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Structural analysis of the glycoprotein allergen Hev b 4 from natural rubber latex by mass spectrometry

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Abstract

The lecithinase homolog (Hev b 4) from *Hevea brasiliensis* (Q6T4P0_HEVBR) is an important natural rubber latex allergen. Hev b 4 is a highly glycosylated protein and its carbohydrate moiety has been implicated in the binding of IgE from natural rubber latex allergic patients. The cDNA for Hev b 4 has recently been cloned and sequenced. Here, we have analyzed the post-translational modifications of natural Hev b 4 by liquid chromatography/electrospray ionization-mass spectrometry of tryptic peptides. Seven of the eight potential glycosylation sites were found to be occupied. One site, however, was only partially glycosylated. Asn224 was substituted by complex type N-glycans with fucose and xylose, whereas all other sites carried either oligomannose glycans or a mixture of oligomannose and complex N-glycans. Glycosylation site Asn308, the most C-terminal one of the eight sites, was only found in the non-glycosylated form. The complex type N-glycans apparently form the molecular basis for the immune reaction with patients' sera. A large fraction of Hev b 4 molecules contains two or more complex N-glycans and thus a physiological reaction against these polyvalent allergens on the basis of the carbohydrate is in theory possible. Aside from allowing glycosylation analysis, the mass spectrometric data defined the N-terminal cleavage site of Hev b 4. This study once more demonstrates the outstanding analytical potential of electrospray ionization-mass spectrometry coupled with liquid chromatographic separation.

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1. Introduction

Natural rubber latex (NRL) is the cytoplasm obtained from the laticifers of the rubber tree *Hevea brasiliensis*. Dried NRL is the raw material of a wide range of rubber products such as vehicle tyres and shoes. Alternatively, NRL can be stabilized and concentrated for processing into “dipped” latex products

such as examination and surgical gloves. Besides rubber elastomer, the NRL cytoplasm contains a multitude of metabolites and proteins. There is a consensus that certain proteins or peptides in the latex products, especially gloves, readily elute by the sweat of the end users. A small proportion of these proteins is responsible for NRL allergy, which is an Immunoglobulin E (IgE)-mediated type I hypersensitivity. Allergy to NRL is prevalent among health care workers and other personnel who routinely wear protective latex gloves in the course of their work. To date, thirteen NRL proteins, Hev b 1 to Hev b 13 are recognized by the International Union of Immunological Societies (IUIS) as the causal agents of NRL allergy. Knowledge of the causative allergens is essential to develop reliable diagnosis of NRL allergy and to develop methods for determination of allergenicity of NRL products [1–4].

Substantial progress has been made over the years in the molecular and immuno-characterization of NRL allergens. IgE reactivity of NRL proteins was sensitive to periodate treatment

Abbreviations: CCD, cross-reactive carbohydrate determinant; Man5, Man6 etc., oligomannosidic N-glycans with 5, 6 etc. mannose residues; MMXF³, Man α -6(Man α -3)Man β -4GlcNAc β -4(Fuc α -3)GlcNAc- (see also Fig. 1); MGnXF³, Man α -6(GlcNAc β -2Man α -3)Man β -4GlcNAc β -4(Fuc α -3)GlcNAc-; GnMXF³, GlcNAc β -2Man α -6(Man α -3)Man β -4GlcNAc β -4(Fuc α -3)GlcNAc-; GnGnXF³, GlcNAc β -2Man α -6(GlcNAc β -2Man α -3)Man β -4GlcNAc β -4(Fuc α -3)GlcNAc- (see also <http://www.proglycan.com> for an explanation of N-glycan abbreviations); LC/ESI-MS, liquid chromatography/electrospray ionization-mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NRL, natural rubber latex; Q-TOF, quadrupole time-of-flight

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(implying carbohydrate IgE epitopes) [5], and IgE binding to the carbohydrate moiety of Hev b 2 and Hev b 13 has been demonstrated [6,7]. More recently, the major IgE binding component of Hev b 4, a latex lecithinase homologue was cloned (Q6T4P0_HEVBR). The predicted protein sequence contains eight potential N-glycosylation sites and hence Hev b 4 potentially constitutes a multivalent glyco-allergen. The latex lecithinase homologue (Hev b 4) natural protein was found to be heavily glycosylated, and by chemical deglycosylation, it was shown that its glycan moiety plays a dominant role in the recognition of the allergen by IgE from latex allergic patients [8]. In the recent years, there has been increasing evidences of IgE binding to allergenic glycoproteins harbouring α 1,3-fucosylated and β 1,2-xylosylated N-glycans of plant and invertebrate origin [9–12]. The role of N-glycan-specific IgE in the clinical manifestation of allergic reactions, however, is still a matter of debate. Although these N-glycans do interact with IgE, it is widely assumed that such cross-reactive carbohydrate determinants (CCDs) commonly have no clinical significance in allergies [13,14]. The latex lecithinase homologue (Hev b 4), on the other hand, is a potent NRL allergen and it has been shown to be active in skin prick tests [15]. Likewise, N-glycans of some plant glycoproteins e.g. Ole e 1 of olive tree pollen [16], Lyc e 2.02 of tomato [17], and Api g 5 of celery [18] were shown to be able of eliciting in vitro effector release from basophil cells.

Recently, the N-glycosylation of several glycoprotein allergens has been determined by MALDI-MS [17,19–22]. This method is particularly useful for the identification of plant N-glycans since they are neutral without exceptions and composed of residues easily discernable by mass alone (pentasaccharide core plus Gal, Fuc, Xyl, GlcNAc). However, MALDI-MS, even if performed with glycopeptides, is usually a poor method for the site specific analysis of glycoproteins. Quenching of signals of some peptides and the difficulty of measuring very large (glyco-)peptides as well as small peptides most often limits the sequence coverage to something around 50%. LC/ESI-MS often achieves superior results in part due to the separation of peptides competing for ionization [23].

In order to assess the types of N-glycans and their distribution on natural Hev b 4 we have thoroughly analyzed its tryptic peptides and glycopeptides by LC/ESI-MS and LC/ESI-MSMS in addition to classical glycan analysis by MALDI-TOF MS.

2. Methods

2.1. Proteolytic digestion of Hev b 4

Hev b 4 was purified from the B-serum fraction of NRL essentially as previously described [24]. The protein was desalted and precipitated by dialysis using SnakeSkin™ (Pierce, IL, USA) tubing with molecular weight cut-off 3000 Da, against distilled water for 48 h at about 5 °C. Hev b 4 was recovered by centrifugation at 20,000 g for 30 min, was then lyophilized and kept desiccated until further use. Due to the insolubility of Hev b 4 in water, the lyophilized protein was dissolved in 4 M urea containing 100 mM ammonium carbonate. An amount of 5 µg of protein was subjected to reducing SDS-PAGE. The major band representing Hev b 4 was excised, destained, carbamidomethylated, digested with trypsin and extracted from the gel pieces as described [19]. Extracts were dried in a Speed Vac concentrator.

2.2. Isolation and identification of Hev b 4 N-glycans

The dried extracts from an SDS-PAGE band were immersed in ammonium acetate buffer of pH 5.0 and the N-glycans were released by peptide:N-glycosidase A (0.3 mU). The released glycans were purified and analyzed by linear MALDI-TOF-MS according to a recently described procedure [19]. Structures of the glycans were deduced from their molecular masses, which is possible as plants exhibit not a too large repertoire of glycan structures [11,25–27].

2.3. MS analysis of the tryptic peptides and glycopeptides of Hev b 4

Dried extracts of SDS-PAGE bands were reconstituted in 0.1% formic acid. The mass spectrometric analysis was performed on a Q-TOF Ultima Global (Waters Micromass) equipped with a standard electrospray unit, a Cap-LC system (Waters Micromass) and a 10-port solvent switch module (Rheodyne). Samples were at first captured on an Aquasil C18 precolumn (30 × 0.32 mm, Thermo Electron) using water as the solvent. The analytical column was held at 5% acetonitrile before solvent switching and then a linear gradient from 5 to 50% acetonitrile was applied at a flow rate of 2 µL/min. All eluents contained 0.1% formic acid. The mass spectrometer had been previously tuned with [Glu1]-fibrinopeptide B to give the highest possible sensitivity and a resolution of ca. 10,000 (full width at half maximum). Mass tuning of the TOF analyzer in the tandem MS mode was done again with [Glu1]-fibrinopeptide B.

At first, a sample containing peptides and glycopeptides with their attached N-glycans was analyzed in the MS mode. Peptide signals that could not be assigned to the in silico tryptic digest of Hev b 4 were selected for targeted tandem MS in order to obtain unambiguous identification. Data analysis was performed with MassLynx 4.0 SP4 Software (Waters Micromass).

Predictions of glycosylation site usage and signal peptide cleavage site(s) were performed with the programs SignalP and NetNGlyc (<http://www.cbs.dtu.dk/services/SignalP/>; <http://www.cbs.dtu.dk/services/NetNGlyc/>).

3. Results

The glycoprotein nature of Hev b 4 has been shown by carbohydrate specific staining and by chemical deglycosylation where the concomitant shift of its band on SDS-PAGE suggested an intense glycosylation of this latex allergen. The predicted amino acid sequence of Hev b 4 (Q6T4P0_HEVBR) contains

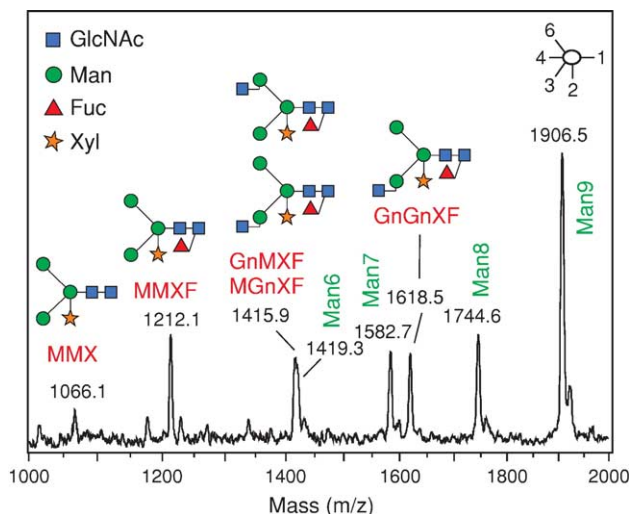


Fig. 1. MALDI-TOF-MS spectrum of N-glycans from whole Hev b 4. Cartoons for the complex type sugar chains are drawn according to the scheme of the Functional Glycomics Consortium. Linkages are indicated as shown in the upper right corner.

eight potential N-glycosylation sites distributed evenly along the polypeptide chain. In an initial survey experiment we released the N-glycans enzymatically from all sites after tryptic digestion of the protein. The MALDI-TOF-MS spectrum revealed the presence of large amounts of oligomannose N-glycans accompanied by ca. 30% of complex type glycans (Fig. 1). The N-glycan species MMXF³, GnMXF³/MGnXF³ and GnGnXF³ occurred in similar amounts (see Fig. 1 for structures). As usual for isolated plant glycoproteins, no larger complex type glycans were seen. The core α 1,3-linkage of the fucose residue, as inferred from work on other plant glycoproteins [25], was corroborated by enzymatic digestion as described below.

A typical global glycan analysis like this will not give information about the site occupancy and the distribution of particular structures to individual sites. Therefore, we undertook to analyze the mixture of peptides and glycopeptides generated by tryptic digestion of Hev b 4 by ESI-MS combined with

reversed phase capillary HPLC. The separation of the peptides in the digest provides ionization of peptides which are prone to suppression effects when ionized in a mixture with other peptides. As a consequence, the sequence coverage is usually higher with LC/ESI-MS than with off-line ESI-MS or MALDI-MS [23]. However, in the present case, a naive approach lacking information about signal peptide cleavage and glycosylation would have given a sequence coverage of 49.7% only. Extensive processing of the data by “little grey cells” increased the coverage to 94.1% and allowed a simultaneous thorough analysis of the protein and the glycans attached to it (Table 1).

Only glycosylation site 224 was found to be occupied solely by complex N-glycans (Fig. 2), whereas four sites carried a mixture of complex and oligomannose glycans (Fig. 2) and two other sites were equipped with only oligomannose glycans (Table 2).

Several glycopeptides were accompanied by notable amounts of the respective unglycosylated peptide. These

Table 1
Masses of peptides and glycopeptides from Hev b 4 as found by LC/ESI-MS

Tryptic fragment	Sequence	Calculated mass (Da)	Charge state	Detected ions (<i>m/z</i>)
1–24	MASLAYSLEFI LSLFTFTLLN PVCT	Signal peptide		
25–44	ELDEYLFSGDGLYDAGNAK	2223.00	3	742.04
45–50	FIYPDK	781.40	1	782.47
51–70	YLPSYHHHPYGTTFFDYPTGR	2418.11	3	807.09
71–75	FSDGR	580.26	1	581.30
76–89	TVVDFVAENVSLPR	1544.82	2	773.46
	N84+Man9	3409.45	3	1137.56
90–94	IPPFK	600.36	1	601.42
97–123	EANFTYGANFASEGATASDSNPLIDFR	2864.29	3	n.d.
	N99+Man9	4728.92	4	1183.31
124–127	SQIR	502.29	1	503.33
128–133	DFGELK	707.35	1	708.41
134–148	LEWAVQLVNVTELAR	1739.96	2	n.d.
	N142+Man9	3604.59	3	1202.62
152–175	KAVYLIISFGADDYLNIEIPSEASR	2720.33	4	681.13
153–175	AVYLIISFGADDYLNIEIPSEASR	2592.24	3	865.14
176–192	EQLESIVDVVLGNISDR	1884.98	2	943.55
	N188+Man9	3749.61	3	1250.96
193–202	IKELYDFGAR	1210.63	2	606.36
195–202	ELYDFGAR	969.45	1	970.53
195–203	ELYDFGARK	1097.55	1	1098.64
203–219	KFVVENVAPLGLIPFIK	1883.13	2	942.63
204–219	FVVENVAPLGLIPFIK	1755.03	2	878.57
220–238	QTSNDSTLFYELASLHAMK	2171.02		n.d.
	N224+MMXF	3325.44	3	1109.56
220–245	QTSNDSTLFYELASLHAM _{ox} KLPQILEK	2992.52		n.d.
	N224+MMXF	4162.94	4	1041.81
239–245	LPQILEK	839.51	1	840.58
246–267	IQDGYLFPEFNNTVFNYFGIIK	2687.33	3	896.84
	N256+Man6	4065.81	3	1356.36
268–279	EIIDAPGEHGFK	1311.65	2	656.87
280–292	YGDIACCGNSTYR	1535.61	2	n.d.
	N288+Man9	3400.25	3	1134.50
293–309	GQACGFLDYEFVCVGNK	2023.82	2	1012.99
	no glycopeptide detected			
310–335	TEYLFFDGTHTNTDAANNLLAELMWDK	3028.39	3	1010.55
310–346	TEY LLA ELM _{ox} WDKESGFISPYGVK	4192.97	4	1049.32
336–346	ESGFISPYGVK	1182.59	2	592.34
347–366	DFFPSPPTTIQTLLTEATALG	2122.08		n.d.

Average mass error was +0.056 with a standard deviation of 0.019. For glycopeptides, only the glycoform with the highest intensity is listed. Cys residues were measured as carboxamidomethyl cysteine; Met_{ox} indicates oxidized methionine. n.d.=not detected.

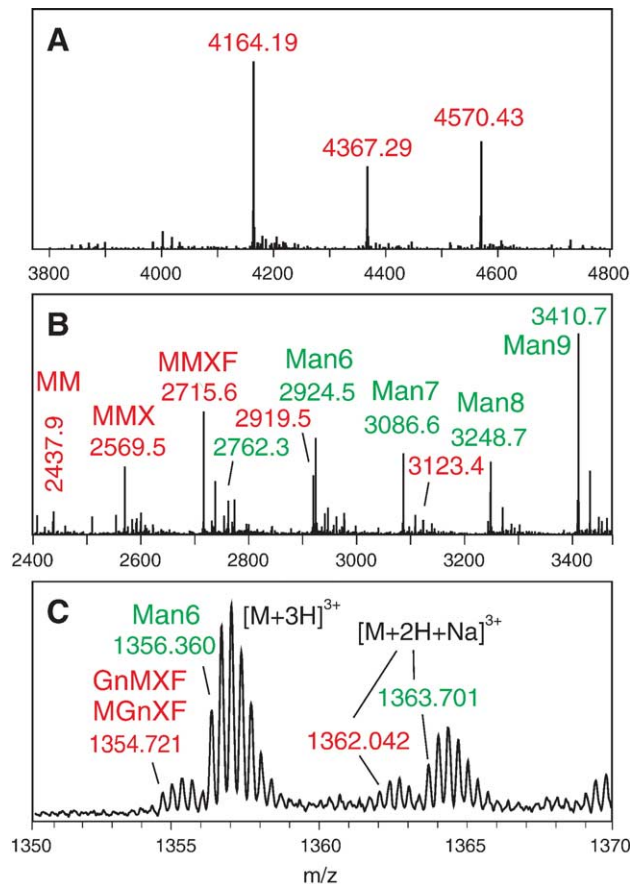


Fig. 2. Examples of ESI mass spectra of glycopeptides. Panels A and B show deisotoped and deconvoluted spectra (=processed with MaxEnt3) of glycopeptides comprising glycosylation site 224 and 84, respectively. Panel C depicts part of the unprocessed spectrum of site N256 where the presence of a small amount of a complex type N-glycan (GnMXF/MGnXF) besides an oligomannose glycan (Man6) can be seen. The mass difference between the observed ion (4066.0 Da) and the non-glycosylated peptide (2687.3 Da) is 1378.5 Da which is exactly the sum of 2 GlcNAc and 6 mannose residues.

peptides in fact have never been glycosylated as they have retained the Asn-residue at the respective position, whereas posterior deglycosylation would have led to the formation of an

Table 2

N-glycan structures on individual glycosylation sites of Hev b 4: for some sites, small amounts of unglycosylated peptide were observed

N-glycan structure	Relative abundance on individual glycosylation sites (%)						
	N84	N99	N142	N188	N224	N256	N288 N308
None	Traces			30	Traces	Traces	100 ^a
MM	4.9					8.5	
MMX	10.9					17.4	
MMXF ³	14.8	6.8	9		46.3	15.1	
GnMXF ³ /MGnXF ³	6.8	13.6	7.5		22.7	4.3	
GnGnXF ³	2.7				31		
Man5	4.6						
Man6	11.2	9.4	6.6	9.8		21.3	10.2
Man7	9.2	13.6	12.7	10.9		16.6	14.4
Man8	10.5	18.9	17	4.1		10.4	21.9
Man9	24.3	37.7	47.2	45.2		6.4	53.5

Site N308 was not found as glycopeptide (see also text). Complex type structures, which potentially bind IgE, are printed in bold.

^a Peaks of low intensity. Partial glycosylation cannot be excluded.

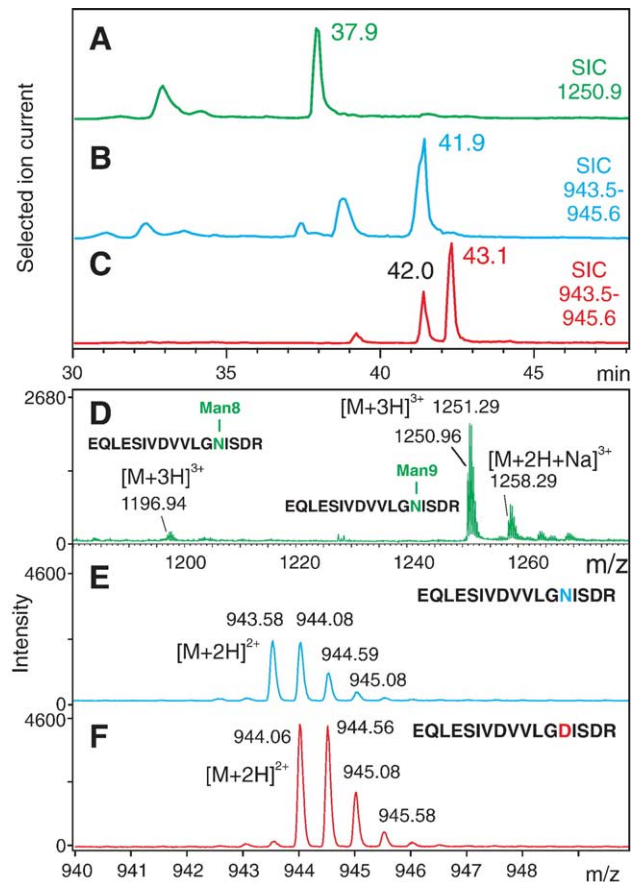


Fig. 3. LC-MS traces and spectra of glycosylated and non-glycosylated peptide containing Asn188. Panel A shows the selected ion chromatogram (SIC) for the mass of peptide 176–192 with the Man9 glycoform. Panel B gives a "broad-range" SIC of the non-glycosylated peptide and C depicts the sample after peptide N-glycosidase A digestion. Incidentally, the non-glycosylated peptide with an Asn and the deglycosylated peptide with an Asp residue were separated on the column used and therefore the undisturbed isotope pattern of the two forms could be obtained (see below). Panel D shows a part of the spectrum of the glycopeptide 176–192 and panels E and F present the isotope pattern of the non-glycosylated and of the de-glycosylated peptide, respectively.

Asp-residue, which is larger by 1 Da. Previous work with human immunoglobulin G indicated that the ionization/detection efficiency of the non-glycoform of a glycopeptide

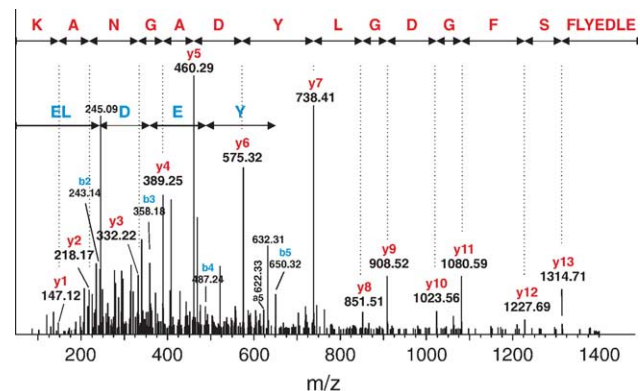


Fig. 4. MSMS spectrum of the N-terminal peptide of Hev b 4. Fragmentation of the putative N-terminal peptide (doubly charged ion at 742 Da) confirmed the sequence with a long y-series (red) and a stretch of the b-series (blue).

may exceed that of the glycopeptide(s) by a factor of 1.5 to 2.0 (J. Stadlmann, unpublished results). Therefore, we based the quantitative estimation of under-glycosylation on a separate LC-MS experiment where the N-glycans had been removed by N-glycosidase A (Fig. 3). In the case of site Asn188, the quantitation was facilitated by the fact that the Asn and the Asp form of this peptide were separated on the BioBasic RP-column, which also has anion-exchange properties. The calculation was performed assuming that the Asn and the Asp form of a peptide do not exhibit relevant differences in their ionization efficiency. In other cases, no quantitation was done as only marginal amounts of maybe 1 or 2% of the respective peptide were found in the unglycosylated form (Table 2).

The most C-terminal potential glycosylation site Asn308 could only be detected as a set of unglycosylated peptides differing in their content of acrylamide-modified cysteines. Since glycosylation would contribute to further splitting of the signal and hence reduction of intensity, we cannot rule out that this site is partially glycosylated such as e.g. Asn188. Remarkably, the web-based prediction tool NetNGlyc 1.0 rather regards site Asn288 than site Asn308 as being non-glycosylated. Admittedly, NetNGlyc 1.0 was designed for human glycoproteins and the oligosaccharyl transferases from mammals and plants may differ in their fine specificity.

LC-ESI MS was also used to verify the assumed core α 1,3-linkage of fucose. Therefore, a tryptic peptide mix was incubated with peptide-N-glycosidase F (PNGase F), an enzyme which is unable to act in the presence of core α 1,3-fucose [28]. In the case of e.g. glycopeptide 76–89, the oligomannosidic glycans were absent after PNGase F treatment, whereas the complex type sugars were still found attached to the peptide, which corroborates the hypothesized core α 1,3-linkage of the fucose residue.

As a side product of the site-specific glycosylation analysis of Hev b 4, its N-terminus could be determined to start at Glu25 as assumed in the EMBL entry (Fig. 4). No indication for the use of the other possible cleavage site before Asn20 which is suggested by the program SignalP 3.0 with similar probability could be found [29]. Unfortunately, the predicted C-terminal peptide could not be found. As the sequence Pro–Ser–Pro vaguely reminds of a plant glycosylation motif [30], we searched for hydroxylation, arabinosylation and galactosylation but without any positive result. However, O-glycosylation of this part of the molecule cannot be entirely ruled out.

Another remarkable observation was made on glycopeptide 220–238/245 where the sequence MetLysLeu was only hydrolyzed by trypsin in the absence of oxidation of Met whereas oxidation led to the incompletely digested peptide 220–245 (which predominated in our analysis). In contrast to its inability to cleave before a prolyl residue, this restriction of the potency of trypsin has not been observed before to the best of our knowledge.

4. Conclusion

Hev b 4 contains complex type N-glycans with xylose and core α 1,3-linked fucose which explain the carbohydrate-based

reactivity with IgE from allergic patients [31]. The (at least partial) multivalency of the Hev b 4 with regard to these cross-reactive carbohydrate determinants (CCDs) could even explain the positive skin prick tests observed with natural (=glycosylated) but not with recombinant, unglycosylated Hev b 4 [8].

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