identified by reverse phase high-performance liquid chromatography (RP-HPLC) analysis using a Vydac (Deerfield, IL, USA) C₈ column and a Beckman System Gold apparatus (Fullerton, CA, USA). Nonspecific lipid transfer protein was further purified by RP-HPLC. Elution was obtained by a linear gradient of eluent B (0.08% trifluoroacetic acid (TFA) in acetonitrile) in eluent A (0.1% TFA in water). The ns-LTP eluted by RP-HPLC was manually collected and dried in a rotary vacuum centrifuge. After several washing with water, the protein samples were dried and stored at –20°C until required.

Protein concentration was estimated on the basis of the molar extinction coefficient at 280 nm (3480 M^{–1} cm^{–1}). Purity of the protein preparations was checked by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), RP-HPLC, and N-terminal amino acid sequencing.

Amino acid sequence determination and analysis

Denaturation and alkylation of the ns-LTP sulfhydryl groups with 4-vinylpyridine was carried out as already described (20). Denatured ns-LTP was divided into three aliquots and subjected to proteolytic cleavage by either trypsin, Asp-N or Arg-C, following manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

Separation of peptides obtained by proteolytic cleavages, amino acid sequencing, and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry measurements were performed as already described (20).

Protein sequence analyses were performed using softwares available on the ExPASy Proteomics Server

Preparation of ns-LTP solutions for skin prick test (SPT)

Patient selection

mulberry and peach. Peach allergic patients enrolled for the CD203c assay denied any mulberry ingestion. This testing was part of the routine diagnostic workup following approved National and International guidelines.

Consecutive patients detected positive for ns-LTPs were invited to take part to the *in vivo* study. Enrolled patients were specifically enquired whether they knew mulberries or not; if yes, whether they have ever ingested them or not, and whether they tolerated the ingestion or not. Skin test was performed according to standard procedure using standardized lancets and by applying the nPru p 3 and nMor n 3 glycerinated solutions (21).

IgE immunoblotting analysis

	1	11	21	31	41	51
	ITCGQVSSSLAPCINYL RAGGVVPANCCNGVRS LNNAAKTTAD RQAACNLCK					
N-term						
Trypsin						
ARG-C						
ASP-N						
	61	71	81	91		
	SAFN SIKGLNLNL AAGLPGKGVSPYKISPST DCKSVK					
N-term						
Trypsin						
ARG-C						
ASP-N						

3

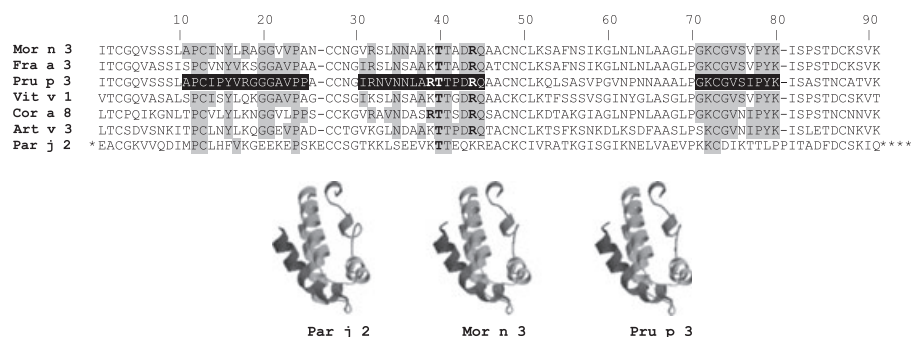


Figure 3 Multiple sequence alignment. Alignment of the amino acid sequences of Mor n 3 (accession number P85894), Fra a 3 (accession number Q4PLU0), Pru p 3 (accession number P81402), Vit v 1 (accession number Q850K5), Cor a 8 (accession number Q9ATH2), and Art V 3 (accession number P0C088) allergens. Shaded boxes and amino acid residues highlighted in bold indicate

regions that have been shown to be relevant for IgE-binding activity on the Pru p 3 allergen. Backbone ribbon representation of the Par j 2, Mor n 3, and Pru p 3 allergens were determined by using the services of the Swiss-Model Protein Modelling Server using the structure of a maize lipid transfer protein as a template (PDB entry 1mzm).

dry Trans-Blot Transfer (Millipore). Human sera were diluted 1 : 5 in a buffer containing 1× phosphate-buffered saline (PBS), 0.25% bovine serum albumin, 0.1% Tween 20. IgE complexes were detected using a goat anti-human IgE-horseradish peroxidase conjugated (Biosource International, San Francisco, CA, USA). Reactive bands were visualized by using SuperSignal West Pico Maximum Sensitivity Substrate (Pierce Biotechnology Inc, Rockford, IL, USA) and subsequent exposure to Kodak X-OMAT X-ray film (Kodak, New York, NY, USA).

CD203C basophil activation assay

Heparinized peripheral blood was obtained from allergic and nonallergic subjects ($n = 6$). Blood aliquots (100 μ l) were incubated with serial dilutions of the nMor n 3 allergen (from 1 to 10 000 ng/ml in 1× PBS) for 15 min at 37°C. 1× PBS was used as a negative control. Anti-IgE antibodies were used as a positive control (1 μ g/ml). After incubation, cells were resuspended in 100 μ l of fluorescence-activated cell sorting (FACS) buffer (BD PharmingenTM, Milan, Italy) and incubated with 20 μ l/test of phycoerythrin-labeled anti-CD203c mAb 97A6 (Immunotech, Marseille, France) for 20 min at 4°C in the dark. Samples were subjected to erythrocyte lyses, washed twice in ice cold FACS buffer and analyzed by means of flow cytometry on a FACSCalibur flow cytometer (Becton Dickinson). Basophils were detected on the basis of side-scatter characteristics (y -axis) and expression of CD203c (x -axis). For each sample, 100 000–200 000 cells were analyzed.

ISAC microarray inhibition assay

Ten patients' sera having IgE antibodies to nPru p 3 and/or rCor a 8 (6 of 10 nPru p 3⁺) were used in the inhibition assay. Individual sera (15 μ l) were preincubated over night with equal volumes of purified nMor n 3 (1.3 mg/ml) or nPru p 3 (1.0 mg/ml) solutions, representing the highest inhibitor concentrations available. Buffer was used instead of allergen preparations to obtain the noninhibited specific binding value. The remaining IgE reactivities to rCor a 8 and nPru p 3 were measured by running a new ISAC103 assay. If present, IgE binding to nArt v 3 and rPar j 2 were evaluated as well. The assay was run as single point highest inhibition achievable assay (SPHIAa) and percentage of inhibition was calculated (22). Specificity of the inhibition was assessed by incubation with only the detection antibody. Control IgE values obtained on noncross-reactive molecules showed no inhibition and thus are not shown.

Data and statistics

All the clinical and routine diagnostic data have been saved in an allergy *e*-record (InterAll; Allergy Data Laboratories, Latina, Italy) fully interfaced with the ISAC system as soon as they were available. The same data storage including preliminary statistical evaluation of SPHIAa has been performed by using the same INTERALL software.

GRAPHPAD PRISM version 5.01 has been used for advanced statistics and graphs (Graphpad Software Inc., La Jolla, CA, USA).

Chi-square test has been used to compare prevalence. Values at $P < 0.01$ have been accepted as statistically significant.

Results

nMor n 3 and nPru p 3 purification

The recovery of the pure protein was 1 mg/100 g and 8 mg/100 g for black mulberry and peach peel, respectively. Protein preparations resulted pure upon analysis by SDS-PAGE and Coomassie staining (see Fig. 1, lane B), RP-HPLC and *N*-terminal amino acid sequencing.

Primary structure elucidation of nMor n 3 and homology search

The direct sequencing of the *N*-terminal region of the entire protein produced 27 identifiable amino acid residues. The complete primary structure of nMor n 3 was determined by automated sequencing of peptides deriving from the enzymatic digestion of denatured and *S*-pyridylethylated protein. Most of the primary structure was elucidated by aligning the amino acid sequence of peptides from trypsin digestion, whereas the sequence of the regions corresponding to the residues 40–44 and 86–91 were obtained by sequencing peptides from Arg-C and Asp-N digestions, respectively. In Fig. 2, only the peptides necessary to elucidate the complete amino acid sequence are indicated.

The sequence of black mulberry ns-LTP comprises 91 amino acids producing a molecular mass of 9246 Da. This value is in good agreement with that

obtained by MALDI-TOF mass spectrometry (9235 ± 20 Da).

Homology search in UniProt protein database carried out using the BLAST algorithm (<http://www.expasy.org>) has shown that the amino acid sequence of Mor n 3 displays a high sequence identity with several allergenic ns-LTPs. The sequence identities observed for some of them, showing values ranging from 75% (Fra a 3) to 61% (Art v 3), are shown in Table 1. Figure 3 shows the alignment of the amino acid sequence of Mor n 3 with the sequences of the homologs Fra a 3, Pru p 3, Vit v 1, Cor a 8, Art v 3, and Par j 2. To align Par j 2, displaying significant differences at the primary structure level including the presence of 11 additional residues, it was necessary to insert two gaps in the ns-LTP1 sequences. Nevertheless, 3D modeling (Fig. 3) showed that, as for many other LTPs (2), the overall folding is conserved in Pru p 3, Mor n 3 and Par j 2; therefore, the rational of the low cross-reactivity between Mor n 3 and Par j 2 has to be searched at the primary structure level. In the Fig. 3, three regions, comprising the residues 11–25, 31–45, and 71–80, reported to be relevant for IgE binding in Pru p 3 have been gray shadowed (23), showing that the amino acid residues contained in these regions are only partially conserved in the aligned sequences. The highest conservation with respect to Pru p 3 has been observed in the region comprising the amino acids 71–80, where only one residue out of ten is substituted in Mor n 3, Fra a 3, Vit v 3, and Cor a 8; two residues are substituted in Art v 3 and eight residues are substituted in Par j 2. In the other two regions, different patterns of substitutions can be observed in each one of the analyzed LTPs, and the highest number of replacements with respect to the consensus sequence of Pru p 3 is observed

Table 1 Amino acid sequence identity (%) among nonspecific lipid transfer proteins from different sources

Accession numbers	Allergen	Mor n 3	Fra a 3	Cit s 3	Rub i 3	Pru d 3	Pru p 3	Pru av 3	Mal d 3	Vit v 1	Pru du 8	Pru ar 3	Pyr c 3	Cor a 8	Art v 3	Par j 2
P85894	Mor n 3	100	75	73	72	70	70	70	70	69	68	68	64	62	61	30
Q4PLU0	Fra a 3		100	64	82	73	70	68	73	68	67	69	68	59	56	32
Q8L5S8	Cit s 3			100	64	63	68	62	70	59	64	69	61	59	56	29
Q0Z8V0	Rub i 3				100	74	69	70	75	61	67	68	71	61	54	31
P82534	Pru d 3					100	88	87	82	60	91	91	77	59	51	30
Q5RZZ3	Pru p 3						100	87	79	61	97	91	75	56	52	26
Q9M5X8	Pru av 3							100	83	63	89	85	79	59	53	26
Q5J026	Mal d 3								100	60	79	86	85	61	50	26
Q850K5	Vit v 1									100	60	58	63	53	56	34
B6CQU2	Pru du 8										100	89	78	62	52	25
P81651	Pru ar 3											100	78	60	52	30
Q9M5X6	Pyr c 3												100	56	56	25
Q9ATH2	Cor a 8													100	57	26
P0C088	Art v 3														100	32
P55958	Par j 2															100

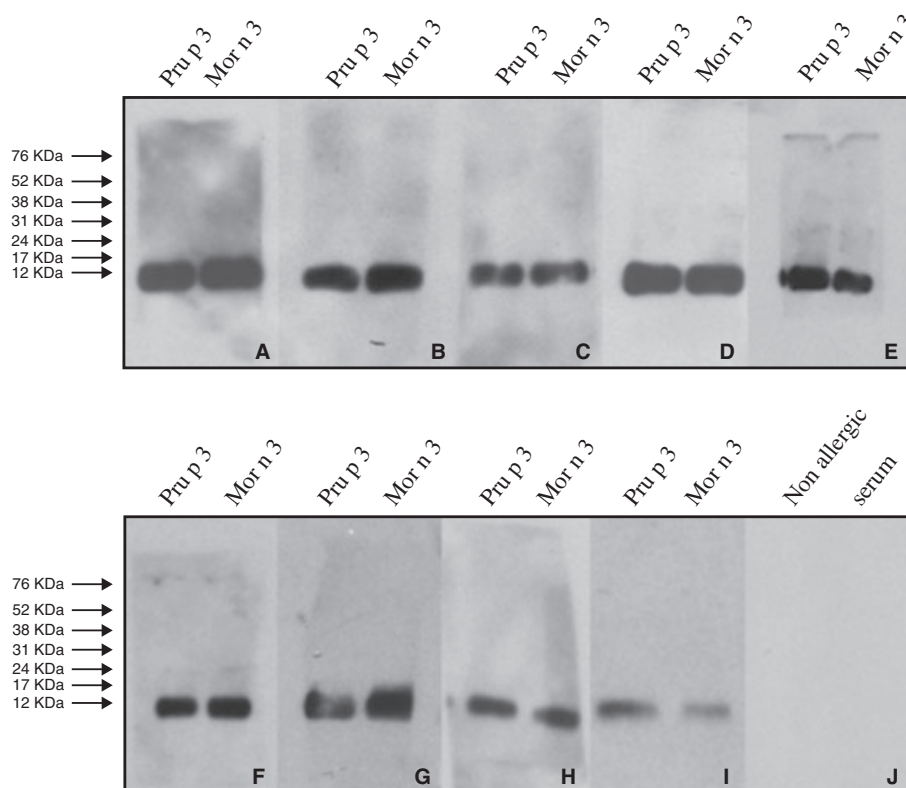


Figure 4 IgE-binding activity of the nMor n 3 allergen. IgE immunoblotting analysis of nine sera from Pru p 3⁺ allergic patients with purified nPru p 3 and nMor n 3 allergens.

in Par j 2. The three residues R39, T40, and R44, reported to be involved in the IgE binding of Pru p 3, are all conserved in Cor a 8. In Mor n 3, Fra a 3, Vit v 1 and Art v 3, T40, and R44 are conserved, whereas the residue in position 39 is conservatively substituted (R39/K39). In Par j 2, only T40 is conserved, whereas R39 and R44 are conservatively replaced by K39 and K44.

IgE immunoblotting analysis

The IgE-binding activity of the purified nMor n 3 was analyzed by immunoblotting. Sera from three mulberry allergic patients were incubated with the purified protein demonstrating that the mulberry ns-LTP displays an IgE reactivity (Fig. 1, lanes C, D, E). No reactivity was observed when a serum from nonallergic subject was tested as negative control (Fig. 1, lane F).

In addition, we performed immunoblotting experiments with nine randomly selected sera from well-characterized Pru p 3 allergic patients using the purified nMor n 3 and nPru p 3 proteins. These set of experiments indicated that all the peach allergic patients present IgE antibodies capable of reacting to the mulberry ns-LTP (Fig. 4).

Basophil activation assay

The anaphylactic activity of nMor n 3 was studied by basophil activation detecting over-expression of the CD203c marker. Figure 5 shows a representative plot of basophil activation (panels A–F) from one out of three mulberry allergic patients stimulated with increasing concentrations of the nMor n 3 allergen. Panels from G to L shows a representative plot of activation of one out of two peach allergic patients who were scored as not mulberry consumers. Panels from M to R display the same assay using blood from a nonallergic control subject. This analysis demonstrated that the nMor n 3 ns-LTP is capable of cross-linking surface-bound specific IgE-activating CD203c over-expression in mulberry allergic patients and in peach allergic patients who are not exposed to mulberry by ingestion.

nMor n 3 and nPru p 3 skin tests in ns-LTP-sensitized subjects within the general allergic cohort

As reported in Table 2, almost all the 26 selected subjects (96.15%) were sensitized to Pru p 3 based on the ISAC IgE results. Just one subject has been enrolled

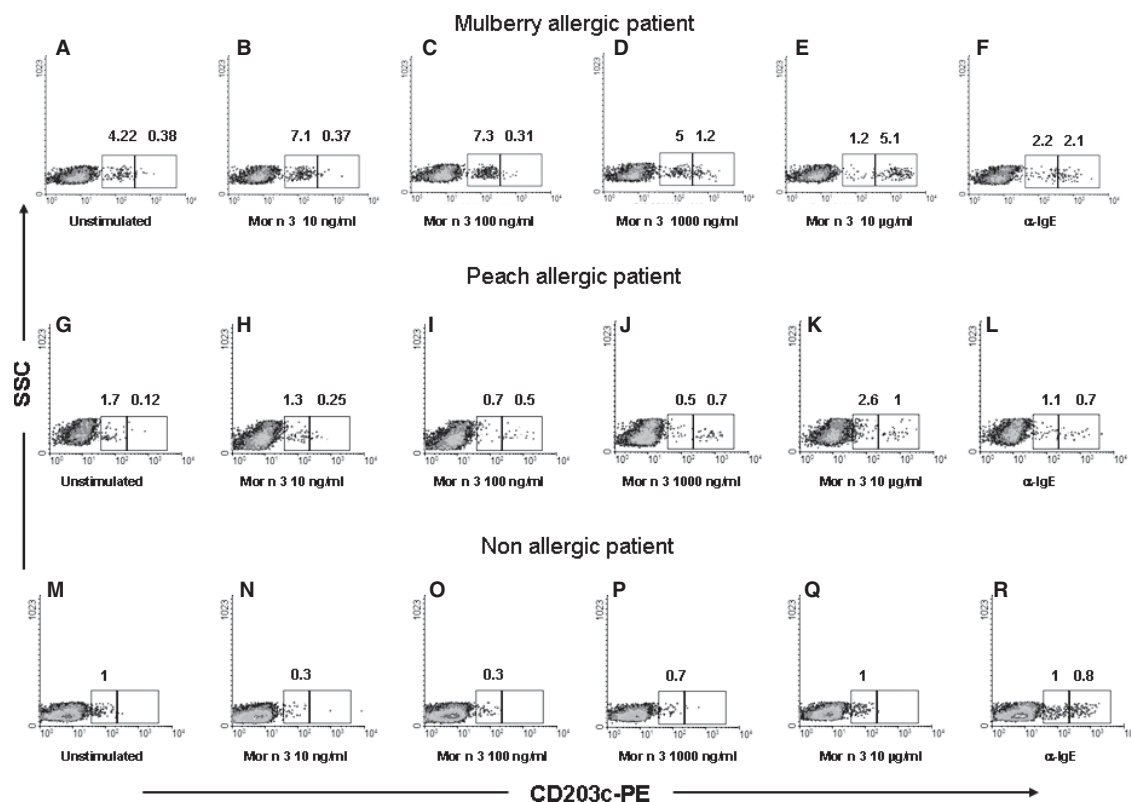


Figure 5 Basophil CD203c surface expression. The biological activity of the nMor n 3 was studied by basophil CD203c activation. Peripheral blood from one mulberry allergic patient (panels from B to E), a peach allergic patient (panels from H to K), and a nonallergic negative control subject (panels from N

to Q) was stimulated with serial dilutions of the nMor n 3 allergen. Panels A, G, and M show the activation induced by the negative control (1× phosphate-buffered saline); panels F, L, and R show the activation induced by the positive control (anti-IgE).

because of Cor a 8 sensitisation without a Pru p 3 IgE positive result. Within this selected group, sensitizations to other ns-LTPs were recorded as follows: Art v 3 $n = 12$ (46.15%); Cor a 8 $n = 13$ (50%); and Par j 2 $n = 5$ (19.23%). One subject was sensitized to Art v 3 but not to Cor a 8, whereas two were sensitized to the hazelnut ns-LTP but not to the mugwort's one. Art v 3 and Cor a 8 prevalence were recorded significantly higher in this small subset of peach ns-LTP-sensitized subjects than in the overall ISAC tested population ($n = 4297$; Art v 3⁺ $n = 329$ [7.65%]; Cor a 8⁺ $n = 243$ [5.65%]); both prevalence were statistically different by chi-square test with $P < 0.0001$, whereas Par j 2 IgE sensitization prevalence was lower and did not statistically differ from that of the general cohort of allergies (Par j 2⁺ $n = 675$ [15.7%]). Pru p 3 prevalence in the general cohort was 12.28% ($n = 528$).

nMor n 3 was applied to the skin test and recorded positive in 23 out of 26 enrolled subjects (88.46%), exerting cutaneous reactions comparable, in terms of

wheel areas, to that of Pru p 3. All the 25 Pru p 3⁺ subjects were scored positive when skin tested with the purified nPru p 3 preparation. The patient with a negative ISAC result on Pru p 3 was confirmed negative by skin testing with the same molecule but was scored positive with the nMor n 3 preparation. In three Pru p 3⁺ patients, nMor n 3 has been recorded negative on skin test. The prevalence of nMor n 3⁺ subjects lacked statistical difference when compared to Pru p 3⁺ patients ($n = 25$, 96.15%).

IgE inhibition on ISAC microarray ns-LTPs using purified natural nMor n 3 and nPru p 3 by means of SPHIAa

Immunological co-recognition of ns-LTPs has been studied by IgE inhibition assay, using the high throughput multiplex inhibition method on ISAC named SPHIAa. As reported in Fig. 6, IgE inhibitions have been performed by detecting residual IgE binding on ns-LTPs available on the ISAC system by incubating

Table 2 Demographics, IgE selection data, and skin test results using natural purified Mor n 3 and Pru p 3

No.	APC code	Age	Gender	ISAC*				Skin test†	
				Art v 3	Cor a 8	Par j 2	Pru p 3	Mor n 3	Pru p 3
1	IDI1799	29	F	9.89	0.78	61.25	20.98	58	47
2	IDI26116	17	F	Neg	Neg	Neg	1	87	11
3	IDI32095	18	F	Neg	0.57	Neg	Neg	22	Neg
4	IDI27343	44	F	Neg	Neg	Neg	0.92	14.4	48.7
5	IDI2444	43	F	4.6	6.41	Neg	5.83	96.7	139
6	IDI8101	26	F	Neg	Neg	Neg	4.53	32	16
7	IDI1206	23	F	2.92	0.9	Neg	1.57	29	61
8	IDI17488	22	F	Neg	Neg	Neg	1.07	Neg	45
9	IDI7531	5	M	Neg	Neg	Neg	0.23	26	8
10	IDI10720	18	M	34.78	5.71	Neg	>100	6.6	51
11	IDI12028	41	F	Neg	Neg	6.48	0.89	Neg	26
12	IDI31384	10	M	Neg	Neg	Neg	0.04	Neg	8.5
13	IDI31558	29	M	0.76	3.37	Neg	3.97	36.6	14
14	IDI21272	39	M	2.31	Neg	Neg	3.27	23	12
15	IDI28837	25	M	0.26	0.16	13.38	1.07	53	78
16	IDI35446	11	M	Neg	0.48	Neg	4.7	34.4	97.4
17	IDI4456	10	M	7.99	5.33	Neg	11.29	18	87
18	IDI30718	16	M	7.59	2.64	23.45	13.47	12	23
19	IDI33186	22	M	0.25	0.41	Neg	0.82	72	84
20	IDI23952	35	F	1.26	0.31	Neg	2.34	64	66
21	IDI2687	11	M	Neg	Neg	Neg	0.33	20.3	14
22	IDI29916	10	M	Neg	Neg	Neg	2.04	33.4	54
23	IDI14196	15	F	Neg	Neg	Neg	2.4	61	45
24	IDI28945	15	M	Neg	Neg	Neg	0.95	8	30
25	IDI4631	31	F	Neg	Neg	2.86	0.11	23	16
26	IDI2126	23	F	0.46	0.37	Neg	2.04	38.3	90.6
<i>n</i> = 26		Mean = 22.62 range = 5–44	F = 13 M = 13	Pos = 12	Pos = 13	Pos = 5	Pos = 25	Pos = 23	Pos = 25
				46.15%	50%	19.23%	96.15%	88.46%	96.15%

ISAC, immunosolid-phase allergen chip technology.

*IgE values tested by ISAC are expressed as kU_A/l.†Skin test results are expressed as wheal area (mm²).

single sera with the highest concentration available of nMor n 3 or nPru p 3. Samples used for SPHIAa had the following features: nArt v 3⁺ *n* = 10, specific IgE mean value = 4.61 kU_A/l (range 0.50–19.57 kU_A/l); rCor a 8⁺ *n* = 10, specific IgE mean value = 2.79 kU_A/l (range 0.39–8.42 kU_A/l); rPar j 2⁺ *n* = 7, specific IgE mean value = 30.4 kU_A/l (range 4.37–43.88 kU_A/l); and nPru p 3⁺ *n* = 12, specific IgE mean value = 14.52 kU_A/l (range 4.84–33.38 kU_A/l). Individual percent inhibition values are reported in Fig. 6. All the 12 nPru p 3⁺ sera were fully inhibited by the homologous natural preparation. nMor n 3 behaved almost the same on the same allergen with inhibition values ranging between 83% and 100%, excepting one sample where a 62% inhibition value was achieved. A similar behavior was observed for the Art v 3⁺ sera where another serum could not be fully inhibited by both allergens (53%), whereas the remaining ranged between 83% and full inhibition. Both nMor n 3 and nPru p 3

allergens were close to full inhibition to all rCor a 8⁺ samples. IgE inhibition on seven Par j 2⁺ samples was negligible when nPru p 3 was used as inhibitor, whereas six out of seven sera showed a slight inhibition using nMor n 3 preparation with value ranging between 15% and 47%.

Discussion

Lipid transfer proteins occur widely throughout the plant kingdom and, recently, several reports have shown an increasing number of allergens belonging to this protein family. Furthermore, it has been shown that ns-LTP allergens from several food as well as pollens have high prevalence in Mediterranean allergic patients, thus the definition of a complete spectrum of allergenic ns-LTPs and their degree of cross-reactivity between species is of paramount importance in the clinic.

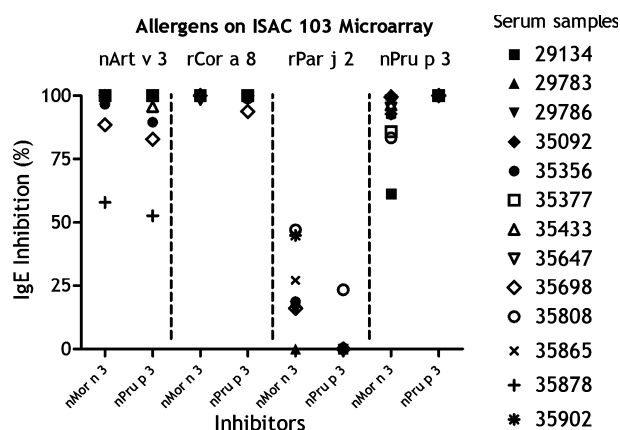


Figure 6 Single point highest inhibition achievable assay (SPHIAa) for defining IgE cross-reactivity. Single samples have been incubated with the highest concentration available of nMor n 3 (1.3 mg/ml) or nPru p 3 (1.0 mg/ml). Samples used for SPHIAa were 13 having the following features: nArt v 3⁺ *n* = 10, specific IgE mean value = 4.61 kU_A/l (range 0.50–19.57 kU_A/l); rCor a 8⁺ *n* = 10, specific IgE mean value = 2.79 kU_A/l (range 0.39–8.42 kU_A/l); rPar j 2⁺ *n* = 7, specific IgE mean value = 30.4 kU_A/l (range 4.37–43.88 kU_A/l); and rPru p 3⁺ *n* = 12, specific IgE mean value = 14.52 kU_A/l (range 4.84–33.38 kU_A/l). Percent inhibition values have been calculated by the InterAll software from data received on line from the immunosolid-phase allergen chip system.

This article describes the first allergen identified in the *Morus* genus, named nMor n 3. On the basis of its sequence and molecular mass of 9146 Da, it has been included in the ns-LTP1 protein family. nMor n 3 has been purified in good amounts from the cell wall fraction of the black mulberry fruit after high ionic strength extraction.

The elucidation of the primary structure, obtained by direct sequencing of the natural protein, showed that mulberry ns-LTP has a high percent of amino acid identity with ns-LTPs from several plant-derived fruits. Fra a 3, the ns-LTP from strawberry, has been recorded as having the highest sequence identity (75%), higher than Pru p 3 (70%), the second ns-LTP tested in the present study.

Immunological and clinical properties of nMor n 3 have been studied in comparison with the homologous allergens from peach fruit (Pru p 3), hazelnut (Cor a 8), mugwort pollen (Art v 3), and *Parietaria* pollen (Par j 2). Immunoblotting analysis and basophil activation assay demonstrated that this protein displays an allergenic activity both in mulberry clinically reactive patients and in patients clinically sensitized to other ns-LTP.

To study the *in vivo* reactivity of nMor n 3, a selected group of characterized ns-LTP allergic patients were studied by skin test showing a high percentage of nMor

n 3⁺ subjects (88.46%). This percentage was comparable to the number of Pru p 3⁺ subjects even in patients who have never eaten mulberry before. This datum is in agreement with the IgE immunoblotting analysis performed with Pru p 3⁺ positive sera where we observed that all the tested subjects showed nMor n 3 specific IgE. In a similar way, the CD203c activation assay showed that peach allergic patients, which are not mulberry consumers, display an activation of basophils after stimulation with the natural purified Mor n 3 allergen. To understand in more details, the degree of IgE co-recognition with other ns-LTPs, an IgE inhibition test was performed using the nPru p 3 and nMor n 3 purified natural allergens toward the nArt v 3, rCor a 8, rPar j 2, and nPru p 3 proteins available on the microarray system. This assay demonstrated an almost complete inhibition of IgE binding to the nArt v 3 and rCor a 8, and a very high percentage of inhibition to nPru p 3 allergens indicating a strong cross-reactivity between these allergens. Therefore, our reported close structural relation, in terms of sequence identity, between Mor n 3 and other LTPs suggests the need to further investigate the degree of cross-reactivity to Fra a 3 and Vit v 1 (24). In line with the low sequence identity, a low level of inhibition was observed in ns-LTP allergic patients having IgE also to Par j 2 LTP, despite the common 3D structure (25, 26). A possible explanation for this observation can be found in the IgE-binding studies performed with the Pru p 3 allergen. Epitope mapping performed by synthetic peptides (23) and phage display (27) identified few regions relevant for the IgE-binding activity. The alignment of the corresponding residues of the Pru p 3, Mor n 3, Fra a 3, Cor a 8, Art v 3, and Vit v 1 allergens has shown that several of these amino acids are conserved between the six allergens. On the other hand, the corresponding region on the Par j 2 allergen showed only a limited number of conserved residues in those areas justifying the low or absent cross-reactivity between this allergen and the other ns-LTPs reported in this study.

All the above reported structural, clinical, and immunological findings seem to describe Mor n 3 as potential strongly cross-reactive allergen in ns-LTP-sensitized patients, mostly Pru p 3⁺ ones. Although the majority of the patients seems to behave in this way, differences have been recorded for some of them where a negative IgE result, either detected on ISAC or by SPT, has been recorded. These findings would suggest the existence of a heterogeneous recognition of IgE epitopes on LTPs not fully shared by these molecules as shown for other LTPs (28).

In conclusion, we have identified and immunochemically characterized the first mulberry allergen, nMor n 3.

This is an ns-LTP1 showing high sequence and structural similarity with other already known allergenic ns-LTP1. Because of the regional or occasional use of mulberries, we found relevant to report cases of clinical relevant reactions among consumers and to describe the potential risk for ns-LTP1 allergic patients representing 12.28% of the examined cohort of this study.

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