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Molecular and immunological characterization of the glycosylated orange allergen Cit s 1

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The IgE of sera from patients with a history of allergy to oranges (*Citrus sinensis*) binds a number of proteins in orange extract, including Cit s 1, a germin-like protein. In the present study, we have analyzed its immunological cross-reactivity and its molecular nature. Sera from many of the patients examined recognize a range of glycoproteins and neoglycoconjugates containing β1,2-xylose and core α1,3-fucose on their *N*-glycans. These reagents also inhibited the interaction of Cit s 1 with patients' sera, thus underlining the critical role of glycosylation in the recognition of this protein by patients' IgE and extending previous data showing that deglycosylated Cit s 1 does not possess IgE epitopes. In parallel, we examined the peptide sequence and glycan structure of Cit s 1, using mass spectrometric techniques. Indeed, we achieved complete sequence coverage of the mature protein compared with the translation of an expressed sequence tag cDNA clone and demonstrated that the single N-glycosylation site of this protein carries oligosaccharides with xylose and fucose residues. Owing to the presumed requirement for multivalency for in vivo allergenicity, our molecular data showing that Cit s 1 is monovalent as regards glycosylation and that the single *N*-glycan is the target of the IgE response to this protein explain the immunological cross-reactive properties of Cit s 1 as well as its equivocal nature as a clinically relevant allergen.

Key words: allergy/glycan/orange

Introduction

Cross-reactive IgE recognizing carbohydrate epitopes is a major complication in the correct laboratory diagnosis of food and pollen allergy; the phenomenon of carbohydrate cross-reactive determinants was first described 25 years ago (Aalberse et al. 1981). Many studies have shown that plant and insect glycoproteins bind the IgE of the sera of allergic patients, but the biological and clinical significance is in some cases unclear. A number of studies do indeed show that glycoconjugates, particularly in multivalent forms, elicit histamine release (Batanero et al. 1999; Bublin et al. 2003; Westphal et al. 2003; Wicklein et al. 2004); however, monovalent glycoconjugates tend not to display such activity (Wicklein et al. 2004). The highly conserved nature of core α1,3-fucosylation of *N*-glycans, though, is the basis for the cross-reaction of many plant and invertebrate glycoproteins to antisera raised against individual plant glycoproteins such as horseradish peroxidase (HRP) (Wilson et al. 1998; Fabini et al. 2001; Wilson 2002; Paschinger et al. 2004). The widespread occurrence of this glycostructural determinant is reflected in the presence of relevant core α1,3-fucosyltransferases in plants (Leiter et al. 1999), nematodes (Paschinger et al. 2004), and insects (Fabini et al. 2001). On the other hand, the presence of enzymes transferring β1,2-xylose to *N*-glycans is primarily a feature of plants (Strasser et al. 2000), snails (Mulder et al. 1995), and trematodes (Faveeuw et al. 2003), but this feature also contributes to antiglycan cross-reactivity. The immune response to such carbohydrate epitopes varies between species, being apparently higher in rats and rabbits than in some mouse strains (Bardor et al. 2003; Jin et al. 2006). However, both core α1,3-fucose and xylose induce Th2 responses and antibody production in *Schistosoma*-infected mice (Faveeuw et al. 2003). It is also interesting that a large proportion of nonallergic human blood donors were found, in one study, to have IgM and IgG_1 recognizing core α1,3-fucose or β1,2-linked xylose (Bardor et al. 2003), whereas core α1,3-fucose is an important epitope for IgE from nematode-infected sheep (van Die et al. 1999).

Recently, the presence of carbohydrate epitopes, recognized by IgE, on the orange protein Cit s 1 has been described (Ahrazem et al. 2006); this protein constitutes one of three recently identified and characterized orange allergens. Whereas Cit s 2 corresponds to orange profilin and represents a major allergen, according to its in vitro and in vivo reactivity in patients with allergy to this fruit (Lopez-Torrejon et al. 2005), Cit s 3 belongs to the lipid transfer protein (LTP) panallergen family and behaves as a minor allergen in the patients studied (approximately 35% prevalence) (Ahrazem et al. 2005). Cit s 1, on the other hand, was first detected in a study of six patients as being a protein of 24 kDa recognized by patients' IgE (Ibañez et al. 2004), which in a larger study was shown to display high in vitro reactivity, with its glycans constituting the major IgE epitopes (Ahrazem et al. 2006). Cit s 1 was also identified in an independent study as being a major orange allergen (Crespo et al. 2006). Interestingly, the presence of Cit s 1-reactive IgE did not generally appear to correlate with the ability to elicit

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positive skin prick tests; only one-eighth of patients showed a significant response in this test with purified Cit s 1 (Ahrazem et al. 2006). Initial N-terminal sequencing data indicated that Cit s 1 may be a germin-like protein (Ahrazem et al. 2006; Crespo et al. 2006); however, the full sequence was not determined and the nature of its N-glycosylation had not been examined. In this study, we present not only the full molecular characterization of Cit s 1, using mass spectrometry (MS), in terms of its peptide and glycan sequence, but also examine the nature of the anticarbohydrate cross-reactivity in more detail.

Results

Immunological analysis

Previous data indicated that the carbohydrate of Cit s 1 is a major epitope for the IgE of orange-allergic patients, because deglycosylation of this protein with trifluoromethanesulphonic acid abolished the binding of IgE from pooled and individual sera (Ahrazem et al. 2006). In order to study this in more detail, orange peel extract, purified Cit s 1, remodeled forms of human transferrin carrying xylose or fucose [i.e. Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc (MMX), Manα1-6(Manα1-3) Manβ1-4GlcNAcβ1-4(Fucα1-3) GlcNAc (MMF), and relevant Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (MM) controls carrying solely $Man_3GlcNAc_2$] and BSA neoglycoconjugates carrying bromelain glycopeptides with and without fucose [Manα1-6 (Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc (MUXF) and Manα1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc (MUX)] on a xylosylated $Man_2GlcNAc_2$ structure were subject to western blotting both with anti-HRP, as a model anticarbohydrate antiserum, and with patients' IgE. The specificity of anti-HRP has been examined in a number of studies, and both xylose and core α1,3-fucose are known epitopes (Kurosaka et al. 1991; Wilson et al. 1998; Bencúrová et al. 2004). Therefore, the MMX, MMF, MUXF, and MUX neoglycoforms were employed as positive controls for the interaction with anti-HRP, whereas their use with patients' IgE was intended to gain a first hint as to the contribution of xylose and fucose to the binding of IgE from orange-allergic patients. Coomassie staining (Figure 1A) was performed to check that the protein content of each sample was approximately equal.

The results indicate that anti-HRP reacts strongly with various orange glycoproteins and Cit s 1 (Figure 1B). As expected from previous data, transferrin carrying xylose or fucose (after remodeling with recombinant forms of either rice xylosyltransferase or nematode fucosyltransferase) reacted with anti-HRP (Fabini et al. 2001; Bencúrová et al. 2004), whereas the corresponding MM controls did not. On the other hand, all transferrin isoforms reacted with antihuman transferrin (data not shown). The two BSA neoglycoconjugates and HRP itself also reacted with this antibody as expected (Wilson et al. 1998). With the patients' serum pool, used in combination with a conjugated antihuman IgE for detection, a similar pattern of reactivity was observed (Figure 1C), indicating binding of IgE to both xylosylated and fucosylated glycans.

Specific IgE determination by direct and inhibition ELISA

Previous enzyme-linked immunosorbent assay (ELISA) data indicated that HRP could inhibit the binding of patients' sera to Cit s 1, whereas deglycosylated forms of Cit s 1 and HRP did not diminish this interaction (Ahrazem et al. 2006); this suggests that the patients' IgE displays glycan-dependent reactivity toward both Cit s 1 and HRP and no significant interaction with the Cit s 1 polypeptide. To examine the role of plant-like glycans further, the transferrin neoglycoforms and the BSA neoglyconjugates were used in various ELISA tests. In direct ELISA (Figure 2), the serum pool showed a strong reaction toward both the MUX and MUXF forms of BSA; three of four individual sera also showed significant reactivity toward both these neoglycoconjugates, which is compatible for a role at least for xylose, if not also for fucose, in IgE binding. As regards the transferrin neoglycoforms, a more complex picture emerged: the pool and the same three sera showed a reaction toward MMX; only one serum showed a significant reaction toward MMF.

The binding characteristics of Cit s 1 with two of the sera as well as the serum pool were investigated further by inhibition ELISA (Figure 3). BSA-MUXF and BSA-MUX were effective in inhibiting the binding of Cit s 1 to the sera and

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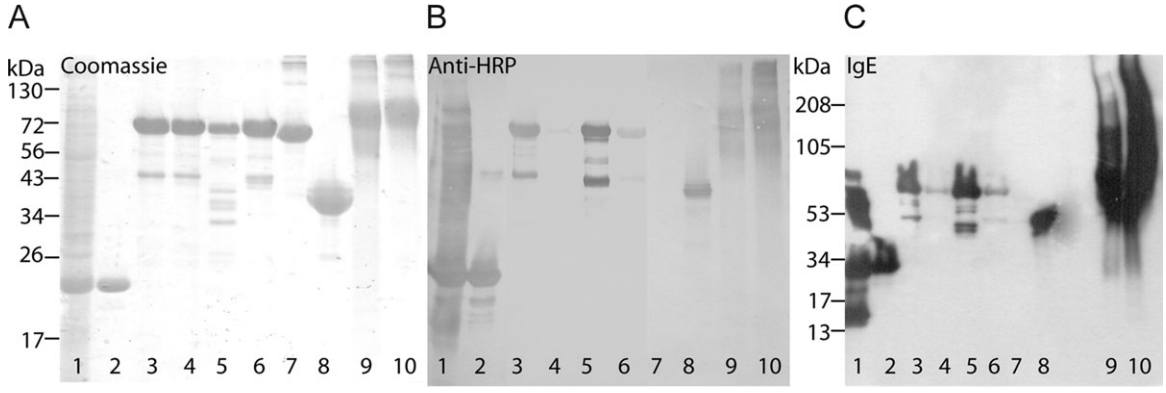

Fig. 1. SDS–PAGE and western blotting of various glycoproteins. Coomassie staining (**A**) or western blotting with either anti-HRP followed by antirabbit IgG (**B**) or a pooled patients' sera followed by antihuman IgE (**C**) was performed with the following protein samples: 1, orange peel extract; 2, purified Cit s 1; 3, MMX-transferrin; 4, MM-transferrin (no UDP-Xyl control); 5, MMF-transferrin; 6, MM-transferrin (no GDP-Fuc control); 7, bovine serum albumin; 8, HRP; 9, BSA-MUX; 10, BSA-MUXF. Definitions of glycoforms are given in the *Abbreviations* section and also in Figure 2.

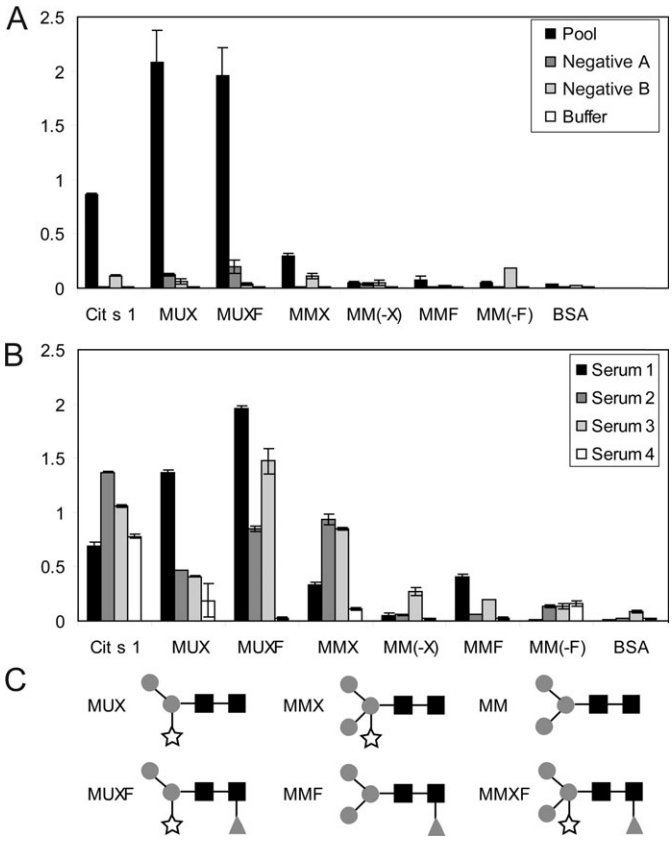

Fig. 2. Direct ELISA of glycoproteins with patients' sera. ELISA plates were coated with either purified Cit s 1, BSA-MUX, BSA-MUXF, MMX, and MMF forms of transferrin (and corresponding controls) or BSA prior to probing with either (**A**) the pool of patients' sera, two sera with no reaction to Cit s 1, and buffer or (**B**) the four individual patients' sera displaying binding to Cit s 1. The degree of binding is expressed as the absorbance obtained at 490 nm after addition of the chromogenic substrate. (**C**) Explanation of the oligosaccharide abbreviations, using the symbolic nomenclature of the Consortium for Functional Glycomics (http://www.functionalglycomics.org), with GlcNAc as a black square, mannose as a gray circle, fucose as a gray triangle, and xylose as a white star; MM indicates the presence of two terminal α-mannoses, MU an unsubstituted 3-hydroxyl on the β-mannose, X a xylose, and F a fucose.

the pool; with serum 3 and the pool, these neoglycoconjugates were as effective as Cit s 1 at all concentrations used (left panel). With the transferrin glycoforms (right panel), the situation was somewhat more complex and it was obvious that, under the conditions used, all glycoforms possessed inhibitory activity regardless of the presence of the xylose or fucose residues. This appears contradictory to the other data (blot and direct ELISA), but may be explained by considering that inhibition with trimannosyl-oligosaccharides may be different from actual specific binding to xylose and fucose. Furthermore, the maximum level of inhibition when employing the native allergen is also attained with the highest neoglycoform concentration; this demonstrates that these reagents are approximately as effective as the allergen itself in inhibiting IgE binding.

MS analysis

The data accumulated to date indicate that Cit s 1 may have a plant-type *N*-glycan, which is an IgE epitope, but actual glycoanalytical data were missing. Furthermore, the exact nature of the peptide was also unknown. Solely on the basis of N-terminal sequencing, yielding the sequence TDPGHLQDVXVAINDPKXGVFVNRK (Ahrazem et al. 2006; Crespo et al. 2006), amino acid homology with a germin-like protein from pepper (*Capsicum annuum*, GenBank accession no. AY391748) was found; the pepper protein was previously found as a major allergen when using sera from patients with mugwort-birch-celery-spice syndrome (Leitner et al. 1998). In order to molecularly define Cit s 1 more fully in terms of both glycan structure and peptide sequence, an MS-based analysis was performed.

After the tryptic digestion of the protein, the peptides were initially analyzed by liquid chromatography electrospray-ionization MS (LC-ESI-MS). After this run, two peptides were selected and MS-MS (collision-induced dissociation, CID) experiments were carried out in order to determine the sequence for an ensuing database search. For this purpose, $[M+H]^+$ ions of m/z 747.5 and 835.5 were fragmented. The analysis was performed in a so-called survey scan. This means that the analyzer was scanning in a defined mass range and that, when an intense signal appeared, the mass analyzer opens an additional channel and fragments this ion. In the case of 747.5, the analyzer sought out not the tryptic fragment T5 itself, but selected an ion which was produced in the mass analyzer by a loss of an N-terminal alanine; the sequence FQLDPK was determined (Figure 4A). For the second ion, the peptide T6 with the sequence DGVFVNGK was found (Figure 4B).

With these sequences and the results of the N-terminal sequencing, a tBLASTn search (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) was performed using the EST database. In this search, sections of a cDNA clone from the peel of *Citrus sinensis* (GenBank accession no. CK937230) were found. Both sequences matched exactly to this cDNA; therefore, the full reconstructed open-reading frame was translated in silico (http://us.expasy.org/tools/dna.html) (Figure 5). Analysis of the putative full-length protein (224 residues) revealed that the previously determined N-terminal sequence of Cit s 1 is probably the result of signal sequence cleavage. Without further posttranslational modification, the protein displays a molecular weight of 21 943.79 (average mass); however, one potential N-glycosylation site appears in the sequence. The theoretical tryptic digest of the protein was then used to aid identification of the other peptides detected by LC-ESI-MS; the theoretical mass of each was compared with that observed (Table I).

The analysis of the tryptic peptides indicated that only the tryptic fragment with the potential N-glycosylation site was not detected. To determine if this was caused by the relatively high molecular mass of this peptide (3155.6874 Da) or because it is glycosylated, the tryptic peptides were further digested with chymotrypsin. The fragments which are important for the sequence coverage are shown in Table II. An unglycosylated peptide containing the potential N-glycosylation site was not detected, which suggested that the occupancy of this site is complete. Indeed, only glycosylated forms of this peptide were detected. Most prominently, a doubly charged ion with m/z 1137.97, glycosylated with a Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc (MMXF) structure, was found with the sequence

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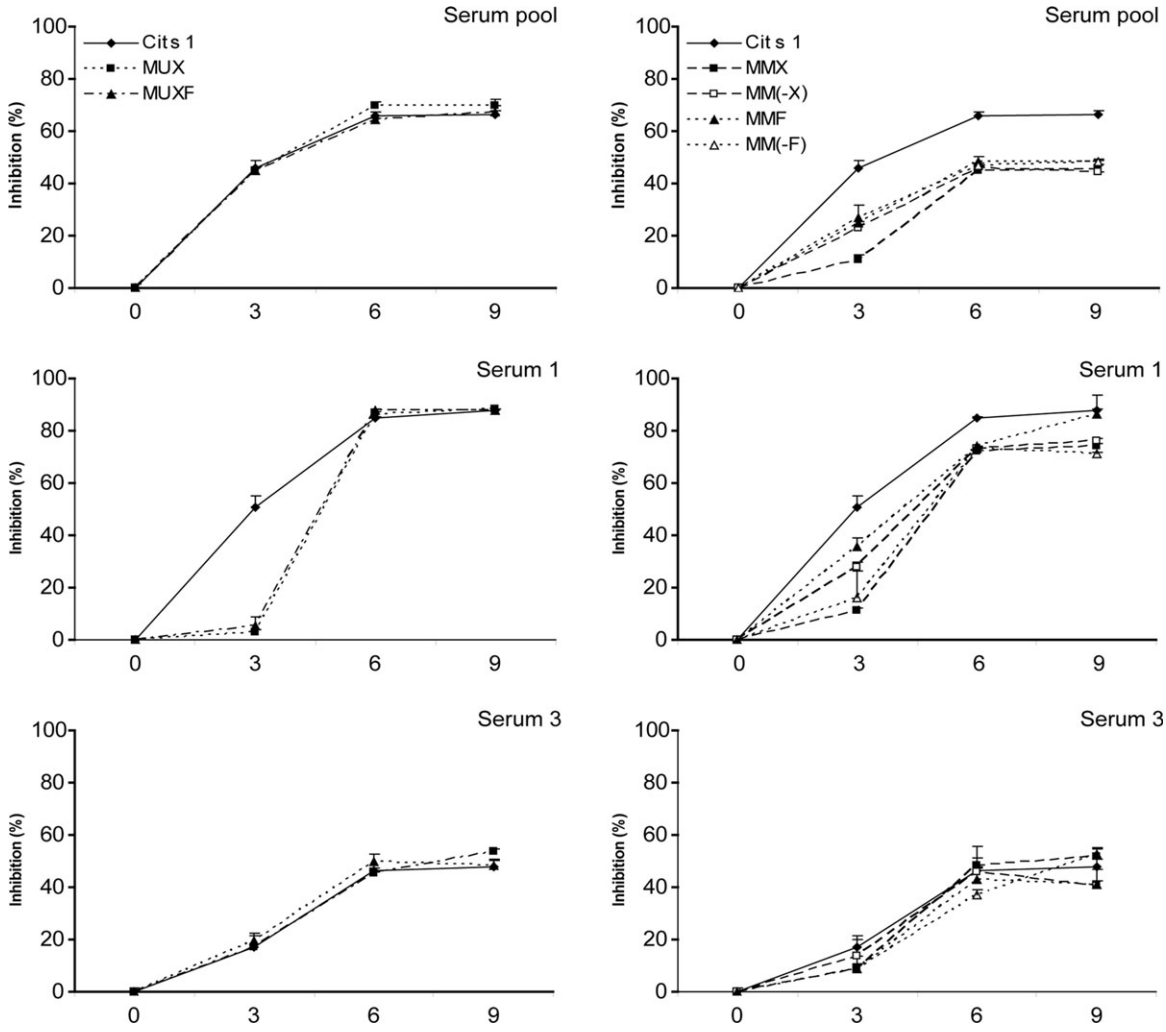

Fig. 3. Determination of inhibition of binding of IgE to Cit s 1. Various concentrations (0–9 μg/mL) of either Cit s 1 or BSA neoglycoconjugates (left panels) or transferrin neoglycoforms (right panels) were incubated with either the patients' serum pool or serum 1 or serum 3 prior to binding to Cit s 1-coated ELISA plates and subsequent IgE detection. Sera 1 and 3 in the present study correspond to the sera 6 and 10 described by Ahrazem et al. (2006); in the case of sera 3 in the present study, immunoblotting showed abolition of IgE binding upon deglycosylation of Cit s 1.

VTSDQLNNTL (peptide CT14); an overlapping glycopeptide (CT13) was also found. As shown in Table III, minor structures, other than MMXF, were also present.

As a final confirmation for the presence of glycosylation, the aforementioned CT14 ion was fragmented by CID (Figure 6). This MS-MS experiment gave fragments of the peptide alone as well as a set of fragments of the glycopeptide sequentially lacking the individual monosaccharide residues; the intact glycopeptide, which was the peptide VTSDQLNNTL modified with an N-linked MMXF glycan, was also observed. Fragments of the carbohydrate alone were detected between 204 and 822 Da. The glycopeptide and glycan fragments confirm the major *N*-glycan structure as being, as expected, MMXF. After treatment of the trypsin/chymotrypsin peptides with PNGase A, the deglycosylated peptide was also found with the sequence VTSDQLDNTL; the glycosylated Asn was converted to Asp. The observed m/z for the singly charged ion was 1105.5687 compared with the theoretical value of 1105.5372. Similarly, a deglycosylated form of CT13 (m/z 774.3902; DNTLIAK) was also observed in the PNGase A digest; thus, the exact location of the single N-glycosylation site of Cit s 1 was verified.

Discussion

The molecular analysis of allergens is a major focus of modern allergology and raises the possibility of more accurate diagnosis and therapy. In the case of plant and insect glycoproteins which bind patients' IgE, cross-reactions are very common (Aalberse et al. 1981; Tretter et al. 1993; Batanero et al. 1996; Mari et al. 1999; Hemmer et al. 2001); this is not unexpected, since the glycan is probably the most conserved aspect of these molecules. The presence of β1,2-xylose and core α1,3-fucose on *N*-glycans is more or less ubiquitous throughout the plant kingdom, whereas these moieties are absent from mammals, thus making them immunogenic. On the other hand, the matter as to whether cross-reactive carbohydrate epitopes are clinically significant has been a matter of controversy and debate (Aalberse and Van Ree 1997; Van der Veen et al. 1997; Fötisch and Vieths 2001; Altmann 2007).

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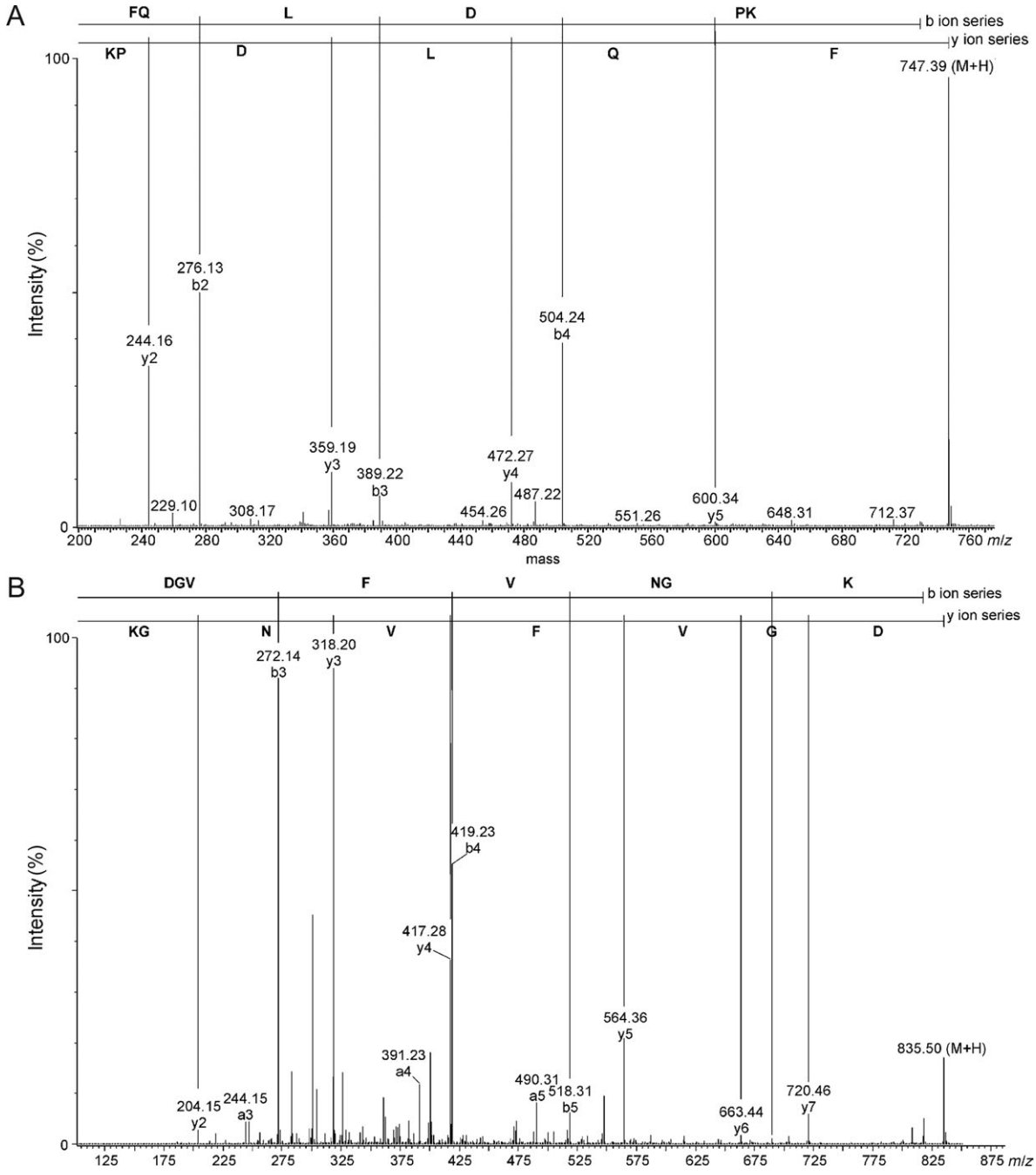

Fig. 4. MS-MS fragment spectra of two selected peptides of Cit s 1. (**A**) Fragment spectrum of the $[M + H]^+$ ion 747.39 with the assignment of the peaks to the sequence FQLDPK (the form of peptide T5 lacking one alanine residue). (**B**) Fragment spectrum of the $[M + H)]^+$ ion 835.5 with the assignment of the peaks to the sequence DGVFVNGK (peptide T6).

However, a number of cases show that glycans can contribute to the allergenicity of glycoproteins as measured by histamine release with, e.g., Api g 5, Cup a 1, Phl p 11, and Lyc e 2 (Iacovacci et al. 2002; Bublin et al. 2003; Foetisch et al. 2003; Westphal et al. 2003; Wicklein et al. 2004) or skin prick tests with HRP (Mari 2002). Such examples of an actual biological activity are at variance with other studies on other glycoproteins, such as Phl p 1 (Wicklein et al. 2004), which is not active in histamine release assays, or bromelain (Mari 2002), which generally does not elicit positive skin prick tests; it must be noted, though, that the "positive" examples tend to be proteins carrying multiple glycans, whereas "negative" examples, such as bromelain or Phl p 1, carry only a single *N*-glycan, and that multivalency is considered a prerequisite for the antigen-induced cross-linking, of IgE bound to its receptors, necessary for histamine release. This would then imply that, for a solely anticarbohydrate response, in vivo allergenicity requires that a protein will carry at least two glycan residues.

The major orange allergen, Cit s 1, has now been molecularly proved by us to possess, however, only a single *N*-glycan, and examination of the tryptic and chymotryptic

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Fig. 5. Translation of the putative Cit s 1 open-reading frame. The gray-highlighted sequence could be confirmed by the MS analysis; the preceding sequence is assumed to be a cleaved signal sequence. The black-highlighted asparagine corresponds to the single N-glycosylation site. Peptides T1–T11 are, in order of size, the observed tryptic peptides, and peptides CT1–CT12 are those observed after digestion with both chymotrypsin and trypsin. CT13 and CT14 are glycopeptides observed after treatment of tryptic peptides with chymotrypsin.

peptides facilitated the final definition of the glycosylation of this protein. The theoretical M_r of the unglycosylated mature protein is 21 943.79 (average mass); with an MMXF type *N*-glycan, the theoretical mass would be 23 114.56. Previous matrix-assisted laser-desorption ionization-time of flight (MALDI-TOF MS) of the intact protein demonstrated the existence of three major species of 23 095, 23 298, and 23 504 Da (Ahrazem et al. 2006); the first corresponds to the protein carrying MMXF, and the second to a form carrying an additional nonreducing terminal GlcNAc residue (either the isomer MGnXF or GnMXF, depending on whether the α1,3- or α1,6-arm carries the nonreducing GlcNAc, or a mixture thereof). Thus, our glycopeptide MS data confirm this microheterogeneity of Cit s 1. However, there are, in addition, minor forms lacking either a fucose (MMX) or a xylose (MMF), but these perhaps occur in too low a concentration to be observed by MS of the intact protein. These types of xylosylated and fucosylated glycans observed have been found on a number of allergens such as Api g 5 (Bublin et al. 2003), Cup a 1 (Alisi et al. 2001), Cyn d 24 (Chow et al. 2005), Hev b 4 (Kolarich et al. 2006), Lol p 11 (van Ree et al. 2000), Lyc e 2 (Westphal et al. 2003), and Phl p 1 (Wicklein et al. 2004). Interestingly, other allergens have been found to possess only or predominantly solely xylosylated species such as MMX; examples of such allergens include Ara h 1 (Kolarich and Altmann 2000; van Ree et al. 2000), Ole e 1 (Kolarich and Altmann 2000; van Ree et al. 2000) and Cor a 11 (Lauer et al. 2004). The presence of xylose and fucose residues on Cit s 1 explains its ability to be recognized by anti-HRP.

The matter of the cross-reactivity of IgE from the sera of patients allergic to orange is somewhat more complicated than the situation with the "model" IgG anti-HRP. In one study, a monovalent MUXF glycopeptide (as opposed to the multivalent conjugates used in the present study) did not inhibit binding of IgE to Cit s 1 (Crespo et al. 2006). Our data, though, indicate that the MUX and MUXF glycans attached to the BSA neoglycoconjugates have approximately the same potency as ligands for the patients' IgE and as inhibitors of IgE binding to Cit s 1: thus, it should be noted that there appears no particular bias toward recognition of fucose as being a major decisive element. Certainly, however, our inhibition data as well as those derived from blots and ELISA previously showing that deglycosylated Cit s 1 does not possess IgE epitopes (Ahrazem et al. 2006) strongly indicate that the glycan component is important for IgE binding to Cit s 1.

The direct binding to the transferrin neoglycoforms is potentially weaker than to the BSA neoglycoconjugates—the former carry only two *N*-glycans, and neither the MMF nor the MMF forms have both xylose and fucose; on the other hand, the BSA can carry many bromelain glycopeptides (on average 3–4) and so has a higher degree of valency (Wilson et al. 1998). It has also been reported that the α1,3-mannose found on, for example, MMX may inhibit IgE binding in some cases compared with glycans lacking this residue, for example, MUX (van Ree et al. 2000). Interestingly, in the case of inhibition experiments, it appears that even MM can prevent binding of IgE to Cit s 1, with approximately the same potency as MMX and MMF; thus, the trimannosyl core may, at a sufficient concentration, sterically hinder access by xylose- and fucose-containing *N*-glycans to Cit s 1-specific

Table I. Detected tryptic fragments of the translated Cit s 1 protein[a]

Peptide	Sequence of tryptic fragment	$[M+H]^+$ calculated	Predominant ion	m/z theoretical	m/z found	Retention time (min)
T1	FCK	397.1904	$[M+H]^+$	397.1904	397.2409	28.96
T2	ALNK	445.2769	$[M+H]^+$	445.2769	445.2050	25.50
T3	FINGN	564.2776	$[M+H]^+$	564.2776	564.2913	21.84
T4	DLQNK	617.3253	$[M+H]^+$	617.3253	617.3528	18.44
T5	AFQLDPK	818.4406	$[M+H]^+$	818.4406	818.4667	28.00
T6	DGVFVNGK	835.4309	$[M+H]^+$	835.4309	835.4840	24.98
T7	TDPGHLQDFCVAINDPK	1869.8799	$[M+2H]^{2+}$	935.4436	935.5186	29.10
T8	AEDFFFSGLGKPGNTANR	1927.9296	$[M+2H]^{2+}$	964.4684	964.4894	34.28
T9	GDVFVFPQGLIHFQFNIGK	2163.1385	$[M+2H]^{2+}$	1082.0728	1082.1140	32.14
T10	LGVDETDANVEQIPGLNTLGISAFR	2629.3467	$[M+3H]^{3+}$	877.1204	877.1707	38.21
T11	TNAAAYSALNSQFPGEVTIADTVFGANPSINPDFLGK	3797.8656	$[M+3H]^{3+}$	1266.6267	1266.7316	40.57

[a]Listed are those peptides predicted by the in silico digest of the expressed sequence tag open-reading frame which could be detected by MS following the tryptic digest. The locations of peptides T1–T10 within the Cit s 1 sequence are shown in Figure 5.

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Table II. Detected tryptic/chymotryptic fragments of the translated Cit s 1 protein[a]

Peptide	Fragment sequence	$[M+H]^+$ calculated	Predominant ion	m/z theoretical	m/z found	Retention time (min)
CT1	LVL	344.2544	$[M+H]^+$	344.2544	344.2494	31.25
CT2	DPK	359.1925	$[M+H]^+$	359.1925	359.1686	27.85
CT3	IDY	410.1922	$[M+H]^+$	410.1922	410.1895	27.92
CT4	YVGF	485.2394	$[M+H]^+$	485.2394	485.2000	40.39
CT5	EGTLY	582.2769	$[M+H]^+$	582.2769	582.2640	28.41
CT6	VTSDQL	662.3355	$[M+H]^+$	662.3355	662.3419	28.69
CT7	ASEIFL	679.3661	$[M+H]^+$	679.3661	679.3511	34.71
CT8	VLEGTLY	794.4294	$[M+H]^+$	794.4294	794.4069	31.25
CT9	LARAEDF	821.4152	$[M+H]^+$	821.4152	821.4072	28.41
CT10	VGFVTSDQL	965.4938	$[M+2H]^{2+}$	483.2505	483.2319	32.77
CT11	APYGQRPPHIHPR	1525.8134	$[M+2H]^{2+}$	763.4103	763.3263	29.86
CT12	QLDPKIVKDLQNK	1538.8900	$[M+2H]^{2+}$	769.9486	769.9529	29.03

[a]Peptides resulting from the sequential digestion with trypsin and chymotrypsin are listed, which were detected and relevant for an increased sequence coverage. The locations of peptides CT1–CT12 within the Cit s 1 sequence are shown in Figure 5.

IgE. This may be similar to the ability of MM to inhibit binding of the monoclonal YZ1/2.23, which recognizes plant-type *N*-glycans, to MMX (Bencúrová et al. 2004). Other previous data have suggested that the mannose residues do have a role in the binding of antibodies to plant-type glycans, but that the trimannosyl core itself is insufficient for recognition by anti-HRP (Wilson et al. 1998; Bencúrová et al. 2004); on the other hand, a monoclonal antibody derived from *Schistosoma*-infected mice could bind MM and MMF equally (van Remoortere et al. 2003). All our experiments suggest that there is no strong difference between those glycans carrying xylose alone (i.e. MMX and MUX) and those carrying either fucose alone (MMF) or both xylose and fucose (MUXF). Perhaps, there may be a pool of fucose-specific IgE and a more dominant pool of xylose-specific IgE in the sera tested. In some studies, fucose-specific IgE seems to predominate (Bencúrová et al. 2004), although a role for xylose in IgE binding has been also observed in some cases (Garcia-Casado et al. 1996; van Ree et al. 2000; Bencúrová et al. 2004).

The presence of a single *N*-glycan on Cit s 1 confers a monovalent status to the protein as regards binding of anticarbohydrate IgE; together with the apparent lack of polypeptide epitopes, this constitutes an explanation of its previously determined low in vivo allergenic activity and of its "equivocal" status as an allergen (Ahrazem et al. 2006). This is not merely an academic problem, since in vitro diagnostics can play a major role in deciding the course of treatment of allergic individuals. Falsely identified positives can lead to overzealous avoidance measures for patients, with a consequent effect on their quality of life; analytical tools, showing whether a glycoallergen is multivalent and so potentially active in histamine release, are therefore a useful accompaniment to the usual laboratory tests. Cit s 1 is a paradigmatic example of a strong IgE-binding protein that can lead to false-positives in in vitro diagnosis, although the positive skin prick test responses in some patients suggest its potential clinical relevance with orange allergy. Nevertheless, its full molecular characterization opens the way for further studies to determine the role of this protein in complicating the

Table III. Glycopeptides of Cit s 1[a]

Glycan structure	$[M+H]^+$ calculated	Predominant ion	m/z theoretical	m/z found	Retention time (min)
CT13 + MMXF	1943.8690	$[M+2H]^{2+}$	972.4381	972.4393	27.16
CT14 + MMXF	2274.9704	$[M+2H]^{2+}$	1137.9889	1137.9729	28.41
CT14 + MMX	2128.9125	$[M+2H]^{2+}$	1064.9599	1064.9653	28.48
CT14 + MMF	2142.9282	$[M+2H]^{2+}$	1071.9677	1071.9607	28.41
CT14 + GnMXF/MGnXF	2478.0500	$[M+2H]^{2+}$	1239.5287	1239.5316	28.27

[a]Listed are the ions detected after combined trypsin/chymotrypsin treatment and their assignment to forms of the overlapping peptides NNTLIAK (CT13) and VTSDQLNNTL (CT14) modified with various *N*-glycan structures.

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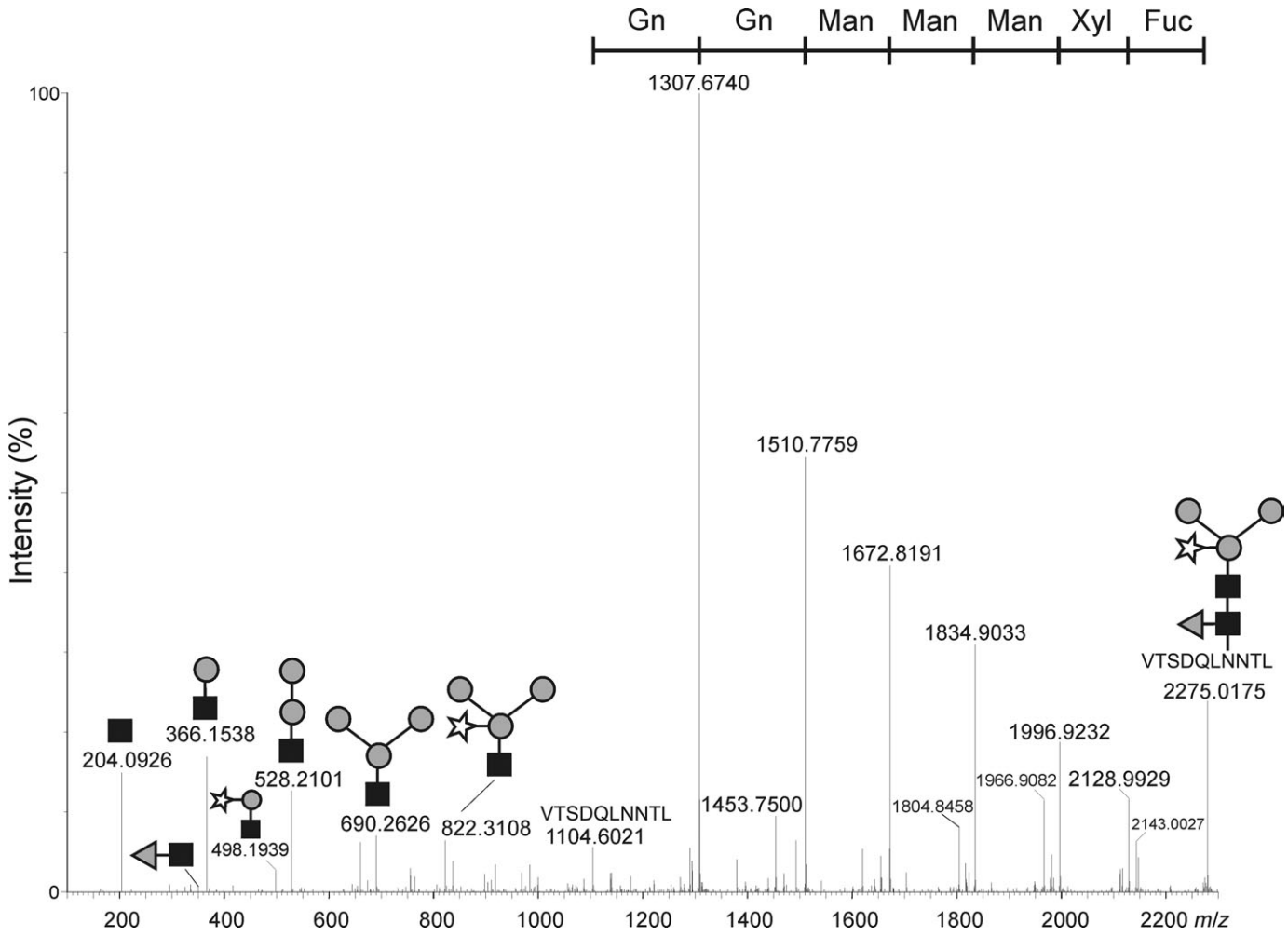

Fig. 6. CID analysis of the major glycopeptide of Cit s 1. CID was performed in the form of the peptide VTSDQLNNTL (peptide CT14) putatively modified with an MMXF glycan; the glycan attached to the peptide could be fully sequenced (fragments with m/z between 1104 and 2279). Smaller fragments of the glycan alone were also observed with m/z between 204 and 822. The glycans are depicted using the nomenclature of the Consortium for Functional Glycomics (http://www.functionalglycomics.org); see also Figure 2.

laboratory-based diagnosis of which proteins are important in orange allergy and in facilitating the preparation of recombinant forms of this protein.

Materials and methods

Patients' sera

A serum pool ($n = 10$) and individual sera (1–4) from orange-allergic patients, corresponding to patients 6, 8, 10, and 11 whose clinical characteristics have been previously described (Ahrazem et al. 2006), were used for IgE immunodetection, specific IgE determination, and ELISA-inhibition assays. All 10 sera of the pool showed specific IgE to purified Cit s 1. Furthermore, as shown by Ahrazem et al. (2006), testing sera 2–4 with trifluoromethanesulphonic acid-deglycosylated forms of either Cit s 1 or HRP resulted in abolition of IgE binding as judged by blotting (sera 3 and 4), whereas in the case of serum 2, IgE binding to Cit s 1 in ELISA was inhibited approximately 80% by HRP.

Orange extract and purified glycoproteins

An orange peel extract was prepared as previously described (Ahrazem et al. 2006), and its protein content quantified according to Bradford (1976). Cit s 1 allergen was isolated as in Ahrazem et al. (2006) and quantified by the commercial bicinchoninic acid test (Pierce, Cheshire, UK). The model neoglycoconjugates, BSA-MUXF and BSA-MUX, were prepared as previously described using, respectively, native and defucosylated bromelain glycopeptides (Wilson et al. 1998). The remodeled forms of transferrin were produced by a modification of previously published procedures (Fabini et al. 2001; Bencúrová et al. 2004): MMX-transferrin, carrying xylose on the core $Man_3GlcNAc_2$ pentasaccharide, was produced by serial incubation of human apo-transferrin with sialidase, galactosidase, a supernatant of *Pichia* expressing rice β1,2-xylosyltransferase (Léonard et al. 2004) with UDP-Xyl and finally hexosaminidase; MMF-transferrin, carrying core α1,3-fucose, was produced similarly using sialidase, galactosidase, hexosaminidase, and finally, partially purified

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Pichia-expressed *Caenorhabditis elegans* core α1,3-fucosyltransferase FUT-1 (Paschinger et al. 2004) with GDP-Fuc. MM controls (-X or -F), carrying only the core pentasaccharide, were prepared in the presence of the relevant glycosyltransferase but in the absence of a nucleotide sugar donor. The presence of the transferred sugar was demonstrated by MALDI-TOF MS analysis of the tryptic peptides.

SDS–PAGE and immunodetection assays

Samples (20 μg of protein of crude orange extract and 3 μg of purified proteins) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) on Bio-Rad Miniprotein II System (Bio-Rad, Hercules, CA) gels (12.5 or 15% polyacrylamide), following the method of Laemmli (1970), and then electrotransferred onto nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK, or Pall Corporation, East Hills, NY). After blocking, membranes were immunodetected with rabbit anti-HRP (Sigma, St Louis, MO; 1:20 000 dilution) and then treated with a goat antirabbit IgG alkaline phosphatase-conjugated antibody (Vector Laboratories, Burlingame, CA; 1:2000 dilution) prior to development with SigmaFAST NBT/BCIP. Alternatively, replica-blocking membranes were incubated with a pool of sera from orange-allergic patients (1:4 dilution), then with mouse antihuman IgE monoclonal antibody HE-2 ascitic fluid (1:3000 dilution) (Sanchez-Madrid et al. 1984), and finally with a rabbit antimouse IgG peroxidase-conjugated antibody (DAKO A/S, Glostrup, Denmark; 1:5000 dilution) prior to enhanced chemiluminescence (Amersham Biosciences).

Specific IgE determination and ELISA-inhibition assays

Specific IgE binding to Cit s 1 and model glycoproteins was determined essentially by a method previously described, using peroxidase-labeled antihuman IgE (DAKO) for detection (Diaz-Perales et al. 2003). Cit s 1 and each purified glycoprotein were used as solid phase (3 μg/mL) and the serum pool and four individual sera (1:2 dilution) from orange-allergic patients were tested. BSA (1% in PBS buffer), dilution buffer, and two sera from patients with orange allergy showing positive specific IgE levels to orange LTP allergen Cit s 3 but negative to Cit s 1 were used as negative controls. ELISA-inhibition assays were carried out by the same method, except that the sera were preincubated with the appropriate inhibitor (3, 6, and 9 μg/mL) for 3 h at 25 °C. All tests were performed in triplicate.

Tryptic digest

About 10 μg of the purified Cit s 1 protein was dried, dissolved in 15 μL 50 mM ammonium acetate, pH 8.4, and denatured for 10 min at 95 °C. After cooling to room temperature, 5 μL of a solution of 50 ng/μL trypsin (bovine pancreas; Sigma) was added and the sample was incubated overnight at 37 °C; the trypsin was then deactivated for 20 min at 95 °C. For a single MS analysis, about one-third of the sample was used.

Trypsin and chymotrypsin digest

An aliquot of the trypsin-digested peptides was further digested with chymotrypsin; dried tryptic peptides were dissolved in 15 μL 50 mM ammonium acetate in water, pH 8.4. Afterward, 5 μL of a 50 ng/μL α-chymotrypsin from bovine pancreas (Sigma) solution (approximately 200 μU) was added and the sample was incubated overnight at 37 °C; the sample was heat-inactivated for 20 min at 95 °C prior to analysis by LC-ESI-MS.

Deglycosylation of the tryptic/chymotryptic peptides

Peptides resulting from combined trypsin and chymotrypsin digestion were dried under reduced pressure in a vacuum centrifuge and dissolved in 20 μL 0.1 M citrate-phosphate buffer, pH 5.0. The deglycosylation was performed overnight with 0.15 mU of PNGase A from almonds (Altmann et al. 1998). Then, 100 μL of 1% acetic acid was added and the peptides were purified using a Phenomenex Strata C18-E 50 mg cartridge previously washed with 75% (v/v) aqueous acetonitrile and equilibrated with 1% acetic acid; the deglycosylated peptides were then collected by elution with 75% (v/v) aqueous acetonitrile, dried and dissolved in water.

LC-ESI-MS analysis

The LC-ESI-MS experiments were carried out using a quadrupole time-of-flight (Q-TOF) Ultima Global mass spectrometer (Micromass, Manchester, UK) equipped with an atmospheric pressure ionization electrospray interface and an upstream Micromass CapLC. The precolumn was a Thermo Aquastar C18 (30 × 0.32 mm) and the analytical column a Thermo BioBasic-18 (150 × 0.18 mm). The flow rate was 2 μL/min, starting with 95% solvent A (aqueous 0.1% formic acid) and 5% solvent B (acetonitrile containing 0.1% formic acid). After loading the sample, the separation gradient (5–50% B; 7–33 min) was applied prior to washing the column with 60% solvent B for 5 min. The MS instrument was calibrated with [Glu^1]-fibrinopeptide B in the mass range of 72–1285 amu. The sampling cone potential was 80 V and the capillary voltage 3.0 kV. The electrospray source temperature was 60 °C and the desolvation temperature 120 °C. In the MS experiments, mass spectra were scanned over the range m/z 100–1900. In the CID MS-MS experiments, the detection range was m/z 50–1990, and, for fragmentation, a collision energy of 35 V was used.

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Conflict of interest statement

None declared.

Abbreviations

CID, collision-induced dissociation; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; LC-ESI-MS, liquid chromatography electrospray-ionization mass

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spectrometry; LTP, lipid transfer protein; MALDI, matrix-assisted laser-desorption ionisation; MM, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc; MMF, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc; MMX, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc; MMXF, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc; MUX, Manα1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc; MUXF, Manα1-6 (Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc; Q-TOF, quadrupole time-of-flight.; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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