

RESEARCH ARTICLE

Novel allergens from ancient foods: Man e 5 from manioc (*Manihot esculenta* Crantz) cross reacts with Hev b 5 from latex

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Scope: Manioc (*Manihot esculenta*) is a tuber mainly consumed in the Southern Hemisphere and used worldwide by food and chemistry industry. We aimed to recombinantly produce and characterize the first manioc allergen and evaluate its IgE reactivity in sera of Brazilian and Italian patients.

Methods and results: The molecule, termed Man e5, was expressed in *E. coli*, characterized by amino acid analysis, mass spectrometry, circular dichroism, HPLC, and dynamic light scattering. A tertiary structural model of the protein was produced using bioinformatics and susceptibility to pepsin digestion was analyzed *in vitro*. Based on its high content of charged residues, heat stability, flexibility and lack of secondary structure elements, the allergen was determined a member of the intrinsically disordered protein family. Brazilian patients were selected based on manioc allergy and Italians based on latex allergy and sensitization to Hev b 5. 71% of Brazilians and 40% of Italians were *in vitro* IgE positive to Man e5. Cross-inhibition assays suggest a possible involvement of this allergen in the latex-fruit syndrome.

Conclusion: Man e5, the first purified allergen from manioc demonstrates IgE cross-reactivity with Hev b 5. Data suggest Hev b 5 might act as primary sensitizer and could therefore lead to allergic manifestations upon manioc consumption without prior exposition.

Keywords:

Food allergy / Hev b 5 / IgE cross-reactivity / Intrinsically disordered protein / Latex-fruit syndrome / New allergen



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Abbreviations: CD, Circular dichroism; IDPs, intrinsically disordered proteins; MW, molecular weight; R_H, hydrodynamic radius

1 Introduction

Manioc (*Manihot esculenta* or *Manihot utilissima*) tuber is consumed worldwide, mainly in South America, Africa, and Asia. It is a staple food and main source of carbohydrates for more than 600 million people in these regions. *M. esculenta* has many common names, e.g. manioc or mandioca (Brazil),

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tapioca (Thailand and Vietnam), cassava (West India), and Yucca (South America). In Brazil, manioc is the ninth cultivated food and the country is the third largest world producer. The root is usually consumed cooked or fried and its different flours can be used to prepare breads, cakes, and pastries.

Anaphylactic reactions after ingestion of manioc have previously been reported in Brazil [1], as well as in Mozambique and Spain [1–3]. More recently, we analyzed IgE reactivity profiles from nine Brazilian manioc allergic patients, revealing a fructose-bisphosphate aldolase, GAPDH, and a glutamic acid rich protein as new IgE-binding proteins from this source [4]. An association between manioc and latex hypersensitivity has been suggested [1–5], however so far investigations enrolling purified allergenic molecules have not been performed.

Approximately 5–15% of healthcare workers show clinical latex allergy [6], while also 1.2% of a random population suffering from respiratory symptoms demonstrate latex specific IgE [7]. Almost 30% of patients experiencing allergy to rubber latex exhibit an associated hypersensitivity to various fresh fruits, the so called “latex-fruit syndrome” [8]. Most frequently involved fruits and vegetables are banana, avocado, kiwi, chestnut, papaya, mango, lychee, and potato [9–12].

Rubber latex was shown to contain several allergens, e.g. Hev b 6 (hevein), Hev b 2 (1,3 glucanase), Hev b 7 (patatin-like protein), and Hev b 8 (profilin) [13–15]. Notably, reactivity to Hev b 5 in healthcare workers ranges from 60 to 90%, thus defining this molecule as relevant latex allergen [16, 17]. The full list of latex allergens can be found at Allergome databank (<http://www.allergome.org>).

We recently described a glutamic acid-rich protein with similarity to Hev b 5 as allergen in manioc tuber [4]. The aim of this study was recombinant production of this molecule, termed Man e 5 according to the IUIS allergen nomenclature subcommittee. The allergen was investigated physicochemically, structurally, and tested for IgE reactivity with sera of patients allergic to manioc and latex.

2 Material and methods

2.1 Cloning, expression, and purification of Man e 5

Manioc roots were purchased at local stores in São Paulo, Brazil and an adapted protocol from Tangphatsornnuang et al. [18] was used for RNA extraction. To obtain the full-length sequence of Man e 5, primers were designed based on the manioc glutamic acid-rich protein Pt2L4 (GenBank AAP57707). A representative construct (Br13) cloned into pHis parallel II expression vector was used for production of soluble nonfusion Man e 5 in *Escherichia coli* BL21 Star (Novagen, Gibbstown, NJ, USA). Recombinant Man e 5 was purified by anion exchange and hydrophobic interaction chromatography. Detailed protocols are given in Supporting Information Materials and Methods.

2.2 Gel electrophoresis, amino acid analysis, and MS

Recombinant Man e 5 was analyzed by denaturing gel electrophoresis and amino acid composition, and protein concentration was determined using the Pico Tag method (Waters, Milford, MA, USA). The identity of the protein was confirmed by MS using ESI-QToF MS. Detailed protocols are described in Supporting Information Materials and Methods.

2.3 Homogeneity

Online-high performance size exclusion chromatography-light scattering for determination of molecular weight (MW) and hydrodynamic radius (R_H) of eluting peaks were performed as previously published [19]. The molecular size and aggregation behavior of Man e 5 in solution was investigated by dynamic light scattering (DLS 802, Viscotek). Reference proteins were used for plotting the MW against R_H and retention volume (v_{ret}) and detailed protocols given in Supporting Information Materials and Methods.

2.4 Secondary structure analysis and prediction of disordered regions

Circular dichroism (CD) spectra and thermal denaturation were recorded with a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan). The secondary structure prediction of Man e 5 was performed using PSIPRED and MUSTER [20]. Detailed protocols are described in Supporting Information Materials and Methods.

2.5 Molecular modeling and dynamics simulations

Man e 5 model structure was generated using MODELLER9v8 based on physicochemical characteristics and for dynamics simulations GROMACS was the software used. All analyses were performed on a Bewolf cluster. Detailed protocols are given in Supporting Information Materials and Methods.

2.6 Simulated gastric digestion

Gastrointestinal digestion was simulated *in vitro* as described elsewhere [21]. Briefly, gastric digestion was performed using 3 μ g of protein and 50 ng of pepsin (Sigma-Adrich, Saint Louis, MO, USA) in 0.1 M HCl pH 2. After 2 h of pepsin treatment, the pH was neutralized by addition of ammonium bicarbonate pH 7.8. Reactions were conducted at 37°C and proteolysis was monitored by SDS-PAGE. The same procedure was done using 20 μ g of manioc extract as matrix spiked with 5 μ g of Man e 5.

2.7 Patient selection and molecule-based diagnosis

All recruited subjects or caregivers signed an informed consent to participate in the study on the basis of protocols approved by the Brazilian Ethic Committee for Analysis and Research Projects, CAPPesq of HCFMUSP, and Italian (IDI-IRCCS) Institutional Review board. Brazilian patients were included based on clinical history of allergic reactions to manioc, whereas Italian patients were preselected for clinical reactivity to latex and IgE reactivity to Hev b 5. Sera of seven Brazilian and eight Italian patients who also reacted to Man e 5 in ELISA were selected to perform cross-reactivity studies. IgE inhibition to solid-phase coated rMan e 5, rHev b 5, manioc, and latex extract was assessed in ELISA and percentage of inhibition was determined. Detailed criteria for patients' selection and molecule-based diagnostics, and inhibition assays are described in Supporting Information Materials and Methods.

3 Results

3.1 cDNA cloning and purification of recombinant Man e 5

Using gene-specific primers, cDNA fragments corresponding to the glutamic acid-rich protein from manioc were cloned into the pHis Parallel II vector. Five clones were subjected to sequencing and showed only minor deviations in their nucleotide sequence that did not translate into differences at the protein level (data not shown); the full-length sequence has been submitted to GenBank (accession number JF710639). Notably, the amino acid sequence identified within this study deviated from a previously reported sequence of a glutamic acid-rich protein from manioc (GenBank AAM55492) in three positions, i.e. P18S, A116T, and V122E and in five positions comparing to an allergenic-related protein from manioc (GenBank ABY19572), i.e. E17K, P18S, G57E, G58A, and addition of G at position 91. Man e 5 presents 40.1% (71/177) conserved residues with Hev b 5 (GenBank AAC49448.1) (Fig. 1A).

Man e 5 was expressed in *E. coli* BL21 Star as soluble nontagged protein and total yield of purified protein was 10 mg per liter culture. Aliquots were freeze-dried and stored at -20°C ; resuspension and repeated freeze and thaw cycles showed no negative impact on protein concentration or solubility. Protein purity was visualized by SDS-PAGE, presenting a single band at approximately 30 kDa (Fig. 1B), while the MW of the protein is 18.8 kDa.

3.2 Primary structure analysis of Man e 5

Amino acid analysis was performed to determine the exact concentration and composition of amino acids and was in accordance with the theoretical values (Fig. 1C). The pro-

tein presents more than 30% of glutamic acid residues, the reason why this protein was termed accordingly in previous reports [20]. Also, there are no basic residues, e.g. histidine or arginine, and therefore the protein is not appropriate for colorimetric concentration determination and stains rather weak with Coomassie blue. In addition, the percentage of prolines was 11.3%, twice as high as commonly reported for proteins (around 5.2%) and the protein is deprived of aromatic residues (Fig. 1C). The intact mass of the protein as determined by MS is $18\,773.9 \pm 1.0$ Da which corresponds to the calculated value of 18 774.0 Da (*N*-terminal methionine cleaved off).

3.3 Homogeneity and aggregation behavior in solution

Man e 5 appeared monomeric in solution, as determined by online high performance size exclusion chromatography-light scattering and DLS analyses (Fig. 2A). Both results were compared to commercially available globular proteins used as reference. The 18 kDa recombinant Man e 5, depicted in Fig. 2B and C, represented an outlier in terms of retention volume and hydrodynamic radius since it eluted at a lower retention volume than human IgG (Fig. 2C). The hydrodynamic radius can be related to the compactness of a protein fold. Therefore, a well-defined protein having a globular tertiary structure results in a smaller hydrodynamic radius than a noncompact or nonstructured protein. Resulting in R_H values of 3.3 nm (by DLS with polydispersity (PD) of 10% RSD) and 3.8 nm (by HPSEC), Man e 5 exceeded the range expected from its MW being in terms of its molecular size more comparable to transferrin ($R_H = 3.0$ nm), a globular well-structured protein 80 kDa (Fig. 2B).

3.4 Circular dichroism (CD) and secondary structure determination

The far-UV CD spectrum for Man e 5 was recorded at 20°C and pH 7.0 presented a curve typically observed for disordered proteins [21] (Fig. 3A). The negative amplitude at 200 nm indicates high content of beta turns and random coils, while typical secondary structure elements like alpha helices and beta sheets are nearly absent. Upon thermal denaturation, an increase in the region corresponding to alpha helices at 222 nm was observed, while the peak at 198 nm was decreased. Notably, structural changes induced by heating were completely reversible, since the spectrum recovered its original shape after cooling. Measurements performed at pH 3.0 resulted in similar spectra as obtained after heating (data not shown). Secondary structure predictions using software PSIPRED showed that only a region of four residues (2.26%), i.e. Man e 5_{169–172} was prone to form an alpha helix while majority of the residues were likely disordered random coils (Fig. 3B). Structure predictions were in agreement with

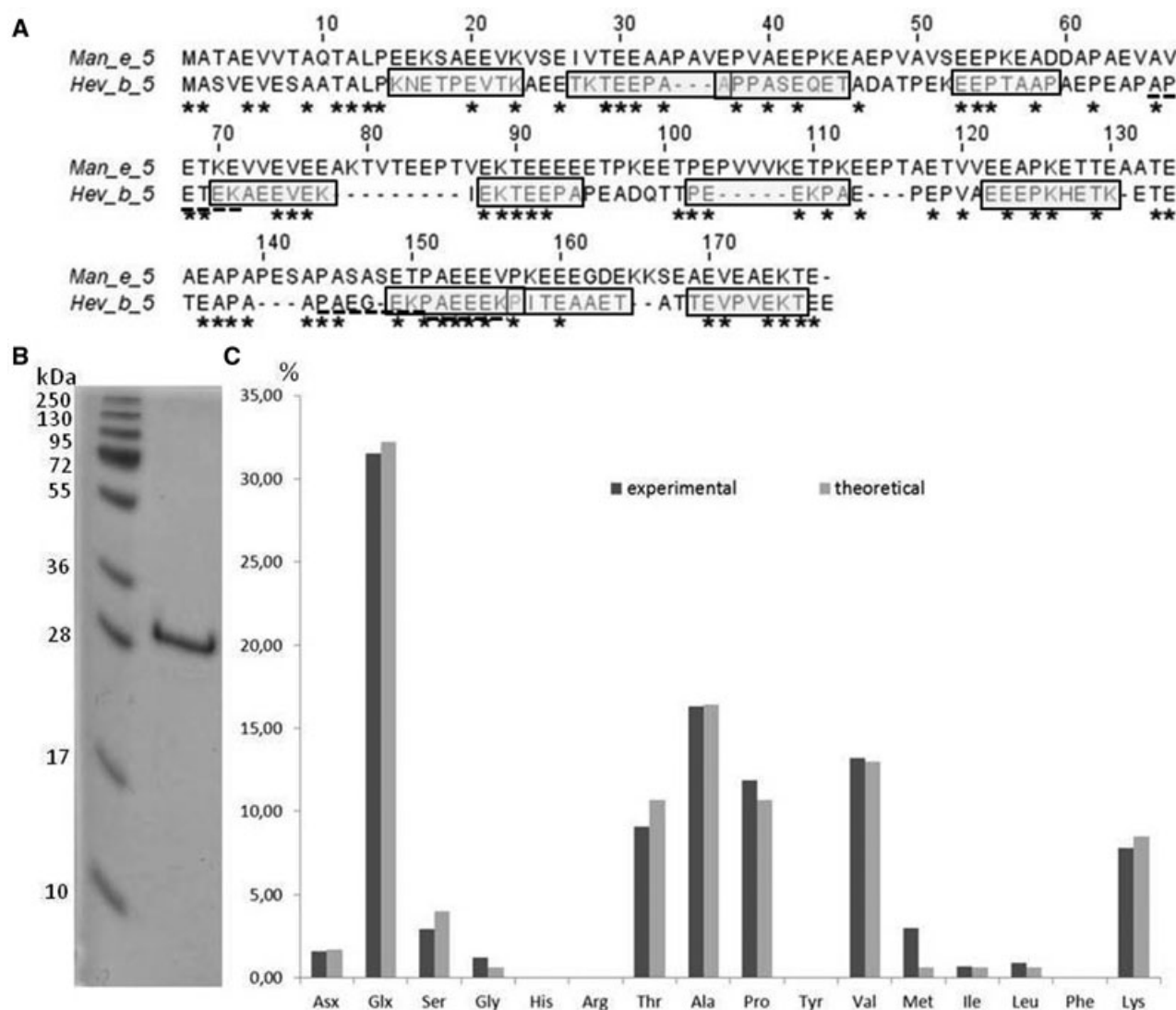


Figure 1. (A) Sequence alignment of the primary sequence of the Man e 5 from manioc and Hev b 5 from latex. The residues marked with an asterisk are showing identical residues. Gray boxes indicate linear human IgE epitopes already determined for Hev b 5 [33] and dashed underlined residues show murine B-cell binding regions [34]. (B) Purified recombinant Man e 5 is migrating as a single band at 30 kDa in denaturing SDS-PAGE. (C) Amino acid analysis of the protein showing a high percentage of glutamic acid content, experimentally determined percentages are well corresponding to theoretical values.

results obtained by molecular modeling and also correspond to experimental data determined by CD analysis.

The prediction using megaPrDOS server indicated that this protein had a high probability to be a disordered protein (Supporting Information Fig. S1A). The analysis of the structural quality of the homology model was performed using PROCHECK for Ramachandran diagram plots (data not shown) and G-factor indicates that this model was adequate for structural studies. Molecular dynamics simulations were carried out for protein in solution. We evaluated the root-mean square deviation of the positions for all backbone C-alpha atoms, radius of gyration, and intermolecular hydrogen

bonds. These analyses suggested that 5 ns of unrestrained simulation was sufficient for stabilizing the protein, a value expected for a protein in this kind of computational simulation. The initial time of stabilization was 1.5 ns, which is longer than generally expected for globular proteins indicating that this protein is very flexible and presents high percentage of random coil secondary structure. Another characteristic of disordered proteins is to present many polar and charged amino acids, which can also be observed in the primary structure as well as in the molecular electrostatic surface in the model of Man e 5 (Supporting Information Fig. S1B).

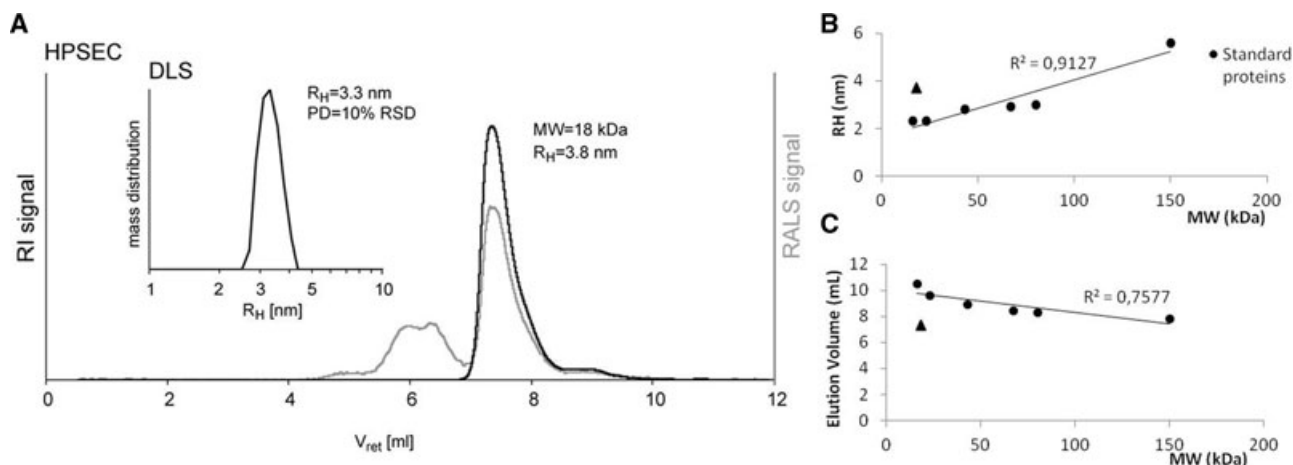


Figure 2. Physicochemical characterization. (A) Aggregation behavior of Man e 5 investigated by HPSEC (large panel) and DLS (small panel). In HPSEC, a molecular weight (MW) of 18 kDa was determined from RI (black) and RALS (gray) of Man e 5 eluting at V_{ret} of 7.3 mL. The RALS signal being very sensitive for high MW compounds showed a double peak at V_{ret} of 6.0–6.4 mL corresponding to less than 0.1% of protein. (B) R_H determined by DLS experiments for standard globular proteins and Man e 5 showing the molecule is out of the range for its MW. (C) Elution volume determined by HPSEC for the same standard globular proteins and for Man e 5 showing it is again out of range.

3.5 Gastrointestinal digestion of Man e 5

In vitro degradation experiments showed that the molecule was rapidly digested by pepsin, resulting in four intermediate degradation fragments upon 10 s of incubation (Fig. 4). Similar results were obtained when the molecule was heated before digestion or submitted to *in vitro* digestion in matrix environment of manioc extract (data not shown), thus neither heating nor matrix conditions had a negative or positive impact on the proteolytic susceptibility.

3.6 Clinical symptoms and molecule-based diagnosis

Detailed information on clinical symptoms and patients' sensitization is given in Table 1. Symptoms after manioc ingestion or latex contact were reported in a broad severity range from mild oral allergy syndrome to anaphylaxis. From 30 Italian latex allergic patients tested in direct ELISA, 12 (40%) had specific IgE to Man e 5, even though exposition to this allergen source is rather unlikely or at least not as usual as in the Brazilian population. Latex allergens were tested using ISAC 89 and all patients tested presented IgE to at least one. Among Brazilian manioc allergic patients, Hev b 1 was positive in 4 (57%), Hev b 3 in 2 (28.5%), Hev b 6 in 6 (85%), Hev b 7 in 3 (43%), and Hev b 11 for 5 (71%) subjects. Among latex allergic patients, Hev b 1 was IgE positive to 4 (33%), Hev b 3 for only one (8.3%), Hev b 6 for 6 (50%), Hev b 8 for 2 (16.6%), and Hev b 11 for 5 (41.6%). Notably, Hev b 5 was the only latex allergen tested positive in all Brazilian patients' sera (Table 1).

3.7 Reactivity in patients' sera allergic to manioc and latex and cross reactivity with Hev b 5

The IgE-binding activity of purified rMan e 5 and rHev b 5 was assessed by ELISA using sera of Brazilian patients allergic to manioc and latex and Italian patients allergic to latex and already known to be sensitized to Hev b 5. Since the IgE-binding properties of Man e 5 could be demonstrated, it was included in the official IUIS allergen nomenclature subcommittee, and currently represents the only allergenic molecule identified in manioc. Among Brazilian patients, 5 out of 7 subjects (71.4%) demonstrated a positive IgE reactivity to the new allergen Man e 5 and all seven were positive to Hev b 5 (see Table 1). From 30 Italian patients initially selected for reactivity against Hev b 5, 12 (40%) were IgE positive to Man e 5 in ELISA (Table 1). IgE levels to Hev b 5 were higher than to Man e 5 in both populations but there was no difference while comparing Brazilian to Italian patients (Fig. 5). Reactivity to Man e 5 was slightly lower for Brazilians but not statistically different. Heat denaturation of the proteins did not show any difference in IgE reactivity (data not shown). Specific IgG4 measurements in ELISA showed very low levels for Man e 5, but considerably higher values for Hev b 5. However, there were no statistically significant differences between reactivity to Man e 5 and Hev b 5 between the two population groups (data not shown).

For cross inhibition assays, sera of five Brazilian and eight Italian patients were selected. IgE cross-inhibition using 0.05 μ g/mL of coated allergen showed that Hev b 5 strongly inhibited Man e 5 (mean 66%) and for two patients, the inhibition was 100% (Fig. 6A). On the other hand, Man e 5 could reduce the IgE binding to Hev b 5 less effectively (mean 7.1%) rising up to 33% only for one patient (Fig. 6B).

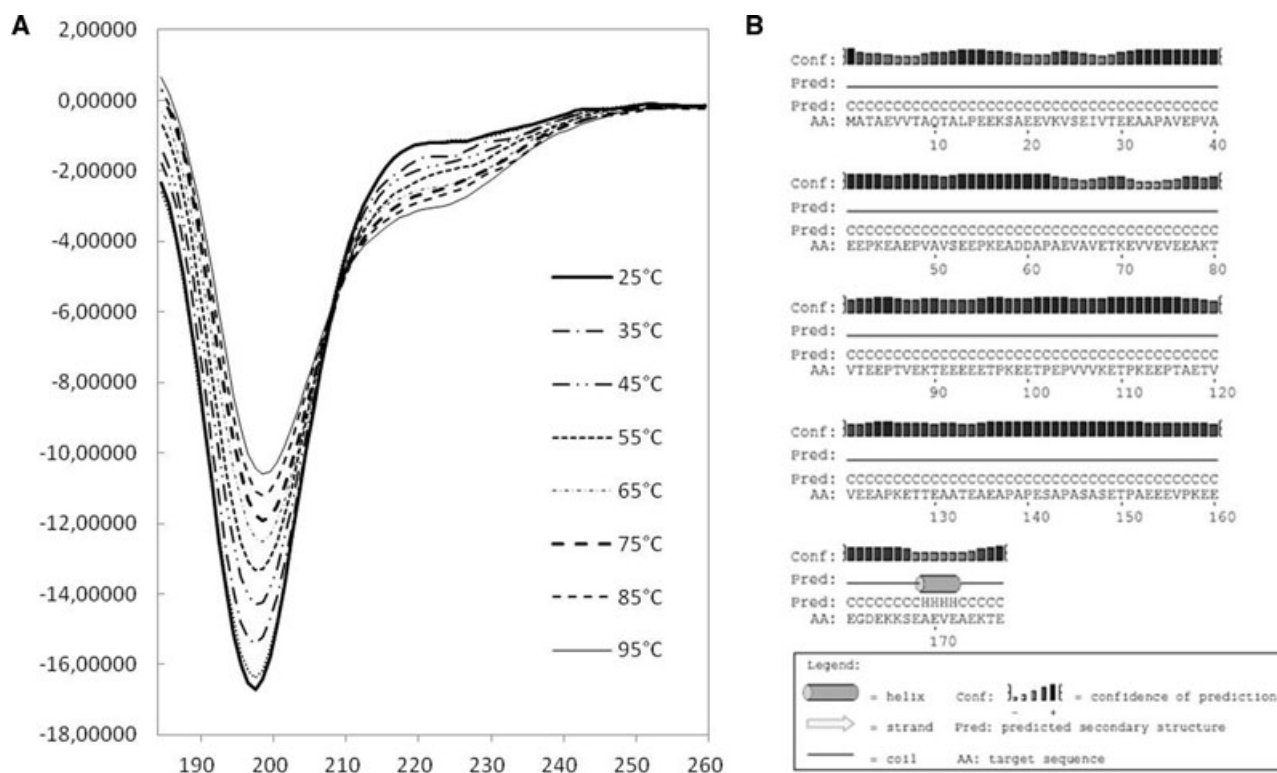


Figure 3. Secondary structure analysis and prediction of Man e 5. (A) Circular dichroism experiments show a typical spectrum of an unstructured molecule, spectra obtained upon heat treatment were recorded from 25 to 95°C. (B) Secondary structure prediction of Man e 5 using the software PSIPRED.

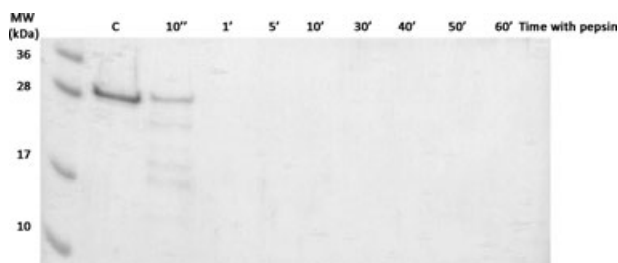


Figure 4. Simulated gastric digestion. Recombinant Man e 5 was subjected to gastric digestion with pepsin and monitored by gel electrophoresis. (C) control of Man e 5 without pepsin.

Man e 5 was able to partially inhibit (mean = 14.26%, range 0 to 46%) the IgE reactivity to coated manioc extract (Fig. 6C) and a similar cross-inhibition was found using Hev b 5 (mean = 18.39%, range 0 to 63%). In addition, SPHIAa inhibition assay in a microarray format showed that Man e 5 could inhibit up to 71% of Hev b 5 IgE, with values dispersed in a broad range. Similar IgE inhibition values, ranging from 43 to 81% were recorded on Hev b 5 using the manioc extract as inhibitor (Supporting Information Fig. S2).

Extract-based inhibition experiments in ELISA using manioc and two fractions of latex clearly showed that latex fraction

C containing Hev b 5 was able to inhibit manioc to the same extend (mean 91.1%) as observed by self-inhibition with manioc extract (mean 89.3%), while inhibition with fraction B was in average 53.4% (Supporting Information Fig. S3). In contrast, manioc represents a weak inhibitor for latex reaching a mean inhibition of 14.6 and 33.8% for latex fraction B and C, respectively.

4 Discussion

Manioc is a staple food in several parts of the world and largely consumed in Brazil, where it has been eaten by native Indians since before the discovery of the country. So why are the first cases of allergy to manioc just emerging now? A simple explanation could be the overlooking of cases and the lack of a rationale diagnostic approach. Our results point out that this phenomenon can be considered as something emerging because of the relatively recent appearance of another problem, namely latex allergy, an epidemic starting few decades ago.

The first case of allergic reactions against manioc was described only in 2001 [22]. In a recent publication from our group, three IgE-binding proteins have been identified [4]. However, the allergenicity of single manioc allergens has not

Table 1. Demographic, clinical, and diagnostic features of manioc and latex allergic patients and molecule-based diagnosis using Man e 5 and latex allergens.

	Patient	Gender/age	Symptoms	Symptoms	ISAC 89						
	ID	(years)	to manioc	to latex	Hev b 5	Hev b 1	Hev b 3	Hev b 6	Hev b 7	Hev b 8	Hev b 11
BRAZILIANS	B1	F/40	Anaphylaxis	U	58.1	Neg	Neg	18.81	Neg	Neg	Neg
	B2	F/55	Anaphylaxis	D, S, U, AE	9.67	0.4	Neg	18.62	Neg	Neg	2.61
	B3	F/45	AE, CU	U, CU	61.81	Neg	Neg	24.94	1.51	Neg	1.88
	B4	F/41	AE, U	U	44.48	2.87	9.8	14.29	12.59	Neg	0.54
	B5	F/45	AE, U	S, U	47.9	0.35	Neg	26.48	Neg	Neg	1.01
	B6	F/41	AE, U	AE, S	61.9	15	64	63.8	65.03	Neg	11.9
	B7	F/48	AE, U	U, CU	21.29	Neg	Neg	Neg	Neg	Neg	Neg
ITALIANS	11	M/63	Unknown	E	3.65	Neg	Neg	Neg	Neg	Neg	Neg
	12	M/41	Unknown	C, R	48.71	Neg	Neg	Neg	Neg	Neg	Neg
	13	F/34	Unknown	R	4.64	Neg	Neg	Neg	Neg	Neg	Neg
	14	F/54	Unknown	C, R, OAS	7.67	Neg	Neg	21.44	Neg	Neg	2.4
	15	F/56	Unknown	R	5.44	Neg	Neg	Neg	Neg	Neg	Neg
	16	M/5	Unknown	A, E, OAS, En	19.92	3.81	0.36	8.13	Neg	Neg	3.5
	17	F/31	Unknown	A	41.75	Neg	Neg	32.68	Neg	1.13	5.3
	18	M/29	Unknown	A, E, R	3.94	Neg	Neg	Neg	Neg	Neg	Neg
	19	F/30	Unknown	AE, U	6.24	1.39	Neg	3.39	Neg	Neg	Neg
	110	M/23	Unknown	AE, U	99.52	1.88	Neg	Neg	Neg	28.7	0.24
	111	M/18	Unknown	A, E, R	6.14	0.47	Neg	Neg	Neg	Neg	Neg
	112	M/II	Unknown	U	25.48	Neg	Neg	10.18	Neg	Neg	4.3

A = asthma; AE = angioedema; C = conjunctivitis; CU = contact urticaria; D = dyspnea; E = eczema; En = enteritis; R = rhinitis; OAS = oral allergy syndrome; U = generalized urticaria.

been determined so far. We have described and characterized here the first recombinant manioc allergen, Man e 5, at the molecular level. Also, we have demonstrated the IgE cross-reactivity with the homologous molecule Hev b 5 from rubber latex. Because extraction and purification of low-abundant allergens from natural sources is generally a limiting process and due to the high content of starch in manioc extract, we sought to produce Man e 5 as a recombinant molecule in *E. coli*. Using recombinant production, we could ensure absence of trace amounts of other allergenic manioc components [4] and thus increase specificity of the immunological assays. The sequence corresponding to Man e 5 was found in five independent clones, thus assuming the presence of one dominant isoallergen in the analyzed *M. esculenta*. The identified amino acid sequence deviates in three and five residues from previous database entries, and might be due to different manioc cultivars. Man e 5 represents a protein with an unusual amino acid composition, with glutamic acid being the predominant residue (32%) and due to the lack of aromatic amino acids the molecule cannot be detected by UV. The glutamic acid-rich protein in manioc seems to be preferentially expressed in storage roots and vascular stem tissues [23], however, the physiological function remains unclear.

Recombinant Man e 5 was purified from *E. coli* as soluble nontagged protein showing an unusual migration pattern in gel electrophoresis, presumably due to the high content of acidic amino acids and proline residues. This finding is in

line with the natural molecule's appearance at 30 kDa in the extract [4] and typical for proteins belonging to this group of molecules [24]. Man e 5 was shown to be labile and easily degraded by proteases probably due to its high amount of exposed residues. Considering also other characteristics such as high thermostability, acidic pI, and anomalous electrophoretic mobility, we can assume this protein to be part of the so-called intrinsically disordered proteins (IDPs) [24–26]. Those proteins, also termed intrinsically unstructured or natively unfolded [24], are characterized by the lack of a well-defined 3D structure under physiological conditions [24, 25]. Since Man e 5 was produced from the soluble protein fraction and never subjected to denaturing conditions, we could verify that lack of obvious secondary structure elements typically observed for globular proteins is not due to production processes. Rather, the disorder of the protein seems to be an intrinsic factor owing to the remarkable amino acid content [25]. In addition, based on the primary sequence, the protein is predicted to be unfolded using *in silico* tools.

The majority of water-soluble proteins have globular and relatively static structures. However, some proteins have natively disordered regions that are flexible, dynamic, and can be partially or completely extended in solution [27]. Disordered proteins are generally depleted in bulky hydrophobic and aromatic amino acids while they are enriched in polar and charged amino acids, exactly as presented for Man e 5. When compared to globular proteins, Man e 5 presented a larger

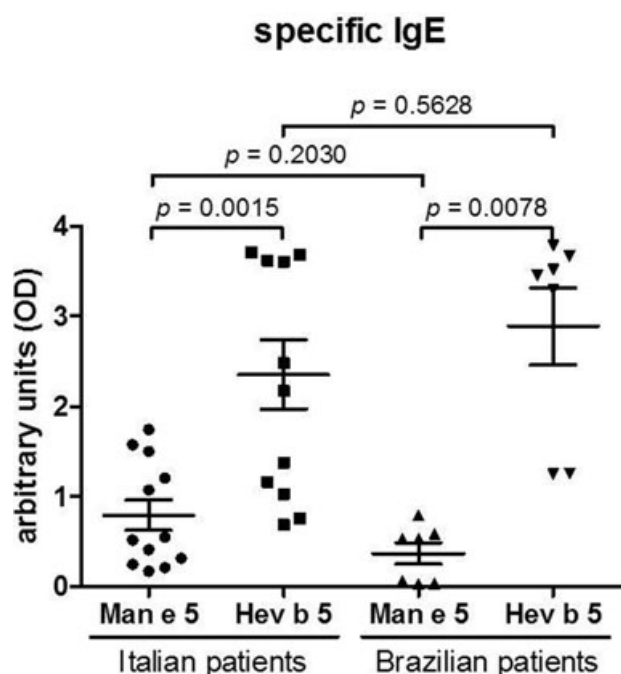


Figure 5. IgE reactivity to purified Man e 5 and Hev b 5 using sera of Brazilian manioc and latex allergic patients and Italian latex allergic patients determined in ELISA experiments. Bars indicate means with SEM. Comparison has been performed using the nonparametric paired Wilcoxon test (Man e 5 versus Hev b 5) or nonparametric unpaired Mann–Whitney test (Italians versus Brazilians).

hydrodynamic radius and apparent bigger MW. Unfolding of proteins results in an essential increase in their hydrodynamic volume thus showing an augmented value relative to native proteins and explaining the increase in their apparent molecular mass [25]. Due to the absence of static structures, IDP cannot be subjected to x-ray crystallography, but investigated using other analysis such as CD and NMR [28]. As also demonstrated for Man e 5, IDP are characterized by very specific shapes of their far-UV CD spectrum, with an intensive minimum in the vicinity of 200 nm and an ellipticity close to zero in the vicinity of 222 nm [29]. As the temper-

ature is increased, there is a shift, and this change in the 220-nm region might indicate generation of some secondary structure at high temperatures [21]. Interestingly, a decrease in buffer conditions from pH 7.0 to 3.0 promoted similar results. Furthermore, the high percentage of proline in Man e 5 is consistent with the low secondary structure content observed by CD.

All the features described above render Man e 5 extremely labile to the action of pepsin in the low pH of gastric juice, leading to its degradation within seconds which was also not prevented in the presence of the food matrix. However, with the elevation of the pH, this manioc allergen remains intact upon gastrointestinal digestion (unpublished data), thereby facilitating its presentation to the immune system. Based on *in vitro* mouse and human studies, it has been proposed that anti-ulcer drugs support clinically relevant sensitization against oral proteins while they increase the pH of gastric juice [30]. The worldwide growing use of anti-acids might have increased the allergenic potential of labile molecules [31], and thus could help to explain the recent reports on allergic reactions to manioc. In addition, the spread of latex allergy could play a major role in causing allergic reactions to manioc through latex homologous molecules.

Recent identification in MS [4] and the apparent inhibition of Hev b 5 using manioc extract verified the expression of Man e 5 in the tuber. Notably, IgE reactivity to Hev b 5 was stronger than to Man e 5 for Brazilian and Italian patients. Both groups had high levels of IgG4 to Hev b 5. Both groups had high levels of IgG4 to Hev b 5 while a very low reactivity to Man e 5 was observed, indicating a limit level of IgG cross-reactivity. Inhibition assays showed that Hev b 5 inhibited Man e 5 (mean 64.17%) stronger than the manioc allergen inhibits the latex allergen (mean 12.10%). Identity between these molecules is 40.1% and eleven linear human IgE epitopes [32, 33] have been described for Hev b 5 and three murine B cell-binding regions [34]. Among these regions, six are partially conserved, as regions 124–132 (78% of conserved residues–7/9) and 76–82 (71% of sequence identity–5/7) in Man e 5 and might constitute cross-reactive epitopes. Since both molecules lack a distinct three-dimensional fold and thus do not present conformational epitopes, high-sequence identities in particular

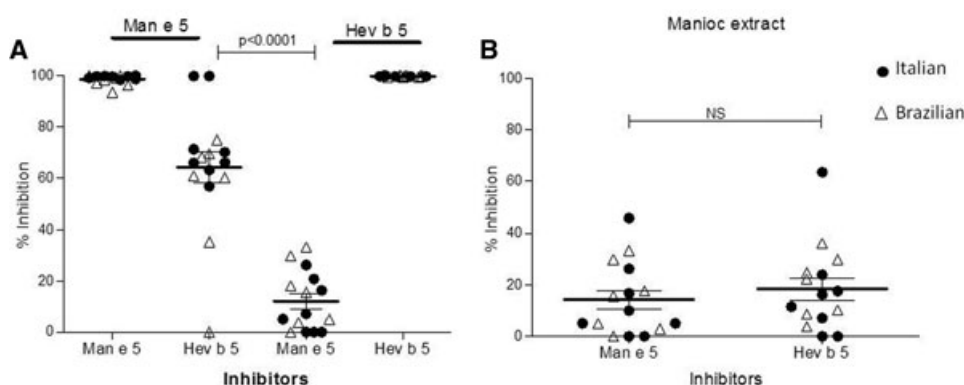


Figure 6. IgE cross-inhibition using sera of Brazilian and Italian allergic patients using 0.05 µg/mL of coating allergen. (A) Self-inhibition of Man e 5 (left) and inhibition of Man e 5 by Hev b 5 (second group); inhibition of Hev b 5 by Man e 5 (third group) and self-inhibition of Hev b 5 (right). (B) Extract inhibition. Man e 5 and also Hev b 5 can partially (mean 14.26 and 18.39%, respectively) inhibit solid-phase coated manioc extract. Bars indicate means with SEM.

amino acid stretches can give rise to the observed antibody cross-reactivity. According to the FAO/WHO report, cross-reactivity should be considered for proteins sharing more than 35% identity in a window of 80 amino acids. Considering this, a search performed in the Structural Database of Allergenic Proteins also suggested a potential cross-reactivity between Man e 5 and Hev b 5. Statistically higher IgE reactivity could indicate that Hev b 5 presents more epitopes than Man e 5. Elucidation of linear epitopes of Man e 5 and comparison with Hev b 5 epitopes will clarify the cross-reactivity. Considering that Man e 5 is highly labile and IgE-binding values in Brazilian patients are lower compared to Hev b 5, Hev b 5 could be the primary sensitizer and manioc allergic reactions occur due to IgE cross-reactivity for the majority of patients. Longitudinal data from the Multicentre Allergy Study clearly indicate that a primary sensitization beginning with foods turning into allergy to natural rubber latex is unlikely [35]. In contrast, there are several examples of the latex-fruit syndrome [9], where latex allergens are acting as primary sensitizing agents. Results for latex extract-based inhibition showed that a fraction of latex containing Hev b 5 is able to inhibit manioc to the same extent as it inhibits itself, whereas manioc is very weak in inhibiting latex, reinforcing our hypothesis that primary sensitization in our patients is via latex. In addition, manioc extract can partially inhibit the latex fraction that does not contain Hev b 5 that indicates the presence of other, yet unidentified allergens.

Knowing the relationship herein described between latex allergy based on Hev b 5-IgE reactivity and the homologous Man e 5 in manioc could prevent allergic reaction by informing visitors coming to countries where manioc is widely used. Our findings could be valuable even for countries where manioc-based products are not so common but manioc-based ingredients could be used in industrial food products. Data extracted from the AllergomeConsumer database where 77 food packages collected in Italy contain manioc-derived ingredients support this alert (Adriano Mari, personal communication). To this regard, manioc as an ingredient could be seen as the cause in otherwise unexplainable allergic reactions. In addition, professionally exposed individuals could be monitored because, as in the case of wheat flour, different kind of allergens could be involved.

In conclusion, Man e 5 is a new allergen from manioc being recognized by more than 70% of patients suffering from adverse reactions to this staple food. Man e 5 is able to partially inhibit IgE reactivity to Hev b 5, whereas it is more strongly inhibited by the latex allergen. The lack of typical secondary structure elements and high susceptibility to proteases indicates Man e 5 to be an intrinsically disordered protein. The study suggests that IgE reactivity to Man e 5 could be a consequence of Hev b 5 sensitization and thus, probably being a new player in the latex-food syndrome.

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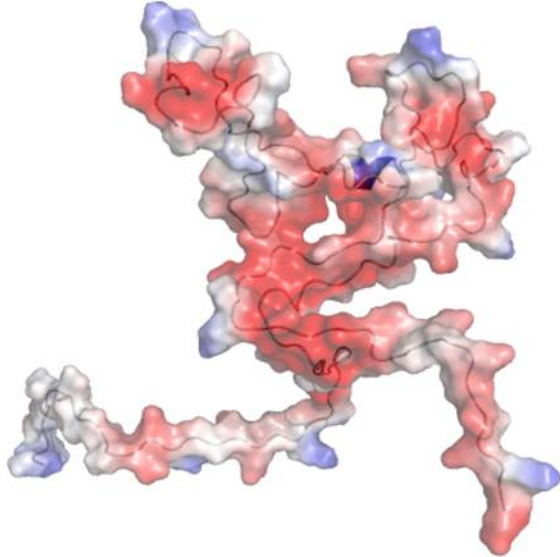
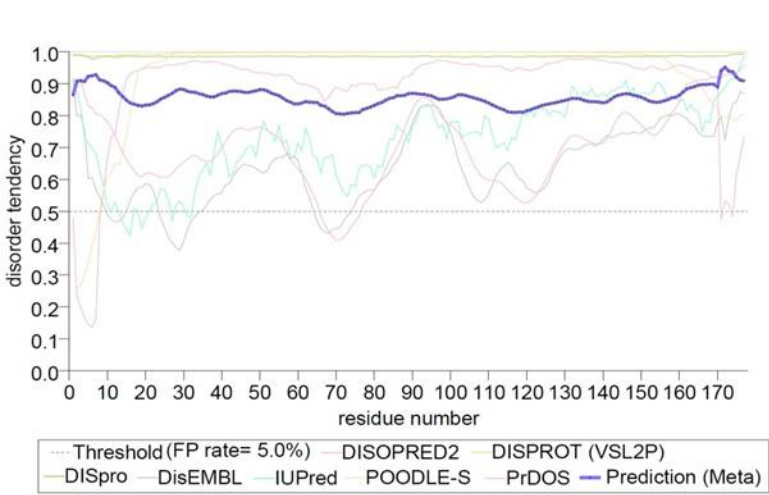
nologia, and Phadia Diagnostics, now Thermofisher Scientific, Brazil. Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Biomay AG, Vienna, Austria and the Italian Ministry of Health, Current Research program 2009–2010. Fatima Ferreira has received research support from the Austrian Science Fund (FWF), the Christian Doppler Research Association and Biomay AG, and provides legal consultation or expert witness testimony for the AllergenOnline Database, Indoor Biotechnologies, and HAL Allergy.

The authors have declared no conflict of interest.

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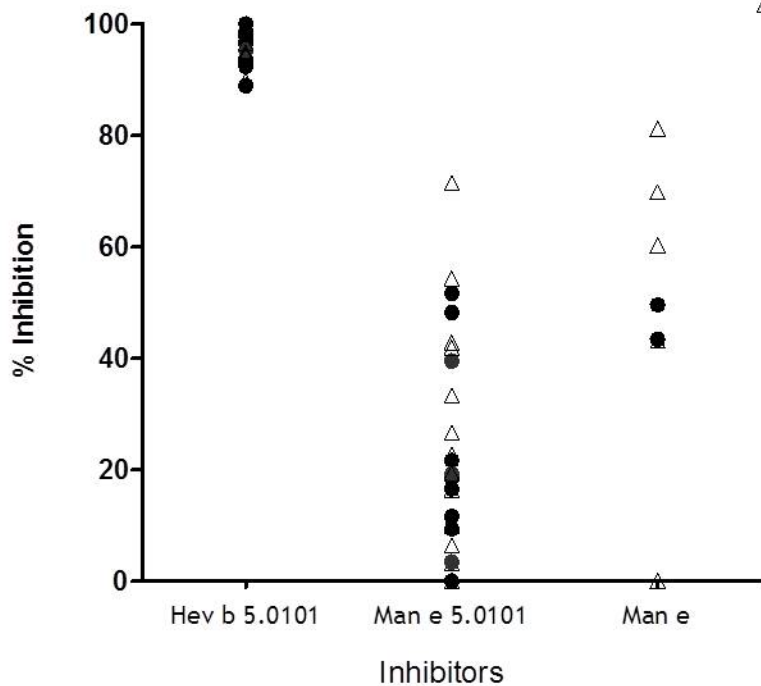
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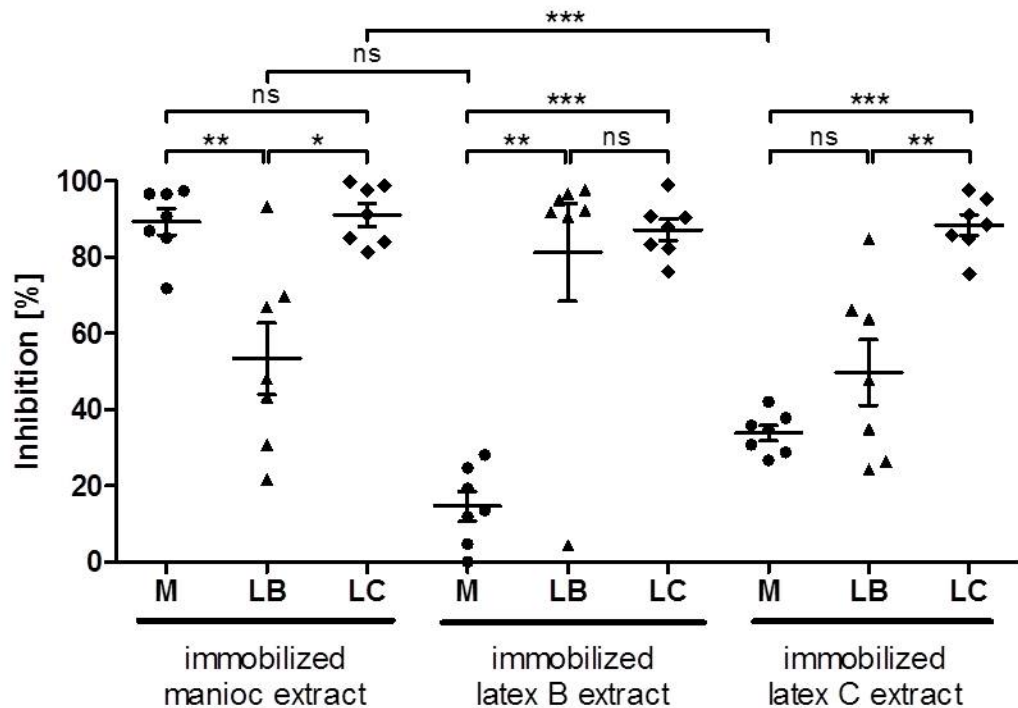
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Hev b 5

● Italians
△ Brazilians





Supporting Information Materials & Methods

Novel allergens from ancient foods: Man e 5 from manioc (*Manihot esculenta* Crantz) cross reacts with Hev b 5 from latex

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Condensed title: Man e 5 from manioc cross reacts with Hev b 5 from latex

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Key words: manioc; new allergen; latex-fruit syndrome; IgE cross-reactivity; intrinsically disordered protein; Man e 5; *Manihot esculenta*; Hev b 5; food allergy.

METHODS

RNA purification and cDNA preparation

An adapted protocol from Tangphatsornnuang et al.[1] was used for RNA extraction. Approximately 4 g of manioc tuber were ground under liquid nitrogen together with glass-sand and dissolved in 5 mL of lysis buffer (10 mM EDTA, 0.2 M sodium acetate, 1% SDS, 1% polyethylene glycol) and 5 mL phenol. The solution was centrifuged at 12.000 g for 4 minutes at 4°C, and the supernatant was re-centrifuged for 10 min under the same conditions. RNA was extracted from the aqueous phase by adding an equal volume of phenol followed by centrifugation at 12.000g at 4°C for 20 minutes. RNA was precipitated with 1/3 volume of 10 M LiCl and incubated overnight at -20°C. After centrifugation for 20 minutes under the same conditions the pellet was washed with 1 ml 2.5M LiCl and 1 ml 75% EtOH, dried and reconstituted in 20µl DEPC-treated deionized water. 0.5 µg total RNA was subjected to oligo dT-primed reverse transcription using the Super-Script III First-strand Synthesis System according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

Cloning and recombinant expression of Man e 5

In order to retrieve the DNA sequence corresponding to the previously described manioc glutamic acid-rich protein, primers were designed based on the published sequence of Pt2L4 (GenBank AAP57707). The PCR product was amplified using forward 5'-GAGACATATGGCTACTGCTGAGG-3' and reverse primer 5'-GAGACTCGAGTCACTCAGTCTTCTC-3' with flanking *Nde*I and *Xho*I restriction sites, respectively. The product was cloned into pHIS parallel II expression vector and five constructs

were sequenced. A representative clone (Br13) was chosen and recombinant Man e 5 was produced as soluble non-fusion protein in *E. coli* BL21 Star (Novagen, Gibbstown, NJ, USA). Briefly, protein expression was induced with 0.4mM isopropyl-b-D-thiogalactopyranosid at $OD_{600} = 0.7$ and cells were grown at 16°C for 18 h. After centrifugation at 5000 *g* for 20 min at 4°C, cells were resuspended in 20mM Tris-HCl pH 8.0 and lyzed by repeated freezing and thawing. The soluble protein fraction was obtained by centrifugation at 20.000 *g* for 30 min at 4°C.

Purification of Man e 5

Recombinant Man e 5 was purified by anion exchange chromatography using a 5 mL Q FF column (Äkta Prime system, GE Healthcare, Uppsala, Sweden) at a flow rate of 1.5 mL/min. After 120 mL gradient elution to 20 mM Tris-HCl pH 8, 500 mM NaCl, fractions containing Man e 5 were pooled. For final purification the protein was loaded onto a 5 mL butyl column (GE Healthcare) in 50 mM sodium phosphate NaP pH 7, 1.5 M ammonium sulphate and eluted with 50 mM sodium phosphate NaP pH 7 at a flow rate of 2.5 mL/min in 40 mL gradient. Fractions containing pure protein were pooled together for desalting through Amicon filters (Merck Millipore, Billerica, MA). The buffer was exchanged for 10 mM sodium phosphate pH 7.0, samples were lyophilized and stored at -20°C for further use.

Amino acid analysis

Approximately, 5–10 µg of protein were dried and hydrolyzed with constant boiling 6 M HCl (Pierce, Rockford, IL, USA) for 18 h at 124°C under vacuum. After neutralization with aqueous

20% v/v triethylamine and 40% v/v ethanol, samples were re-dried and derivatized with 10% v/v phenylisothiocyanate in 70% v/v ethanol and 10% v/v triethylamine for 10 min at room temperature. Dried samples were reconstituted in Pico-Tag Eluent A (prepared according to the manufacturer's instruction and analyzed by reversed phase-HPLC using a Nova-Pak C18 column (Waters, Milford, MA, USA). Measurements were performed in duplicates and concentrations were calculated based on the analysis of the internal amino acid standard A (Pierce).

Mass spectrometry

Intact mass measurement of Man e 5 was performed by ESI-QToF mass spectrometry (Ultima Global, Micromass-Waters, Milford, MA, USA) in aqueous 50% (v/v) HPLC-grade acetonitrile, 0.1% (v/v) formic acid directly infused at a rate of 0.5 μ l/min. Multiple charged peak series were processed by MaxEnt1TM (calibration error horse heart myoglobin 20 ppm).

Homogeneity

Online-high performance-size exclusion chromatography-light scattering (HPSEC-LS) of 100 μ L sample was performed as standard using a 7.8 x 300 mm column TSK2000_{SWXL} (Tosoh Biosciences, Stuttgart, Germany) on a HP1100 analytical chromatography system (Hewlett-Packard, San Jose, CA, USA) at 0.5 ml/min in 100 mM sodium phosphate buffer pH 6.6, 150 mM sodium chloride, 0.05% NaN₃. Using a combination of the built-in UV detector measuring at 280 nm and a sequential refractive index (RI), intrinsic viscosity (IV), and right-angle light scattering detection (TDA 302, Viscotek Corp., Houston, TX, USA) the molecular weight (MW) and hydrodynamic radius (R_H) of eluting peaks were determined as previously published (Himly, 2009). Reference proteins, *i.e.* horse heart myoglobin (16 kDa), alpha casein (23 kDa),

trypsinogen (24 kDa), ovalbumin (43 kDa), hemoglobin (68 kDa), transferrin (80 kDa) and human IgG (150 kDa) were purchased from Sigma-Aldrich and used as reference for plotting the MW against R_H and retention volume (v_{ret}), respectively.

The molecular size and aggregation behavior of Man e 5 in solution was investigated by dynamic light scattering (DLS 802, Viscotek) at 1 mg/mL in 5mM potassium phosphate pH 7.5.

The protein was centrifuged at 14,000 *g* for 10 min and for the measurement the same buffer was used as blank to prevent buffer influences. The solvent settings for water were used. Data were accumulated for 10 x 10 sec and the correlation function was fitted into the combined data curve, from which the intensity distribution was calculated. The determined intensity distribution was weighted statistically by mass using the mass model for proteins (OmniSizeTM, Viscotek) displaying molecular size R_H [nm] and polydispersity (% RSD).

Secondary structure analysis and prediction of disordered regions

Circular dichroism (CD) spectra and thermal denaturation were recorded in 5 mM potassium phosphate pH 7.4 or alternatively pH 3.0 with a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan). Far-UV CD spectra (185–260 nm) were baseline subtracted and results are presented as mean residue molar ellipticity. Thermal denaturation and refolding were made through a ramp temperature starting with 25°C going to 95°C (~1°C/min) and back to the initial temperature. Constant monitoring at 222 nm was performed to determine changes in the alpha helical regions. The secondary structure prediction of Man e 5 was performed using PSIPRED and MUSTER¹ to identify the structurally equivalent segments of Man e 5 with templates in PDB (Protein Data Bank – www.rcsb.org) [2] which was solved experimentally by NMR (Nuclear Magnetic Resonance) (PDB ID: 1MV3) [3]. The prediction of disordered regions of Man e 5 was made

using megaPrDOS server [4] using seven predictors into megaPrDOS server: PrDOS, DISOPRED2, DisEMBL, DISPROT (VSL2P), DISpro, IUpred and POODLE-S.

Molecular dynamics simulations

The molecular dynamics (MD) simulation was performed with the GROMACS 4.5 software package [5]. The protein was immersed in a water box of truncated cubic in which the minimum distance between the protein surface and the box face was 1.0 nm length in all directions and neutralized counter-ions. The final MD simulation was performed under the same conditions except that the position restraints were removed with an interval of 5000 psec, which exhibited a tendency to stabilize from 1500 psec. The convergence of the different simulation was analyzed in terms of radius of gyration, root mean-square deviation from the initial model structures, and intermolecular hydrogen bonds. All analyses were performed on the ensemble of system configurations extracted at 0.5 psec time intervals from the simulation and MD trajectory collection was initiated after 1 nsec of dynamics to guarantee a completely equilibrated evolution and model generated visualized using PyMOL software [6]. All optimization process was performed on a Beowulf cluster with 16 nodes (BioComp - <http://izalco.incor.usp.br/ganglia/>).

Statistics

Differences between Man e 5 and Hev b 5 were assessed with nonparametric Wilcoxon paired test while differences considering the same allergen between the two populations were assessed with nonparametric Mann-Whitney unpaired test. Probability of $p < 0.05$ was considered significant.

Patient selection and molecule-based diagnosis

All recruited subjects or caregivers signed an informed consent to participate the study on the basis of protocols approved by the Brazilian Ethic Committee for Analysis and Research Projects, CAPPesq of HCFMUSP (n. 0637/0) and Italian (IDI-IRCCS) Institutional Review board (106/CE/2005).

Seven patients from Brazil (7 female subjects; age range 40-55 years; median 48 years) were selected based on obvious immediate clinical history of severe allergic reaction upon manioc exposure and prick test using raw manioc [7]. All these patients were evaluated through ISAC 89 microarray IgE testing (Phadia Multiplexing Diagnostic, PMD, Vienna, Austria). A randomized sample of 30 Italian subjects among those tested Hev b 5 IgE positive as detected by ISAC 89 microarray (PMD) and clinically reacting on latex exposure but unknown history of manioc consumption were IgE tested in addition against Man e 5. Amongst those Italians twelve patients (5 female subjects, 7 male subjects, age range 5 – 63 years; median 30.1 years) reacted to Man e 5 and were selected for inhibition assays.

ELISA and cross inhibition assays

Sera of the 7 Brazilian patients and 30 Italian patients were tested for IgE reactivity to Man e 5 and Hev b 5 in ELISA experiments. Sera of patients selected for cross-inhibition assays, twelve Italians and seven Brazilians were also tested for specific IgG4 reactivity. For ELISA, maxisorp plates (Nalge Nunc, Rochester, NY) were coated with 200 ng of allergen/well in PBS o/n at 4°C. Unspecific binding was blocked with TBS pH 7.4, 0.05% (v/v) Tween-20 and 1% (w/v) BSA and incubated with 1:4 diluted patients' sera o/n at 4°C. Bound IgE was detected with

alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (BD Biosciences, Franklin Lakes, NJ) and bound IgG4 was detected with mouse anti-human IgG4-AP (Southern Biotech). Measurements were performed in duplicates and values exceeding 3x standard deviation of background were considered positive. In addition, purified recombinant allergens at concentrations of 1-2 mg/ml in 50 mM phosphate buffer were heat denatured (15 min at 95°C) prior to coating on solid phase and IgE reactivity was assessed as described above.

For inhibition experiments, manioc extract was prepared using manioc powder obtained by grinding cut pieces under liquid nitrogen. Manioc proteins were extracted in 100 mM sodium phosphate pH 7.4 at a manioc powder to buffer ratio of 1:5. The suspension was mixed briefly and centrifuged for 10 min at 13,000 g and 4°C. The protein extract was filtered through 0.45 µm filters (Millipore). For latex extract preparation fresh *Hevea brasiliensis* latex was collected in chilled containers from rubber trees (clone RRIM 600). The latex was centrifuged at 44 000g at 4°C for 1h resulting in 3 fractions: a top fraction containing the rubber particles, an aqueous phase, the C-serum, and a bottom fraction containing specialized organelles, called lutoids. The aqueous phase was collected and centrifugation repeated. The clear C-serum was freeze dried and stored at -20°C. Hev b 5 was already proven to be in this fraction. The bottom fraction containing the lutoids was resuspended in 0.4 mol/L mannitol, resedimented and subjected to repeated alternate freezing and thawing to rupture the lutoids. The fluid content of the lutoids, the B-serum, was then recovered by centrifugation [8].

Dose-dependent IgE inhibition to solid phase coated rMan e 5, rHev b 5, manioc or latex extract was assessed in ELISA using sera of seven Brazilian and twelve Italian allergic patients that were IgE positive to Man e 5. Cross-inhibition was performed with increasing concentrations of purified Man e 5 and Hev b 5 (0.05; 0.5 and 5 µg/well) and most suitable

concentration used for plotting results. In addition inhibition studies were performed as “single point highest inhibition achievable assay” (SPHIAa) in a microarray format as described previously [9] using undiluted sera of Brazilian patients and of two Italian patients pre-incubated overnight with purified allergens at a concentration of 0.1 mg/ml or 1 mg protein/ml of the whole extract (4 mg of protein/g of manioc extract) and percentage of inhibition was determined. For determination of manioc and latex extract cross-reactivity, sera of seven manioc allergic patients were pre-incubated with manioc, latex fraction B and C extracts at a concentration of 50 µg/ml and cross-inhibition to solid phase manioc, and both latex fractions was determined and expressed as percentage of inhibition.

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Legends to figures in the Supporting Information

Fig S1. Disorder prediction and modelling of tertiary structure. A, The disorder profile plot shows a disorder tendency over 50% for each residue in the Man e 5 primary sequence using different software predictors. B, Electrostatic potential surfaces for Man e 5, where blue indicates positive residues and red negative residues.

Fig S2. IgE cross-inhibition using sera of Brazilian and Italian allergic patients in SPHIAa microarray. Self inhibition of Hev b 5 (left), inhibition of Hev b 5 by Man e 5 (centre) and inhibition of Hev b 5 by manioc extract (Man e, right).

Fig S3. Cross-inhibition ELISA using manioc and latex extracts. A decrease of IgE-binding to manioc and latex protein extracts was tested using sera of manioc sensitized/allergic patients (n = 7) after pre-incubation with manioc (M), latex B (LB) or latex C (LC) protein extracts. Mean values and standard error of mean are shown as bar and whiskers, respectively. Statistical evaluation was performed using the Student's t-test. ns, not significant

