

Molecular characterization and allergenic activity of Lyc e 2 (β -fructofuranosidase), a glycosylated allergen of tomato

Sandra Westphal¹, Daniel Kolarich², Kay Foetisch¹, Iris Lauer¹, Friedrich Altmann², Amedeo Conti³, Jesus F. Crespo⁴, Julia Rodriguez⁴, Ernesto Enrique⁵, Stefan Vieths¹ and Stephan Scheurer¹

¹Department of Allergology, Paul-Ehrlich-Institut, Langen, Germany; ²Institute of Chemistry, University of Agriculture, Vienna, Austria; ³CNR-ISPA c/o Bioindustry Park, Colletterto Giacosa, Italy; ⁴Servicio de Alergia, Hospital Universitario Doce de Octubre, Madrid, Spain; ⁵Institut Universitari Dexeus, Barcelona, Spain

Until now, only a small amount of information is available about tomato allergens. In the present study, a glycosylated allergen of tomato (*Lycopersicon esculentum*), Lyc e 2, was purified from tomato extract by a two-step FPLC method. The cDNA of two different isoforms of the protein, Lyc e 2.01 and Lyc e 2.02, was cloned into the bacterial expression vector pET100D. The recombinant proteins were purified by electroelution and refolded. The IgE reactivity of both the recombinant and the natural proteins was investigated with sera of patients with adverse reactions to tomato. IgE-binding to natural Lyc e 2 was completely inhibited by the pineapple stem bromelain glycopeptide MUXF (Man α 1–6(Xyl β 1–2)Man β 1–4GlcNAc β 1–4(Fuc α 1–3)GlcNAc). Accordingly, the nonglycosylated recombinant protein isoforms did not bind IgE of tomato allergic patients.

Hence, we concluded that the IgE reactivity of the natural protein mainly depends on the glycan structure. The amino acid sequences of both isoforms of the allergen contain four possible N-glycosylation sites. By application of MALDI-TOF mass spectrometry the predominant glycan structure of the natural allergen was identified as MMXF (Man α 1–6 (Man α 1–3)(Xyl β 1–2)Man β 1–4GlcNAc β 1–4(Fuc α 1–3)GlcNAc). Natural Lyc e 2, but not the recombinant protein was able to trigger histamine release from passively sensitized basophils of patients with IgE to carbohydrate determinants, demonstrating that glycan structures can be important for the biological activity of allergens.

Keywords: Lyc e 2; tomato; food allergen; IgE reactivity; glycoprotein.

To date, only few attempts have been made to identify and characterize tomato allergens. In most reports, allergy to tomato is linked to other allergies such as grass pollen [1] and latex allergy [2,3]. The prevalence of tomato allergy ranges from 1.5% to 16% among food-allergic patients indicating that tomato is a relevant allergenic food in selected populations.

The first reports on IgE-reactive glycoproteins in tomato extract by Bleumink *et al.* [4,5] described a heat resistant protein fraction between 20 and 30 kDa showing enhanced reactivity in skin prick tests (SPT). Darnowski *et al.* [6] investigated the distribution of profilin in tomato tissues. Recently the cDNA sequence of tomato profilin was published (GenBank accession no. AY061819/AJ417553) and the protein was designated as tomato allergen Lyc e 1.

Cross-reactive carbohydrate determinants (CCD) are found in many allergenic sources such as pollen and insect

venom, but the highest rate of serological reactions to CCD has been observed to plant food extracts. Immunoblot analyses of electrophoretically separated food allergen extracts revealed that IgE-reactive carbohydrate structures are present on many different glycoproteins from one allergen source [7,8]. Examples for IgE-reactive glycoproteins are phospholipase A₂ from bee venom [9], Cup a 1 from cypress pollen [10], Ara h 1 from peanut [11] as well as a vicilin-like protein from hazelnut [12].

The analysis of free [13] and linked [14] N-glycans of tomato revealed the presence of a plant-characteristic glycan core with xylose and fucose participating in an IgE-binding epitope. The N-terminal sequencing of a 52-kDa glycoprotein of tomato extract gave hints for the existence of β -fructofuranosidase as a relevant allergen in tomato [15,16]. β -Fructofuranosidase, also known as acid invertase (EC 3.2.1.26) catalyses the hydrolysis of sucrose into glucose and fructose. A variety of these enzymes is found in plants showing differences between pH optima, isoelectric point and subcellular localization [17]. Soluble invertases are known to be vacuolar [18], but cytosolic forms also exist [19]. The β -fructofuranosidase of tomato was shown to play an important role in the regulation of hexose accumulation during fruit ripening [20]. Two isoforms of the tomato protein were identified that differed only in their C-termini. One isoform with a molecular mass of 51 kDa (GenBank accession no. D11350) has an 86-bp insertion in its sequence, a stop codon in this insertion reduces the open reading frame and thus the length of the protein. It was

Correspondence to S. Scheurer, Paul-Ehrlich-Institut, Department of Allergology, Paul-Ehrlich Str. 51–59, D-63225 Langen, Germany. Fax: + 49 6103 77 1258, Tel.: + 49 6103 77 5200, E-mail: schst@pei.de

Abbreviations: CCD, cross-reactive carbohydrate determinants; HIC, hydrophobic interaction chromatography; RT, reverse transcribed; SPT, skin prick testing; DBPCFC, double blind placebo controlled food challenge.

(Received 10 October 2002, revised 8 January 2003, accepted 5 February 2003)

found that the second isoform without the insertion sequence and a molecular mass of 60 kDa (S70040) exists at a much higher expression level in the tomato fruit [21].

The allergenicity of β -fructofuranosidase of tomato was further confirmed by Foetisch *et al.* [22]. The aim of the present study was to analyze the role of N-linked glycans in the IgE-response of tomato-allergic patients using the β -fructofuranosidase as a model allergen. For this purpose, purified natural as well as recombinant proteins were investigated concerning their IgE-binding capacity and their ability to induce histamine release from human basophils. The glycan structure of the natural β -fructofuranosidase was determined. Our results indicate an important role for N-glycans containing xylose and fucose residues in the IgE-response of tomato-allergic patients.

Materials and methods

Preparation of allergen extract

Extracts from tomato and low fat milk were prepared by a low-temperature method as previously described [23]. In brief, pieces of fresh fruit were frozen in liquid nitrogen, and ground in a mill without thawing. The obtained powder was homogenized in prechilled acetone and stored overnight at -20°C . The precipitate was filtered, washed twice with ice-cold acetone and once with acetone/diethylether (1 : 1, v/v) and lyophilized. Extraction of proteins from this powder was done with NaCl/P_i (0.15 M $\text{NaCl}/0.01$ M NaH_2PO_4) at 4°C . After centrifugation the supernatant was collected, filtered and freeze-dried. The lyophilized extract was stored at -80°C .

Purification of N-linked glycopeptides

N-linked glycopeptides with the glycan structure $\text{Man}\alpha 1-6(\text{Xyl}\beta 1-2)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4(\text{Fuc}\alpha 1-3)\text{GlcNAc}$ (MUXF) coupled to two to four amino acids were prepared from pineapple stem bromelain by digestion with pronase followed by a series of chromatographic steps as described elsewhere [24]. Glycopeptides containing the pentasaccharide core $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$ (MM) were prepared from bovine fibrin.

Purification of natural Lyc e 2 from tomato fruit

To purify the natural β -fructofuranosidase, lyophilized tomato extract was dissolved in starting buffer (1 M $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris/HCl, 1 mM EDTA, pH 8.0) to a protein concentration of 2 mg mL^{-1} . After filtration through a $0.45\text{-}\mu\text{m}$ filter (Sartorius, Göttingen, Germany) the protein solution was applied to a 1-mL phenyl superose column (Amersham Pharmacia Biotech, Uppsala, Sweden) to perform hydrophobic interaction chromatography (HIC). Bound proteins were eluted with distilled water at a flow rate of 0.5 mL min^{-1} . Further purification of the eluted fractions containing the IgE-reactive 50-kDa band was performed by gel chromatography using a Superdex 75 Column, HR10/30 (Amersham Pharmacia Biotech). Elution was done with NaCl/P_i , pH 7.4 at a flow rate of 0.5 mL min^{-1} . Fractions were collected in 0.5 mL steps and analyzed by SDS/PAGE and immunoblotting.

N-terminal amino acid sequencing

Partially purified Lyc e 2 eluted from the HIC column was electroblotted onto a poly(vinylidene difluoride) membrane. After staining with Coomassie Brilliant Blue the protein band was excised from the membrane and analyzed on an Applied Biosystems 492 Procise sequencer (Applied Biosystems, Foster City, CA, USA) in pulse-liquid mode to determine the N-terminal partial sequence of the IgE-reactive protein. All chemicals were from Applied Biosystems.

Cloning the cDNAs of two isoforms of β -fructofuranosidase from tomato fruit

Total RNA was isolated from tomato fruit using the RNeasy Plant RNA Mini Kit (Qiagen, Hilden, Germany). DNA contaminations were removed by using the RNase-free DNase set (Qiagen). The RNA was reverse transcribed (RT) with the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions using $1\text{ }\mu\text{g}$ total RNA for each transcription and the *NotI*-d(T)₁₈ oligonucleotide for priming. To obtain the complete coding region, the RT products were amplified using gene specific 5'- and 3' primers selected on the basis of the published sequences for tomato β -fructofuranosidase (GenBank accession no. D11350 and S70040). Primers for the short isoform of β -fructofuranosidase were FF5SP, matching with the N-terminal sequence of the coding region: 5'ATGGCCACTCAGTATGACC, FF5, matching with the N-terminal sequence of the mature protein: 5'TATGCGTGGTCCAATGCTATGC, and FF3A, matching with the C-terminal sequence of the coding region: 5'TTACAAGGACAAATTAATTGTGCCAG. For amplification of the long isoform the same 5' primers were used, the 3' specific primer was FF3B: 5'TTACAAGTCTTGCAAAGGGAAGGAT. For amplification the Expand long template DNA Polymerase Set (Roche, Mannheim, Germany) was used. The PCR conditions were the following: 94°C , 5 min, followed by 30 cycles: 94°C , 30 s, 50°C , 30 s, 68°C , 2 min. The final extension was 7 min at 68°C . The obtained cDNA was cloned into the pCRII-TOPO vector (Invitrogen, Groningen, the Netherlands).

For protein expression in *E. coli* the coding regions without signal sequences were cloned into the pET100D vector containing a six histidine tag using the pET Directional TOPO expression Kit (Invitrogen). The DNA was amplified using the same 3' primers as for cDNA cloning, whereas the 5' primer contained the sequence CACC for directional cloning. FF5-CACC: 5'CACCTATGCGTGGTCCAATGCTATGC. The PCR was carried out using Vent DNA polymerase (New England Biolabs, Frankfurt, Germany) under the following conditions: 94°C , 5 min, followed by 30 cycles: 94°C , 30 s, 50°C , 30 s, 72°C , 2 min. The final extension was 7 min at 72°C .

DNA sequencing

The sequence analysis was carried out with an ABI 373 automated fluorescent sequencer (Applied Biosystems) using vector or gene specific primers and the ABI PRISM

BigDye Terminators v3.0 CycleSequencing Kit according to the manufacturer's instructions.

Recombinant protein expression and purification

For expression, the pET100D constructs were transformed in *E. coli* BL21 star (Invitrogen) and protein synthesis was induced with 1 mM isopropyl thio- β -D-galactoside for 5 h at 37 °C. After induction, bacteria were harvested by centrifugation and stored at -80 °C. Purification was carried out by electroelution from SDS/PAGE gels. Electroelution was performed as described elsewhere [25]. Briefly, the pellet from 100-mL bacterial culture was resuspended in non-reducing 1 $\times$ SDS loading buffer Rotiload 2 (Roth, Karlsruhe, Germany) and proteins were separated by SDS/PAGE using a 10% resolving gel with 1.5-mm spacers. Desired bands were excised from the gel after staining with 0.3 M CuCl₂ and the protein was eluted using a Centrilotur electroelution device (Millipore, Bedford, MA, USA). Elution of the proteins was done at 25 mA for 3 h directly into Centricon centrifugal filter devices with an exclusion size of 30 kDa. The purity of the eluted fractions was controlled by SDS/PAGE followed by staining with Coomassie Brilliant Blue and the protein content was determined according to Bradford using the Roti-Quant protein assay (Roth).

Patients' sera

Serum samples were taken from a group of 78 patients with a positive case history of immediate type reactions to tomato fruit. Most of the patients (49) were from Germany, the others were from Spain (Table 1). Only adults were included in the study, the age ranged between 19 and 65 years; 20% were male. All Spanish and some of the German patients underwent skin prick testing (SPT) with commercial tomato extract. Four Spanish patients were tested with DBPCFC (double blind placebo con-

trolled food challenge) and showed positive reaction. Serum from a nonallergic subject was taken as a negative control.

Determination of specific IgE

Measurement of allergen-specific IgE was performed with the CAP FEIA system (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions.

In addition, a covalink-ELISA was performed in 96 well Covalink-plates (Nunc GmbH & Co. KG, Wiesbaden, Germany) as previously described using 250 ng natural or recombinant protein per well instead of glycopeptides [8]. For detection of IgE reactivity, streptavidin conjugated with horseradish peroxidase instead of alkaline phosphatase was used. After visualization of the enzymatic activity with tetramethylbenzidine as substrate at 37 °C for 20 min the reaction was stopped by addition of 50 μ L 3 M H₂SO₄ and absorption was measured at 450 nm [26].

IgE immunoblot and IgE immunoblot inhibition

Allergen extracts (20 μ g $\cdot$ cm⁻¹), *E. coli* lysates as well as purified natural and recombinant allergens (0.5 μ g $\cdot$ cm⁻¹) were separated by SDS/PAGE under reducing conditions as described by Laemmli *et al.* [27] in a Mini-Protein 3 cell (Bio-Rad, Munich, Germany). For immunoblot analysis, proteins were transferred onto 0.45 μ m nitrocellulose membranes (Schleicher und Schuell, Dassel, Germany) by tank blotting using the Bio-Rad Mini Trans blot cell for 1 h at 300 mA. Before application of the 1 : 10 diluted patients' sera the membrane was blocked in NaCl/Tris/0.3% Tween20 and cut into 3 mm wide strips. Immunostaining of bound IgE antibodies was performed with an alkaline phosphatase conjugated anti-(human IgE) Ig (Pharmingen, Hamburg, Germany, 1 : 750 dilution, 4 h) and the Bio-Rad alkaline phosphatase conjugate substrate kit (Bio-Rad).

Table 1. Clinical data of patients investigated in this study. OAS, oral allergy syndrome; ND, neurodermatitis; *n*, number of patients investigated; SPT pos., patients with positive skin prick test/patients tested.

Country	Symptoms			
	Mild (OAS)		Systemic (Urticaria, ND, Nausea, Anaphylaxis)	
	CAP	SPT pos.	CAP	SPT pos.
Germany	0 (<i>n</i> = 4)	1/2	0 (<i>n</i> = 10)	3/3
1	(<i>n</i> = 2)	0/0	1 (<i>n</i> = 2)	0/1
2	(<i>n</i> = 13)	3/7	2 (<i>n</i> = 2)	1/1
3	(<i>n</i> = 8)	3/4	3 (<i>n</i> = 3)	2/3
4	(<i>n</i> = 2)	1/1	4 (<i>n</i> = 2)	1/2
5	(<i>n</i> = 1)	0/0	5 (<i>n</i> = 0)	0/0
Spain	0 (<i>n</i> = 1)	0/0	0 (<i>n</i> = 1)	0/0
1	(<i>n</i> = 3)	2/2	1 (<i>n</i> = 1)	1/1
2	(<i>n</i> = 6)	1/1	2 (<i>n</i> = 1)	0/1
3	(<i>n</i> = 5)	3/3	3 (<i>n</i> = 5)	3/3
4	(<i>n</i> = 2)	2/2	4 (<i>n</i> = 3)	3/3
5	(<i>n</i> = 1)	1/1	5 (<i>n</i> = 0)	0/0

For inhibition of IgE-binding 1 : 10 diluted sera were preincubated with 10 µg of purified glycopeptide and 100 µg of allergen extract before incubation of the blot strips.

Circular dichroism (CD) spectroscopy of natural and recombinant β -fructofuranosidase

The CD spectra of the natural Lyc e 2 as well as of the larger recombinant isoform designated as rLyc e 2.02 were recorded on a Jasco J-810S spectropolarimeter (Jasco, Groß-Umstadt, Germany) at 20 °C with a stepwidth of 0.2 nm and a bandwidth of 1 nm. The spectral range was 190–260 nm at 50 nm·min⁻¹. Six scans were accumulated. The protein concentration was 5.5 µM in a 10 mM KH₂PO₄, pH 7.0.

Analysis of N-linked glycans and peptides of Lyc e 2 by MALDI-TOF mass spectrometry

Eight micrograms of HIC-purified Lyc e 2 was excised from a Coomassie-stained SDS/PAGE gel after electrophoresis under reducing conditions and subjected to tryptic digestion as described elsewhere [28]. The extracted and dried peptides were taken up in water/acetonitrile/trifluoroacetic acid (95 : 5 : 0.1, v/v/v) and analyzed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Further preparation and mass spectrometry analysis of N-glycans was performed according to Kolarich and Altmann [29]. Briefly, the peptides were dried and redissolved in ammonium acetate before deglycosylation with N-glycosidase A. To remove salts and peptides the digest was purified using a triphasic column consisting of Dowex W 50, C-18 reversed phase and an AG 3-X4A (Dow Chemical Company, Edegem, Belgium). Analysis and identification of the glycans was carried out by mass spectrometry using a DYNAMO MALDI-TOF (Thermo-BioAnalysis, Santa Fé, NM, USA).

Basophil histamine release

The histamine-release was performed as described previously [30] with several modifications. Peripheral blood was drawn from nonallergic donors and PBMCs were isolated using Ficoll-Hypaque centrifugation. The conditions for stripping of the nonspecific IgE and for the passive sensitization procedure were chosen according to the recommendations of Pruzansky *et al.* [31]. Cells sensitized with a nonallergic serum served as negative control. Stimulation of the cells was performed using a histamine kit (Immunotech, Marseille, France) according to the manufacturer's instructions with tenfold dilutions of the allergens starting at 10 µg·mL⁻¹. For testing, self-prepared tomato extract, nLyc e 2, rLyc e 2, horseradish peroxidase, deglycosylated horseradish peroxidase, the glycopeptide MUXF and MUXF conjugated to BSA as well as BSA alone were used. The histamine releases were measured by an enzyme immunoassay (Immunotech). After subtraction of the spontaneous release of the basophils, the allergen-induced histamine release was calculated as percent of the total amount of histamine determined after lysis of the basophils by twofold freezing and thawing of the cells. A histamine release of more than 10% was considered positive. Duplicate determinations were performed in all cases.

Results

Screening of patients' sera

Sera from patients with a history of adverse reactions to tomato were investigated by immunoblotting. Special attention was drawn to IgE reactions to protein bands in the high molecular mass range frequently found to be glycoproteins with ubiquitous carbohydrate epitopes [8,22]. Out of 49 sera from German patients with tomato-related symptoms such as OAS, nausea, urticaria, abdominal pain and dyspnea (Table 1), 18 (37%) recognized several bands above 20 kDa (Fig. 1A).

From the Spanish group, 10 out of 29 (34.5%) sera showed reactivity in the high molecular mass range (Fig. 1B).

Hence, there was no significant difference in IgE reactivity to glycoproteins between both groups. Besides binding to protein bands larger than 20 kDa we also observed reactivity to proteins with a molecular mass of 15 and 9 kDa. IgE binding to carbohydrates was confirmed by blot inhibition of a patient's serum with known sensitization against CCD. Tomato extract as well as the glycopeptide MUXF obtained from pineapple stem bromelain almost completely inhibited the IgE reactivity except for one band at 55 kDa assuming that either this protein does not contain such glycosylation or the IgE reactivity is based on the protein backbone alone. No inhibition was observed with the fibrin glycopeptide MM and extract from low fat milk (data not shown). These results indicated that the IgE-binding to most of the tomato proteins in the high molecular mass range is mediated by the cross-reactive glycan structure MUXF typically existing in plants but not in mammals.

The 28 patients showing IgE reactivity in the high molecular mass range were selected for further studies on the IgE-binding capacity of Lyc e 2.

Two step purification of Lyc e 2 from tomato extract

The elution profile of the first chromatographic step (HIC) is shown in Fig. 2A. A 50-kDa band corresponding to Lyc e 2 was detected in the four water elution fractions E1–4. After size exclusion chromatography of pooled fractions E3 and E4 the proteins were nearly homogeneous. In the elution fractions 30–33 Lyc e 2 with a molecular mass of 50 kDa was eluted, fractions 34–37 contained a band of 36 kDa and the fractions 38–41 a protein with a molecular mass of about 20 kDa (Fig. 2B).

Immunoblot analysis with a polyclonal anti-profilin serum from rabbit confirmed that another important tomato allergen, profilin, did not contaminate the purified Lyc e 2-fractions. In contrast to tomato extract that showed a profilin band at 14 kDa, no bands were visible in the fractions 30–33 from the second purification step (not shown).

N-Terminal amino acid sequencing

For N-terminal sequencing fraction E3 from the HIC step was used. The sequence of the 50 kDa band excised from the poly(vinylidene difluoride) membrane was YAXSNAMLXX. A search in the protein database

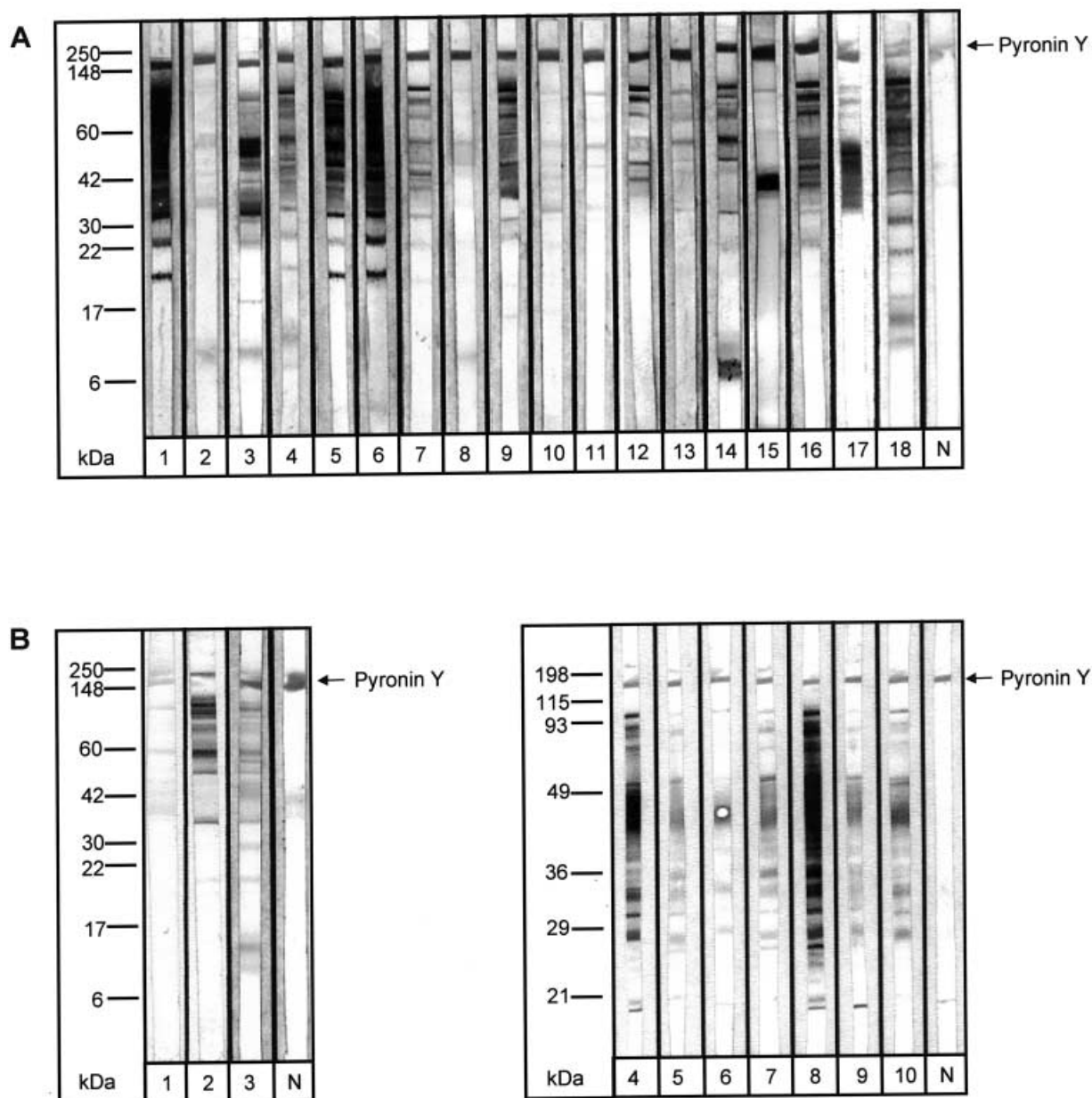


Fig. 1. IgE binding to glycoproteins in tomato extract. IgE-binding of sera from German (A) and Spanish (B) patients to glycosylated tomato extract proteins separated by SDS/PAGE and transferred to nitrocellulose (20 µg protein per cm). N, negative control, serum from nonallergic subject.

revealed this protein to be β -fructofuranosidase (YAW SNAMLSW). From the N-terminal sequence we were not able to distinguish between the two isoforms of the protein, only the molecular mass of 50 kDa would suggest that we had purified the truncated isoform.

Cloning of the cDNA of two isoforms of tomato β -fructofuranosidase and recombinant expression in *E. coli*

For protein expression in *E. coli*, only the cDNA coding for the mature proteins without signal peptide sequence was amplified and cloned in the pET100D expression vector. Because the proteins completely accumulated in insoluble

inclusion bodies, they were purified by electroelution and refolded. The truncated isoform, designated as Lyc e 2.01 had an apparent molecular mass of 51 kDa. The other isoform, Lyc e 2.02 migrated as a 60-kDa band. Both proteins were highly pure (Fig. 3). The CD spectra of natural Lyc e 2 and recombinant Lyc e 2.02 (rLyc e 2.02) were highly superimposable and clearly showed the existence of secondary structures (not shown).

Comparison of IgE-reactivities of the purified natural and recombinant Lyc e 2

HIC-purified natural and electroeluted recombinant proteins (both isoforms) were separated by SDS/PAGE (0.5 µg

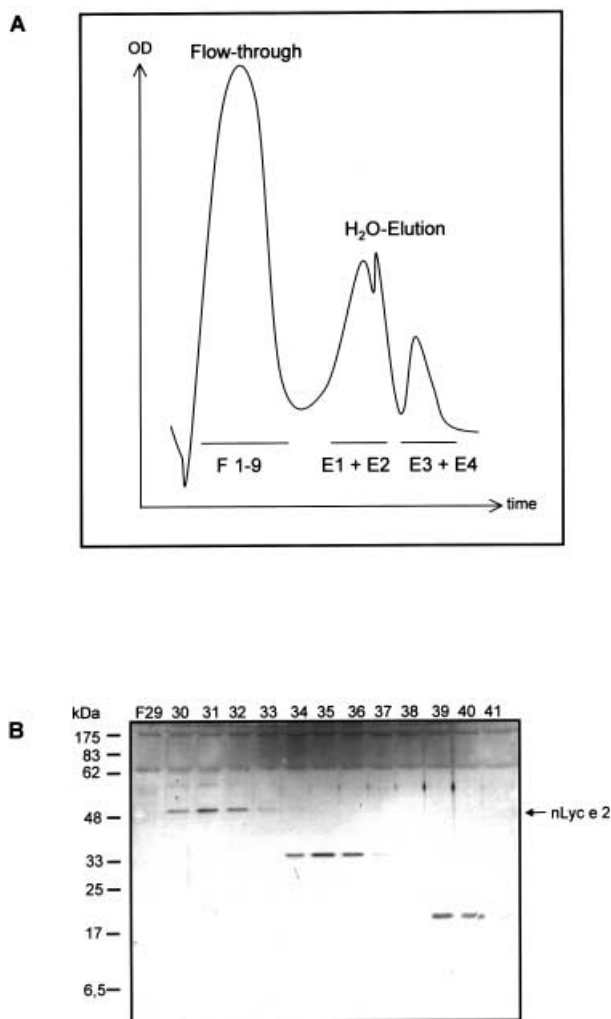


Fig. 2. Purification of natural Lyc e 2. (A) Elution profile of FPLC purification of tomato extract after hydrophobic interaction chromatography (HIC) using a Phenyl Superose column. (B) Silver-stained SDS/PAGE gel of fractions 29–41 eluted from the second purification step with Superdex S 75. The arrow indicates Lyc e 2.

protein per cm) and blotted onto nitrocellulose. As a control for the recombinant proteins, an antibody reacting with the histidine tag (Qiagen) was used. Out of 28 sera preselected by IgE reactivity to high molecular mass proteins in tomato extract, 13 (46%) reacted with the natural protein nLyc e 2 (Fig. 4A) whereas no reaction was observed with the recombinant protein isoforms rLyc e 2.01 (not shown) and rLyc e 2.02 (Fig. 4B). The purified nLyc e 2 fractions contain a contaminating band at 90 kDa that was not detected in the silver stained SDS/PAGE gel but seems to be IgE reactive with almost all sera tested. N-Terminal sequencing analysis failed because this protein was N-terminally blocked.

Besides immunoblotting we also performed a Covalink-ELISA to determine IgE binding to the natural Lyc e 2 and the recombinant protein. All sera reacting with the natural protein in the ELISA were positive in the immunoblotting experiments. For the recombinant protein, 24 of 28 sera were negative in both assays, four of the investigated sera

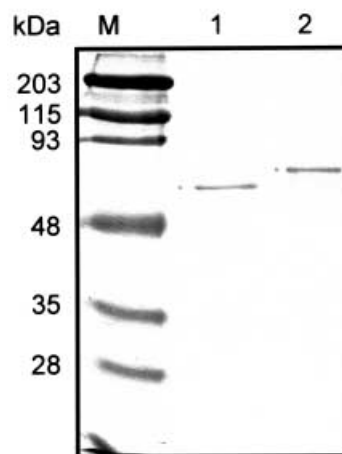


Fig. 3. Purification of recombinant Lyc e 2. SDS/PAGE analysis of electroeluted recombinant Lyc e 2 isoforms rLyc e 2.01 (lane 1) and rLyc e 2.02 (lane 2), Coomassie stain. M, molecular mass marker.

reacted with the recombinant protein in the ELISA, but not in the immunoblot (not shown).

IgE reactivity of the native allergen is completely inhibited by the bromelain glycopeptide MUXF

To confirm the role of the glycan moieties of nLyc e 2 in IgE-binding, blot inhibition studies with purified glycopeptide MUXF from pineapple stem bromelain were performed. MUXF is a typical plant glycan structure that was shown to exist in a high percentage on tomato proteins, namely 17–22% [14]. It is known to act as an IgE reactive structure [4,5,8,15,22] whereas the clinical significance of this reactivity is still unclear [7,8,32]. A pool of three patients' sera recognizing nLyc e 2 was preincubated with tomato extract (100 µg protein), 10 µg MUXF as well as extract from low fat milk (100 µg protein) and 10 µg MM from bovine fibrin as negative controls. Binding to β -fructofuranosidase was almost completely inhibited by tomato extract and the glycopeptide MUXF. The MM glycopeptide as well as low fat milk extract as negative controls showed no inhibition at all (Fig. 5A). Binding to the contaminating 90-kDa band was also inhibited by MUXF, so this protein may be an IgE reactive glycoprotein as well. Preincubation of a non-CCD binding serum with MUXF had no effect on the IgE-binding to low molecular mass protein allergens in tomato extract (not shown).

Peptide map and glycan analysis of the natural β -fructofuranosidase

Investigation of the carbohydrate moieties of the purified allergen from tomato was carried out by MALDI-TOF mass spectrometry. From the sequence it was known that both isoforms of Lyc e 2 contain four putative N-glycosylation sites. The inhibition experiments performed with the MUXF peptide gave a strong hint for the existence of either xylose or fucose or both being components of the glycan structure of the protein. The

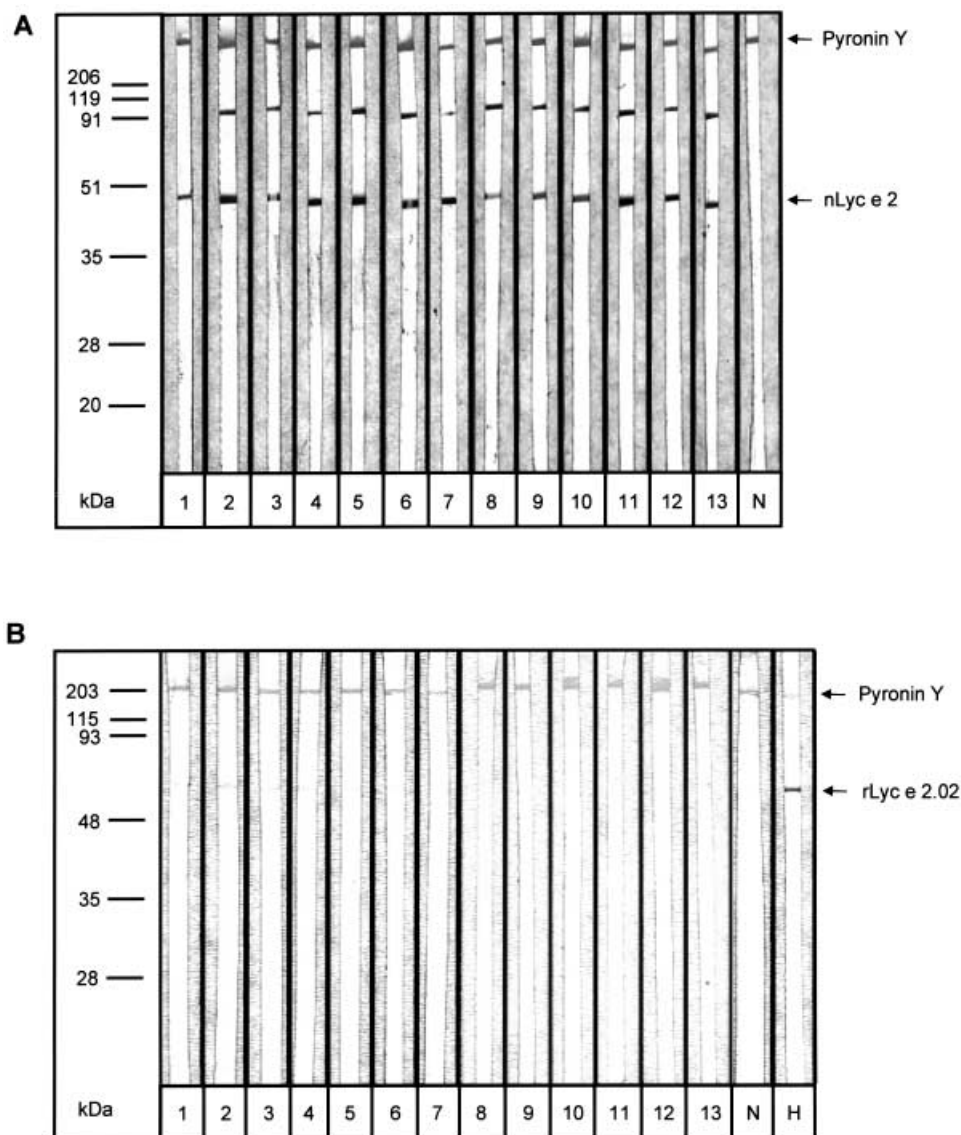


Fig. 4. IgE-binding of sera from tomato allergic patients to Lyc e 2. Patients were preselected for IgE reactivity in the high molecular mass range (≥ 20 kDa) and only positive reacting sera are shown. (A) Binding to natural Lyc e 2. (B) IgE reactivity of sera from tomato allergic patients to recombinant Lyc e 2.02. $0.5 \mu\text{g}\cdot\text{cm}^{-1}$ of purified protein were separated by SDS/PAGE and blotted onto nitrocellulose. H, Anti-histidine-tag Ig; N, negative control, serum from nonallergic subject.

glycan analysis of the natural protein from tomato revealed that MMXF is the dominating glycan with about 84% of all sugar structures. The structures of the N-glycans of nLyc e 2 and their molecular percentages are presented in Table 2.

The peptide analysis of nLyc e 2 identified 21 peptides of the natural allergen. One of four peptides containing a potential glycosylation site was determined by this approach, but the other potentially glycosylated peptides were not detected in the mass spectrum. For the peptide GWYHLFYQYNPDSAIWGNITWGHAVSK, the N-linked glycan bound to asparagine was identified as MMXF. This result is in accordance with the glycan analysis of natural Lyc e 2 that revealed this structure to be the main glycan moiety of the protein.

The carbohydrates of nLyc e 2 are able to trigger histamine release from human basophils

In order to confirm the clinical relevance of the tomato allergen nLyc e 2, its ability to induce histamine release from human basophils was investigated. We performed histamine release experiments with stripped basophils from nonallergic donors, passively sensitized with serum from tomato-allergic patients. Natural Lyc e 2 was further purified by electroelution to almost 100% purity as it was carried out with the recombinant proteins, to eliminate effects of the contaminating protein detected by Western blotting, and its purity was confirmed by Western blot with patients' sera (Fig. 5B). Using serum of a German patient with IgE reactivity to CCD, it was shown that nLyc e 2 as

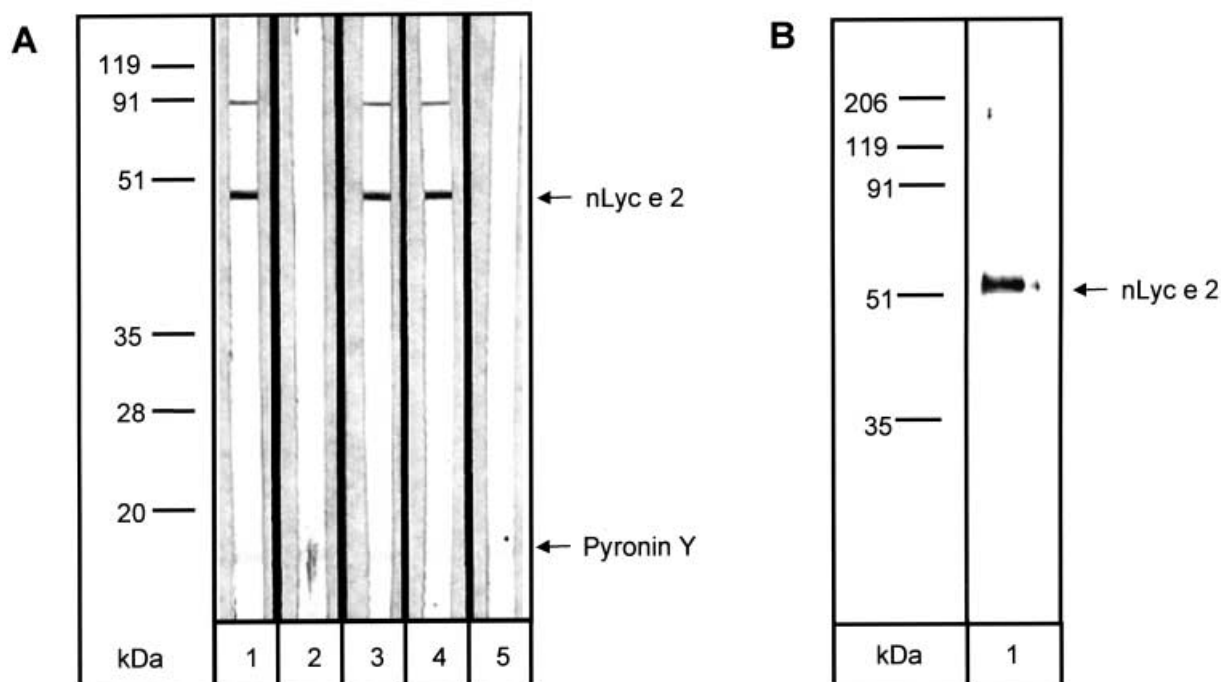


Fig. 5. Immunoblot inhibition of IgE reactivity of a serum pool ($n = 3$) to purified nLyc e 2 (A) and IgE binding to nLyc e 2 after further purification by electroelution (B). 1, No inhibitor; 2, 100 μ g protein of tomato extract; 3, 100 μ g protein of extract from low fat milk; 4, 10 μ g glycopeptide MM; 5, 10 μ g glycopeptide MUXF.

Table 2. Glycan structures identified on natural Lyc e 2 from tomato.

Sugar moiety	Mol-% in nLyc e 2
MMXF ³ (Man α 1-6(Xyl β 1-2) Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc)	5.3
MMX (Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4GlcNAc)	8.2
MMXF ³ (Man α 1-6(Man α 1-3)(Xyl β 1-2) Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc)	83.6
GnMXF ³ (GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc)	2.3
GnGnMXF ³ (GlcNAc β 1-2Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc)	0.6

well as tomato extract, BSA-conjugated pineapple stem bromelain glycopeptide MUXF and horseradish peroxidase containing the glycopeptide MMXF induced dose-dependent histamine release from basophils passively sensitized with serum from a patient reacting with CCD and nLyc e 2. No reaction was observed with the recombinant protein rLyc e 2.02, BSA, deglycosylated horseradish peroxidase and the nonconjugated glycopeptide MUXF (MUXF-GP) which were applied as control antigens (Fig. 6A,B). The short isoform rLyc e 2.01 reacted in the same way as rLyc e 2.02 (not shown). In contrast, with serum from a German patient who did neither react with CCD nor nLyc e 2, no histamine release was induced with the glycoproteins after sensitizing the basophils. Sensitization with serum of this patient only revealed histamine release with tomato extract. (Fig. 6C,D).

Discussion

The present study describes for the first time the purification and detailed characterization of a glycosylated

tomato allergen, Lyc e 2 and the comparison with the nonglycosylated recombinant protein from *E. coli*. In contrast to Ara h 1 from peanut [11], Lyc e 2 has multiple glycosylation sites and was thus investigated as a model of multivalent glycoprotein allergens from plant food. The natural protein was purified from tomato extract using FPLC. Two different isoforms of Lyc e 2 were cloned and expressed in *E. coli* and purified by electroelution. Sera from German and Spanish patients with adverse reactions to tomato were used for investigation of IgE reactivity to glycoproteins in tomato extract and to natural and recombinant Lyc e 2. A subgroup of these patients reacted with proteins in the high molecular mass range, presumably glycoproteins. We also found reactivity of some sera to a 9- and a 15-kDa band. We could show that the 9-kDa band in the tomato extracts reacts with a specific antibody against the LTP from cherry, Pru av 3 and the 15-kDa band shows reactivity using a polyclonal rabbit serum against profilin from pear, Pyr c 4 (data not shown). These results indicate that also LTP and profilin may be relevant allergens of tomato.

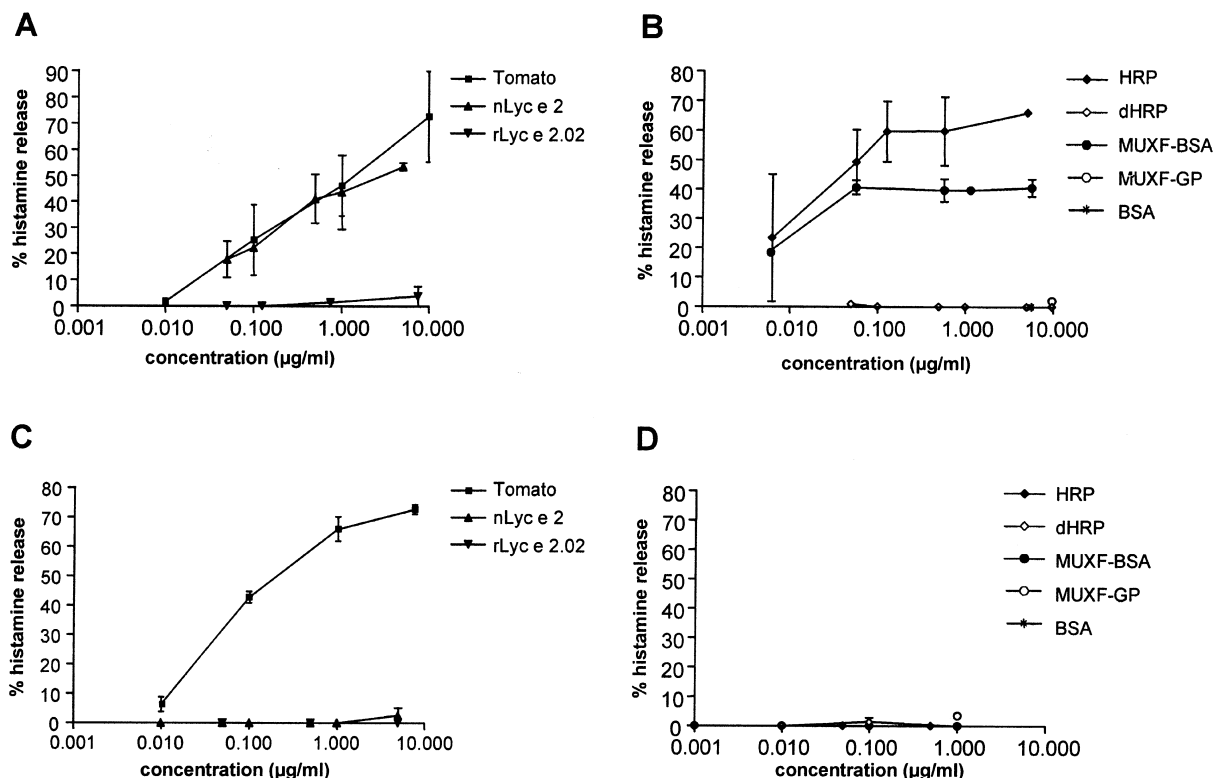


Fig. 6. Induction of histamine release from stripped human basophils passively sensitized with sera from tomato-allergic patients. (A, B) Patient 1, showing IgE reactivity to nLys e 2 and CCD. (C, D) Patient 2, showing no IgE reactivity to nLys e 2 and CCD. Standard deviations are shown for each measurement. horseradish peroxidase: horseradish peroxidase, dhorseradish peroxidase, deglycosylated horseradish peroxidase, MUXF-GP: glycopeptide MUXF from pineapple stem bromelain, MUXF-BSA: glycopeptide conjugated to BSA in a ratio of 1 : 9.

Sera from 17% of the investigated tomato allergic patients reacted with nLys e 2 on immunoblots. We have clearly demonstrated that the IgE-binding capacity of nLys e 2 mainly depends on the glycan structure MMXF that was identified as the main glycan structure on the protein. The IgE-binding to the allergen was completely blocked by the glycopeptide MUXF from pineapple stem bromelain, and recombinant nonglycosylated proteins from *E. coli* with an intact secondary structure were not detected by the human IgE antibodies. Because *E. coli* is not able to perform post-translational modifications such as glycosylation, this is further evidence for the almost exclusive IgE reactivity to glycan structures that found only on the natural tomato protein. In addition, inhibition experiments with tomato extract and MUXF as inhibitor indicated that identical or structurally very similar carbohydrate epitopes were present on many high molecular mass proteins in the tomato extract.

In the covalink ELISA we found good correlation with the immunoblots except for four sera that reacted with the recombinant protein in the ELISA, but not in the immunoblot. We hypothesize that these patients recognize a protein epitope on the allergen that is only accessible under native conditions in the ELISA system.

Interestingly, only 46% of the patients showing IgE reactivity to glycoproteins recognized the allergen Lys e 2 in the immunoblot studies, suggesting that more than 50% of the selected patients are sensitized to other tomato allergens

containing different IgE reactive glycan structures. For example, the glycan moiety MMX ($\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)(\text{Xyl}\beta 1-2)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$) was identified as main glycan of the vicilin-like protein from hazelnuts [12]. The allergen Lol p 11 from ryegrass, *Lolium perenne*, contained MUXF as well as MMXF as main structures [11].

Interestingly, the β -fructofuranosidase from carrot cell wall, which has not been described as an allergen so far, contains only three glycosylation sites in contrast to the four sites detected in the nLys e 2 sequence. The detailed characterization of the carrot protein by Sturm [33] revealed that all three sites are glycosylated. On the first site a high mannose type glycan was identified; the others carry three different complex type glycans. One of these was identified as the same structure found on nLys e 2, MMXF. It would be interesting to investigate the IgE reactivity and allergenic activity of this carrot protein in comparison to the tomato allergen.

As the IgE reactivity of nLys e 2 was inhibited by the pineapple stem bromelain glycopeptide MUXF and not by MM, one could assume that the xylose and/or fucose residues are responsible for the IgE reactivity to the allergen. It seems that often the $\beta 1,2$ -xylose is the important IgE reactive component, but that recognition of the xylose appears to be dependent on the mannose substitution influencing the conformation of the epitope. Van Ree *et al.* [11] suggested that the additional $\alpha 1,3$ -mannose on MMXF

leads to steric hindrance and lowers the IgE reactivity of the xylose epitope. This may be another reason why only a subgroup of sera reacting with glycoproteins recognized nLyc e 2 on the immunoblots.

Besides the main structure MMXF, the bromelain glycopeptide MUXF was also identified on nLyc e 2. Hence, for the IgE reactivity of the natural protein it is also possible that the low amount of MUXF detected on the allergen contributes to the IgE reactivity of this protein. However, the histamine release data with horseradish peroxidase clearly show that the MMXF structure can act as an IgE epitope.

We have shown that the ability to trigger histamine release from human basophils is mediated by the glycan structure of the nLyc e 2. No mediator release was measured using the nonglycosylated recombinant protein isoforms. These results indicate that the glycan structure on nLyc e 2 has allergenic activity because it is able to elicit an essential event of the type I allergic reaction, i.e. specific degranulation of basophils. The protein backbone of the allergen does not seem to play a role because the recombinant protein did not induce the mediator release even at a high concentration of $10 \mu\text{g mL}^{-1}$. The CD spectra of the natural and the recombinant Lyc e 2 proteins were highly superimposed and revealed the existence of secondary structures, thus suggesting correct folding of the electroeluted proteins. Therefore we could exclude a loss of the allergenic activity of the recombinant Lyc e 2 due to unfolding of the protein.

Our observation was further confirmed by use of horseradish peroxidase and a BSA-conjugate containing multiple MUXF glycans that both also induced histamine release in a patient monosensitized to CCD in tomato, thus fully simulating allergenic activity of tomato extract (Fig. 6A,B). To further confirm the clinical relevance of CCD on nLyc e 2, sera from other tomato allergic patients are currently being investigated in histamine release experiments; to date our results give strong evidence for the allergenic activity of the carbohydrates in a subgroup of tomato allergic patients.

Therefore the presence of highly cross-reactive glycan structures has to be taken into account if recombinant allergens are applied for allergy diagnosis. Information about patients' reactivity to CCD-containing allergens would be lost if serological diagnosis were based exclusively on recombinant allergens from *E. coli*. Here, the application of recombinant proteins from plant hosts such as *Arabidopsis thaliana* or *Nicotiana tabacum* would be an attractive approach to produce antigens for detection of anti-CCD IgE molecules.

Acknowledgments

The authors are grateful to Katrin Lehmann, University of Bayreuth, Bayreuth, Germany, for performing the CD spectroscopy. This work was supported by a grant from the Deutsche Forschungsgemeinschaft, DFG SCHE-637/1-1.

References

- de Martino, M., Novembre, E., Cozza, G., de Marco, A., Bonazza, P. & Vierucci, A. (1988) Sensitivity to tomato and peanut allergens in children monosensitized to grass pollen. *Allergy* **43**, 206–213.
- Beezhold, D.H., Sussman, G.L., Liss, G.M. & Chang, N.S. (1996) Latex allergy can induce clinical reactions to specific foods. *Clin. Exp. Allergy* **26**, 416–422.
- Kim, K.T. & Hussain, H. (1999) Prevalence of food allergy in 137 latex-allergic patients. *Allergy Asthma Proc.* **20**, 95–97.
- Bleumink, E., Berrens, L. & Young, E. (1967) Studies on the atopic allergen in ripe tomato fruits. II. Further chemical characterization of the purified allergen. *Int. Arch. Allergy Appl. Immunol.* **31**, 25–37.
- Bleumink, E., Berrens, L. & Young, E. (1966) Studies on the atopic allergen in ripe tomato fruits. I. Isolation and identification of the allergen. *Int. Arch. Allergy Appl. Immunol.* **30**, 132–145.
- Darnowski, D.W., Valenta, R. & Parthasarathy, M.V. (1996) Identification and distribution of profilin in tomato (*Lycopersicon esculentum* Mill.). *Planta (Germany)* **198**, 158–161.
- Aalberse, R.C. (1998) Clinical relevance of carbohydrate allergen epitopes. *Allergy* **53**, 54–57.
- Foetisch, K., Altmann, F., Hausteine, D. & Vieths, S. (1999) Involvement of carbohydrate epitopes in the IgE response of celery-allergic patients. *Int. Arch. Allergy Immunol.* **120**, 30–42.
- Tretter, V., Altmann, F., Kubelka, V., Marz, L. & Becker, W.M. (1993) Fucose alpha 1,3-linked to the core region of glycoprotein N-glycans creates an important epitope for IgE from honeybee venom allergic individuals. *Int. Arch. Allergy Immunol.* **102**, 259–266.
- Alisi, C., Afferni, C., Iacovacci, P., Barletta, B., Tinghino, R., Buteroni, C., Puggioni, E.M., Wilson, I.B., Federico, R., Schinina, M.E., Ariano, R., Di Felice, G. & Pini, C. (2001) Rapid isolation, characterization, and glycan analysis of Cup a 1, the major allergen of Arizona cypress (*Cupressus arizonica*) pollen. *Allergy* **56**, 978–984.
- van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J., Milazzo, J.P., Loutelier-Bourhis, C., Rayon, C., Villalba, M., Koppelman, S., Aalberse, R.C., Rodriguez, R., Faye, L. & Lerouge, P. (2000) Beta (1,2)-xylose and alpha (1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. *J. Biol. Chem.* **275**, 11451–11458.
- Mueller, U., Luettkopf, D., Hoffmann, A., Petersen, A., Becker, W.M., Schocker, F., Niggemann, B., Altmann, F., Kolarich, D., Hausteine, D. & Vieths, S. (2000) Allergens in native and roasted hazelnuts (*Corylus avellana*) and their cross-reactivity to pollen. *Eur. Food Res. Technol.* **212**, 2–12.
- Priem, B., Gitti, R., Bush, C.A. & Gross, K.C. (1993) Structure of ten free N-glycans in ripening tomato fruit. Arabinose is a constituent of a plant N-glycan. *Plant Physiol.* **102**, 445–458.
- Zeleny, R., Altmann, F. & Praznik, W. (1999) Structural characterization of the N-linked oligosaccharides from tomato fruit. *Phytochemistry* **51**, 199–210.
- Petersen, A., Vieths, S., Aulepp, H., Schlaak, M. & Becker, W.M. (1996) Ubiquitous structures responsible for IgE cross-reactivity between tomato fruit and grass pollen allergens. *J. Allergy Clin. Immunol.* **98**, 805–815.
- Kondo, Y., Urisu, A. & Tokuda, R. (2001) Identification and characterization of the allergens in the tomato fruit by immunoblotting. *Int. Arch. Allergy Immunol.* **126**, 294–299.
- Sturm, A. & Chrispeels, M.J. (1990) cDNA cloning of carrot extracellular beta-fructosidase and its expression in response to wounding and bacterial infection. *Plant Cell* **2**, 1107–1119.
- Leigh, R.A., Rees, T., Fuller, W.A. & Banfield, J. (1979) The location of acid invertase activity and sucrose in the vacuoles of storage roots of beetroot (*Beta vulgaris*). *Biochem. J.* **178**, 539–547.
- Fahrendorf, T. & Beck, E. (1990) Cytosolic and cell wall-bound acid invertases from leaves in *Urtica dioica* L. Comparison. *Planta* **180**, 237–244.

20. Yelle, S., Chetelat, R., Dorais, M., DeVerna, J.W. & Bennet, A.B. (1991) Sink metabolism in tomato fruit. *Plant Physiol.* **95**, 1026–1035.
21. Ohyama, A., Hirai, M. & Nishimura, S. (1992) A novel cDNA clone for acid invertase in tomato fruit. *Jpn. J. Genet.* **67**, 491–492.
22. Foetisch, K., Son, D.Y., Altmann, F., Aulepp, H., Conti, A., Hausteiner, D. & Vieths, S. (2001) Tomato (*Lycopersicon esculentum*) allergens in pollen-allergic patients. *Eur. Food. Res. Technol.* **213**, 259–266.
23. Vieths, S., Schoning, B. & Petersen, A. (1994) Characterization of the 18-kDa apple allergen by two-dimensional immunoblotting and microsequencing. *Int. Arch. Allergy Immunol.* **104**, 399–404.
24. Wilson, I.B.H., Harthill, J.E., Mullin, N.P., Ashford, D.A. & Altmann, F. (1998) Core alpha1,3-fucose is a key part of the epitope recognized by antibodies reacting against plant N-linked oligosaccharides and is present in a wide variety of plant extracts. *Glycobiology* **8**, 651–661.
25. Vieths, S., Janek, K., Aulepp, H. & Petersen, A. (1995) Isolation and characterization of the 18-kDa major apple allergen and comparison with the major birch pollen allergen (Bet v, I). *Allergy* **50**, 421–430.
26. Holzhauser, T. & Vieths, S. (1999) Indirect competitive ELISA for determination of traces of peanut (*Arachis hypogaea* L.) protein in complex food matrices. *J. Agric. Food Chem.* **47**, 603–611.
27. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
28. Katayama, H., Nagasu, T. & Oda, Y. (2001) Improvement of in-gel digestion protocol for peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **15**, 1416–1421.
29. Kolarich, D. & Altmann, F. (2000) N-Glycan analysis by matrix-assisted laser Desorption/Ionization mass spectrometry of electrophoretically separated nonmammalian proteins: application to peanut allergen ara h 1 and olive pollen allergen ole e 1. *Anal. Biochem.* **285**, 64–75.
30. Kleine Budde, I., de Heer, P.G., van der Zee, J.S. & Aalberse, R.C. (2001) The stripped basophil histamine release bioassay as a tool for the detection of allergen-specific IgE in serum. *Int. Arch. Allergy Immunol.* **126**, 277–285.
31. Pruzansky, J.J., Grammer, L.C., Patterson, R. & Roberts, M. (1983) Dissociation of IgE from receptors on human basophils. I. Enhanced passive sensitization for histamine release. *J. Immunol.* **131**, 1949–1953.
32. Aalberse, R.C. & van Ree, R. (1996) Cross-reactive carbohydrate determinants. In *Highlights in Food Allergy* (Wüthrich, B. & Ortolani, C., eds), pp. 78–83. Karger, Basel.
33. Sturm, A. (1991) Heterogeneity of the complex N-linked oligosaccharides at specific glycosylation sites of two secreted carrot glycoproteins. *Eur. J. Biochem.* **199**, 169–179.