

Characterisation of Horse Dander Allergen Glycoproteins Using Amino Acid and Glycan Structure Analyses

A Mass Spectrometric Method for Glycan Chain Analysis of Glycoproteins Separated by Two-Dimensional Electrophoresis

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Key Words

Amino acid composition · Glycan analysis · Horse dander allergens · Lipocalin proteins · Mass spectrometry

Abstract

Separation of horse dander allergens using two-dimensional PAGE resulted in the identification of 16 proteins that react with allergic patient sera. A sensitive method has been developed for analysing the structures of the glycan chains of individual glycoprotein allergens transferred to blots following two-dimensional PAGE, and has allowed the structural identification of the glycan chains of the most abundant isoforms of Equ c 1, a glycosylated horse dander major allergen. The method involves separation of the allergens by two-dimensional PAGE, transfer to polyvinylidene difluoride membranes, release of the glycan chains using peptide N-glycosidase F, permethylation and mass spectrometric analysis of the derivatised glycans. The amino acid compositions of the 16 horse dander allergens separated by two-dimensional

PAGE have been determined, allowing the identification of the various isoforms of Equ c 1. These results also confirmed that the two non-glycosylated major allergens, Equ c 2.0101 and Equ c 2.0102, belong to the lipocalin family, and support the idea that these two allergens are most probably isoforms of the same protein. The glycan structures identified using the mass spectrometric method are common biantennary and triantennary glycan chains. These carbohydrate moieties may have a role in the binding of IgE; however, it is more likely that the overall glycoprotein structure involving both the glycan and protein moieties, rather than the structure of the glycan chains alone, is responsible for eliciting allergic responses.

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Introduction

Horse dander preparations contain a number of proteins and glycoproteins that cause allergic responses. Several papers have described the partial purification and

identification of horse allergens [1–5], but the report from Dandeu et al. [6] is to date the only one describing purification to homogeneity of a horse major allergen, Equ c 1. The full-length amino acid sequence of this allergen has been determined [7], identifying Equ c 1 as belonging to the lipocalin family, a large group of low-molecular-mass extracellular proteins responsible for the binding and transport of small hydrophobic molecules. Recently, two isoforms from horse dander preparations, named Equ c 2.0101 and Equ c 2.0102 according to International Allergen Nomenclature recommendations [8], were also shown to be members of the lipocalin family, by a combination of techniques including two-dimensional electrophoresis, immunoblotting with patient sera and N-terminal sequencing [9]. Several other allergens have been described as lipocalin proteins and have been included in the official list of allergens published by the IUIS Allergen Nomenclature Subcommittee (Hørsholm, Denmark). This is the case, for example, for the bovine dander major allergen (Bos d 2 [10, 11]), bovine α -lactalbumin (Bos d 4) and β -lactoglobulin (Bos d 5 [12]) and the mouse major urinary protein (Mus m 1 [13]). Although the reason for an allergic response to proteins is as yet unclear, it has been suggested that lipocalin allergens may have in common a structure responsible for the IgE response [14], and, in general, that there may be a relationship between the structure and function of proteins and their allergenicity [15]. However, this has still to be demonstrated, and the purification and complete characterisation of more allergens is necessary in order to understand what differentiates an allergen from a classical antigen.

In a previous work, we identified 16 allergens in a horse dander preparation [9]. Equ c 2.0101 and Equ c 2.0102 were the only non-glycosylated IgE-binding proteins and were recognised by 50% of the patient sera tested. None of the patient sera that reacted positively on immunoblots with Equ c 2.0101 and Equ c 2.0102 bound glycosylated allergens. Reciprocally, the patient sera that recognised glycosylated allergens did not cross-react with Equ c 2.0101 and Equ c 2.0102. We also reported that the carbohydrate moiety of the glycosylated allergens is likely to interact, at least partially, with IgE from patient sera. However, it has also been shown that a non-glycosylated recombinant form of the glycoprotein Equ c 1 can bind IgE from allergic patients in immunoblot experiments [7]. A first essential step in further investigating the importance of the glycans in the immunogenicity of glycoprotein allergens is the determination of the structure of these glycans.

In order to be able to determine the glycan structures of individual immunoreactive proteins in the microheterogeneous preparation, we have developed a method for analysing minute amounts of N-glycan moieties of glycoproteins directly from Western blots. The successful strategy requires excision of the immunoreactive spot from a Coomassie blue-stained polyvinylidene difluoride (PVDF) membrane, treatment of the membrane fragment with peptide N-glycosidase F (PNGase F; EC 3.2.1.96) to release the N-glycan from the immobilised protein and the subsequent isolation of the released glycans from the dried reaction mixture following permethylation. The permethylated glycans so isolated were identified using mass spectrometry. We report here the identification of sialylated bi- and triantennary N-glycans from the four glycosylated horse dander proteins that gave the strongest positive response on immunoblots with patient sera.

The amino acid compositions of the 16 protein allergens from horse dander preparations separated using two-dimensional PAGE [9] were also determined, and 13 of them were identified as possible members of the lipocalin family. Amongst these 13 putative lipocalins, 7 are most likely isoforms of Equ c 1, 2 are the isoforms of Equ c 2 (Equ c 2.0101 and Equ c 2.0102) and 4 other proteins do not seem to be related to known allergens. Three of the 16 allergens studied do not seem to belong to the lipocalin family.

Materials and Methods

Chemicals and Biological Material

Chemicals used for two-dimensional PAGE, Western blotting and deglycosylation with PNGase F were from Pharmacia Biotech and Boehringer Mannheim as indicated in our previous study of horse dander allergens [9]. All other chemicals were from Sigma Chemical Co. Horse dander extracts were prepared as described [9].

Two-Dimensional PAGE and Determination and Analysis of Amino Acid Compositions of Horse Dander Proteins

Semi-preparative two-dimensional PAGE was performed using the Desaphor gel tube electrophoresis apparatus (Desaga) for IEF and the Protean II xi cell (Bio-Rad) for SDS/PAGE. The technique described by Dunbar [16] was modified as previously described [9]. After two-dimensional electrophoresis, proteins were transferred to PVDF membranes using a Bio-Rad Trans-blot apparatus at 60 V (constant voltage) for 1 h at 4 °C, and 7.5 mM Tris buffer containing 1.2 mM boric acid. Proteins were stained with Coomassie brilliant blue R-250 (0.025% in 40% methanol) and spots from 10 membranes were excised and pooled for amino acid analysis. Amino acid compositions of isolated proteins were determined as previously described [9].

Table 1. Amino acid compositions of horse dander proteins 1–16 (numbered as described in fig. 1)

Amino acid residue	Amino acid compositions (molar percent) of horse dander proteins															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Asx	16.9	16.2	14.9	15.9	15.1	13.3	12.3	13.3	17.1	16.2	17.9	14.5	17.0	16.0	18.2	12.1
Thr	2.8	3.0	2.8	2.9	2.9	3.6	3.9	2.8	3.0	3.2	2.0	2.8	3.7	6.3	6.1	5.3
Ser	8.6	8.8	11.0	7.5	7.2	9.1	12.0	6.6	6.1	6.7	6.8	6.8	7.0	7.0	6.8	7.3
Glx	17.8	17.9	16.8	17.4	18.3	16.0	14.7	15.6	19.6	18.6	17.4	15.6	12.0	18.7	16.1	21.1
Pro	1.8	1.7	1.3	1.7	1.7	2.4	2.6	2.0	1.8	1.8	1.9	2.2	4.9	2.2	2.5	2.4
Gly	10.6	9.2	9.3	6.8	6.4	9.9	11.1	6.2	5.8	5.9	6.5	6.0	6.5	8.6	7.7	3.6
Ala	4.5	4.8	5.1	4.7	4.6	5.6	5.9	4.8	4.1	4.4	4.3	5.0	5.4	7.0	7.0	11.0
Cys	0	0	0	0	0	0	0	0.9	0.2	0.2	0.3	1.3	0.2	0	0.3	0.6
Val	7.7	7.5	7.9	8.3	8.4	7.6	7.3	9.7	8.0	8.0	7.8	7.8	5.3	4.4	4.0	4.7
Met	0	0	0.5	0.6	0.6	0.5	0	0.8	0	0	1.0	0.5	0.5	0.6	0.7	2.3
Ile	5.7	5.4	5.4	6.2	6.4	5.8	4.9	7.8	6.5	6.6	7.2	8.5	7.0	6.9	7.0	3.8
Leu	6.0	5.9	5.6	6.5	6.6	6.3	5.1	7.6	6.6	6.7	7.0	7.8	22.5	6.6	6.5	9.9
Tyr	2.7	2.8	3.0	3.5	3.4	2.5	2.8	3.1	3.7	3.5	3.5	3.5	0.8	1.7	1.6	3.4
Phe	4.7	4.4	4.9	5.3	5.4	4.8	4.7	6.5	5.9	5.9	6.5	6.2	2.0	4.7	4.9	3.7
His	0.6	0.8	0.8	0.6	0.6	1.2	2.0	0.4	0.6	0.7	0.4	0.5	0.5	1.7	1.8	1.9
Lys	4.9	6.2	6.3	6.8	6.7	6.0	5.5	6.4	6.0	6.1	5.3	6.0	1.9	5.0	5.4	5.5
Arg	4.8	5.5	4.5	5.5	5.8	5.5	5.0	5.6	5.0	5.5	4.4	5.2	3.0	3.1	3.2	1.4

The proteins separated by two-dimensional PAGE and transferred to PVDF membranes were hydrolysed with 6 M HCl prior to determining their amino acid compositions. The method used for amino acid analysis did not allow the quantification of Trp residues.

Amino acid compositions of horse dander proteins were compared with amino acid compositions of the proteins from the Swiss-Prot database, using the AACompliment program from the ExPASy Molecular Biology Server (University of Geneva).

Deglycosylation of Glycoproteins with PNGase F

The excisions from the blots were pre-wetted in a minimal volume of methanol and blocked for 1 h at room temperature with 0.5% fish gelatin in 20 mM phosphate buffer, pH 7.4. The membranes were washed three times for 10 min in 20 mM phosphate buffer, pH 7.4, followed by incubation at 37°C in 500 µl of phosphate buffer containing 10 mM EDTA, 1% 2-mercaptoethanol and 20 U of PNGase F. After 24 h, complete deglycosylation was achieved by adding more PNGase F (20 U) to the preparation and incubating the mixture again for 24 h at 37°C. The buffer containing the released glycans was removed, the excisions washed several times with water and all water washes pooled with the buffer, which was subsequently lyophilised.

Permethylation of the Released Glycans

The dried samples were re-dissolved in 250 µl of anhydrous dimethyl sulphoxide (DMSO). Then, 1–2 pellets of NaOH were rapidly ground using a glass pestle and mortar and added to the sample solution. Immediately afterwards, 100 µl of methyl iodide was added, and after 10 min, a second 100-µl aliquot was added. After 20 min, 300 µl of methyl iodide was added, and after a final 20 min, the reaction was terminated by adding firstly 1 ml of 10 mg/ml sodium thiosulphate solution, followed immediately by 1 ml of dichloromethane. The mixture was shaken thoroughly, and after centrifugation, the aqueous layer was removed and discarded. The organic layer was

washed 3 times with 1 ml of water, and dried under a stream of nitrogen. The samples were dissolved in DMSO prior to analysis by mass spectrometry.

Mass Spectrometry

Positive ion fast atom bombardment (FAB) mass spectra were obtained using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer. The FAB gun was operated at an accelerating voltage of 6 kV and with an emission current of 10 mA, using xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on a Hewlett Packard HP9000 data system running JEOL Complement software.

Permethylated glycan samples were re-dissolved in 10 µl of DMSO, and 1.5-µl aliquots of sample solution were loaded into a matrix of mono-thioglycerol.

Results

Amino Acid Composition of Horse Dander Proteins

The amino acid compositions of the 16 allergens present in a horse dander preparation (fig. 1; [9]) were determined (table 1). The total number of residues in the non-glycosylated major allergens Equ c 2.0101 and Equ c 2.0102 (spots 14 and 15, fig. 1) is identical (152), and their amino acid compositions are very similar, except for the Glx and Asx contents. The major difference between the

Fig. 1. Separation of horse dander proteins by two-dimensional PAGE (reproduced with permission from Bulone et al. [9]). Proteins were successively separated using a pH gradient of 2.5–5 and by PAGE under denaturing conditions. The gel was stained with silver.

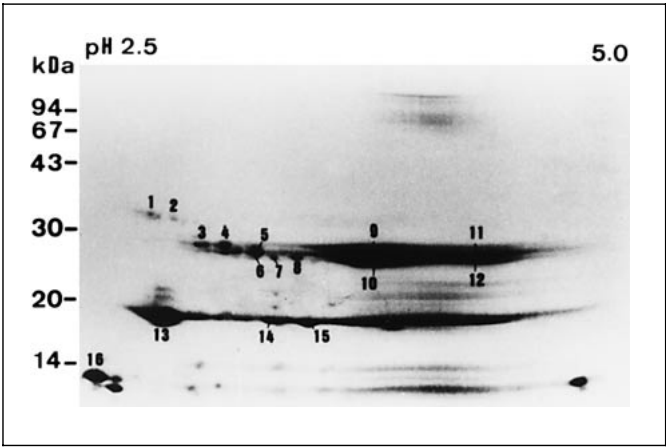


Table 2. Comparison of amino acid compositions of the horse dander proteins and the mammalian proteins in the Swiss-Prot database

Horse dander protein No.	Scores obtained with the AA ComplIdent tool	Similar proteins in the Swiss-Prot database	Horse dander protein No.	Scores obtained with the AA ComplIdent tool	Similar proteins in the Swiss-Prot database
1	66 78	OBP PIG Equ c 1	9	37 56 66	Equ c 1 OBP PIG MUP 5
2	62 64	OBP PIG Equ c 1	10	27* 52 54 66	Equ c 1* OBP PIG MUP 5 MUP 6
3	70 71	Equ c 1 OBP PIG	11	29* 50 68	Equ c 1* OBP PIG MUP 5
4	28* 52 59 65 67 67	Equ c 1* OBP PIG MUP 5 MUP 1 MUP 2 MUP 6	12	15* 37 44 45 47	Equ c 1* MUP 5 MUP 1 MUP 2 OBP PIG
5	27* 56 57 67 68 69	Equ c 1* OBP PIG MUP 5 MUP 1 MUP 6 MUP 2	13	–	–
6	65 65	Equ c 1 OBP PIG	14	47 61 62 67	OBP PIG MUP 6 MUP 1 MUP 2
7	–	–	15	49 53 54 59	OBP PIG MUP 1 MUP 6 MUP 2
8	16* 51 63 64	Equ c 1* MUP 5 MUP 2 MUP 1	16	–	–

The amino acid compositions presented in table 1 were compared with those of mammalian proteins in the Swiss-Prot database having pI values and molecular masses comparable to those of the horse proteins. Proteins other than Equ c 1 are identified as in the Swiss-Prot database: odorant-binding protein from pig (OBP PIG) [17] and mouse major urinary proteins 1, 2, 5 and 6 (MUP1, MUP2, MUP5 [19] and MUP6 [18], respectively). Scores were obtained with the AA ComplIdent program (ExPASy Molecular Biology server, University of Geneva). Score values below 30 indicate a high degree of similarity between the horse dander proteins and the proteins in the Swiss-Prot database. The most significant scores and the corresponding proteins in the

database are marked with an asterisk. Scores higher than 30 indicate increasing difference between the query protein and a protein in the database. Score values of about 70 and higher indicate weak similarities and were considered not significant. Horse dander proteins 7, 13 and 16 did not give significant scores with known proteins. Amino acid compositions of proteins 9 and 10 were determined in our previous work [9], and at that time no significant scores were obtained with Equ c 1 [7] and OBP PIG [17], most probably because the data on these two proteins were not available in the database when the searches were performed.

Table 3. Proposed structures of the glycan chains of allergens 9, 10, 11 and 12, as determined by mass spectrometry

Protein	[M+H] ⁺ (<i>m/z</i>)	Fragment ions (<i>m/z</i>)	Assignment
9	3579	825, 793	triantennary NeuAc ₃
	3218	825, 793, 464	triantennary NeuAc ₂
	2769	2492, 825, 793	biantennary NeuAc ₂
	2408	2131, 825, 793, 464	biantennary NeuAc ₁
	2047	1770, 464	biantennary NeuAc ₀
10	2769	2492, 825, 793	biantennary NeuAc ₂ (major)
	2408	2131, 825, 793, 464	biantennary NeuAc ₁
	2047	1770, 464	biantennary NeuAc ₀
11	3579	825, 793	triantennary NeuAc ₃
12	3579	825, 793	triantennary NeuAc ₃
	3218	825, 793, 464	triantennary NeuAc ₂
	2769	825, 793	biantennary NeuAc ₂ (major)

Proteins separated by two-dimensional PAGE and transferred to PVDF membranes were incubated in the presence of PNGase F to release the N-glycans. After permethylation, the N-glycans were identified by mass spectrometry. The *m/z* values for the [M+H]⁺ pseudomolecular ions, their aminoglycosidic fragment ions and the methanol elimination ions present in the spectra are given.

two proteins is that Equ c 2.0101 has more Glx residues, while Asx residues are more abundant in Equ c 2.0102. These data explain the different apparent pIs observed on two-dimensional PAGE for Equ c 2.0101 and Equ c 2.0102, and lend support to the idea that these two allergens are isoforms of the same protein. The amino acid compositions of Equ c 2.0101 and Equ c 2.0102 were compared with the amino acid compositions of the mammalian proteins in the Swiss-Prot database (AA CompIdent tool, ExPASy Molecular Biology Server, University of Geneva) having molecular masses and pI values similar to those of Equ c 2.0101 and Equ c 2.0102. The proteins showing the highest similarity, i.e. the lowest score values, were the pig odorant-binding protein (OBP PIG [17]) and mouse urinary proteins (MUP MOUSE [18, 19]) (table 2), all belonging to the lipocalin family. These data confirm that Equ c 2.0101 and Equ c 2.0102 are lipocalin proteins. However, the function of these allergens remains to be determined.

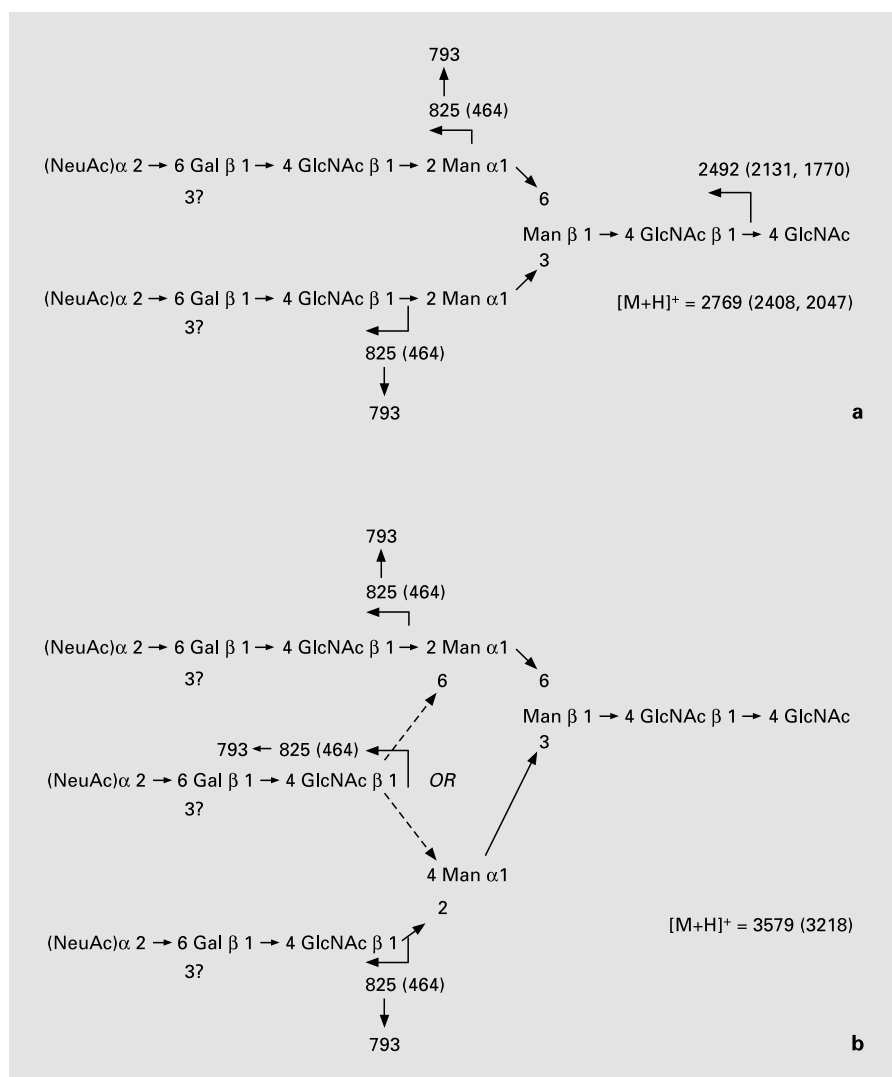
In our previous work, we were unable to sequence the glycosylated allergens (spots 1–13 and 16, fig. 1), either because the amounts available were too low or the N-termini were blocked [9]. However, it has been possible to determine their amino acid compositions (table 1). The proteins in spots 4, 5 and 8–12 gave very similar amino acid composition results, with, however, some variations in the amount of Asx and Glx (table 1). When the compo-

sitions of these spots were compared to those in the Swiss-Prot database of mammalian proteins having acidic pIs, the highest similarity was obtained for a horse dander major allergen belonging to the lipocalin family, Equ c 1 [7] (table 2). These data indicate that these proteins are most probably isoforms of Equ c 1. They suggest that their different mobilities on two-dimensional PAGE, and especially their microheterogeneous pIs, are a consequence of both variation in the relative amounts of acidic amino acids and, as demonstrated below, microheterogeneity arising from differences in the degree of sialylation of their N-glycan chains. It is worth noting that the amino acid compositions obtained from spots 4, 5 and 8–12 also showed similarity with other lipocalins, such as the pig odorant-binding protein [17] and the mouse urinary proteins [18, 19] (table 2). This confirms our hypothesis [9] that these allergens belong to the lipocalin protein family.

Amino acid composition analysis of spots 1, 2, 3 and 6 (tables 1 and 2) revealed that these proteins may also correspond to lipocalin proteins, although they gave rather weak scores with proteins from this family by comparison with spots 4, 5, and 8–12.

Proteins 7, 13 and 16 were also recognised by IgE from allergic patients, but the number of sera that reacted positively with these proteins on immunoblots was low and the intensities of the signals obtained were much lower than those from the glycoprotein allergens analysed above

Fig. 2. Possible structures of the N-glycans released from allergens 9, 10, 11 and 12. The linkages were assigned either from the mass spectrometric data, from lectin-binding experiments [9] as indicated in the text, or from a knowledge of commonly occurring N-linked glycan structures. The positions of N-acetylneuraminic acids are shown in brackets, to indicate that glycan chains may be either fully or partially sialylated. N-acetylneuraminic acids are most probably linked $\alpha(2\rightarrow6)$ to galactose, but they can also be linked $\alpha(2\rightarrow3)$, as indicated by the question mark. **a** Expected fragment ions are indicated for a biantennary structure, with fragments relevant to sialic acids indicated in brackets. **b** Possible triantennary structures and expected fragment ions. Arrows with broken lines indicate that the structures might be 2,6 or 2,4 branched.



[9]. The amino acid compositions of proteins 7, 13 and 16 were also determined; they did not give significant scores with lipocalins or with any other known proteins (tables 1 and 2).

Mass Spectrometric Analysis of the N-Glycan Chains of the Glycosylated Horse Dander Allergens

The results of the mass spectrometric experiments on the permethylated glycans released directly from allergens 9–12 are summarised in table 3. In addition to the observation of $[M+H]^+$ pseudomolecular ions, fragment ions arising from the preferential cleavage of the aminoglycosidic linkages with charge retention on the non-reducing terminal fragments were also present in the spectra and are crucial for structural identification [20]. The observa-

tion of such ions at m/z 825 ($\text{NeuAc-Gal-GlcNAc}^+$) and the absence of ions at m/z 1274 ($\text{NeuAc-Gal-GlcNAc-Gal-GlcNAc}^+$) and m/z 1635 ($\text{NeuAc}_2\text{Gal}_2\text{GlcNAc}_2^+$) are important in the identification of the biantennary and triantennary glycans (fig. 2a, b). In addition, the presence of an ion at m/z 793 (table 3), arising from elimination of the $-\text{O}-\text{CH}_3$ on C-3 of the charge-bearing GlcNAc residue [20], demonstrates that C-3 of the GlcNAc is unsubstituted, consistent with a type 2 N-acetyllactosamine backbone. These results show that the glycans from horse dander major allergens are fully or partially sialylated bi- and triantennary glycans, as illustrated in figure 2. Because FAB mass spectrometry does not yield information on the type of linkage between all monosaccharides, we cannot conclude whether the triantennary structures are 2,4 or

2,6 branched. By combining the results of the lectin-binding experiments performed previously [9] with the results obtained from the method described here, the salient features of the structures of the glycan chains linked to the glycosylated allergens (fig. 2) may be defined: binding experiments with the lectin from *Sambucus nigra* gave a clear positive reaction with spots 9–12 on Western blots, indicating that the terminal sialic acid residues are linked $\alpha(2\rightarrow6)$ to galactose; the much weaker positive reaction with the lectin from *Maackia amurensis* indicates that some glycan chains might also bear sialic acid molecules linked $\alpha(2\rightarrow3)$ to galactose. The linkages between the Gal and GlcNAc sugars are of the $\beta(1\rightarrow4)$ type, as judged by the binding of the *Datura stramonium* lectin to spots 9–12, consistent with the FAB mass spectrometry results.

Discussion

Horse dander preparations are composed of up to 50 proteins having acidic pI values in the range 2.5–5 (fig. 1; [9]). Of these proteins, 16 were found to bind IgE from allergic patient sera and were identified as allergens [9]. The major allergens Equ c 2.0101 and Equ c 2.0102 (spots 14 and 15, fig. 1) were the only non-glycosylated proteins in the preparation, and analysis of their N-terminal sequences revealed that they belong to the lipocalin family [9]. The results presented here confirm that Equ c 2.0101 and Equ c 2.0102 are lipocalin proteins, and allow identification of the different possible isoforms (proteins 4, 5 and 8–12) of Equ c 1, the glycosylated major allergen from horse dander [7], as belonging to the same family. They lend support to the idea that allergens found in mammalian mucosa or epithelial cells and their secretions are often members of the lipocalin family. However, the function of these proteins has yet to be determined, even though it is well known that lipocalins are extracellular proteins that bind and transport small hydrophobic molecules, and that may also bind cellular receptors and be involved in the mediation of cell regulation [21].

The major horse allergens were found to be either non-glycosylated proteins (Equ c 2.0101 and Equ c 2.0102 [9]) or glycoproteins bearing N-glycan chains [7, 9] (isoforms of Equ c 1, as found in this study), each group of molecules being specifically recognised by a different set of patient sera [9]. We previously hypothesised that the glycan chains of the glycosylated allergens might interact with IgE from allergic patients [9]. It was therefore important to characterise these glycan chains structurally. This was achieved after developing a method for isolating the gly-

can chains from the allergens that had been separated by two-dimensional PAGE and transferred to PVDF membranes for Western blot identification of the immunoreactive proteins. In our previous work, the glycoproteins studied appeared to be exclusively N-glycosylated [9], although the presence of a very low proportion of O-glycan chains has been suggested for Equ c 1 [7]. For this reason, our analyses were performed on N-glycan chains only, but the method we developed could also be used to study O-glycan chains. However, the isolation of O-glycans would be more difficult, since no enzyme is known which would remove O-glycans from glycoproteins irrespective of their structure. Following release of the glycans from the blotted proteins using PNGase F, the oligosaccharides were permethylated in order to enhance sensitivity of the mass spectrometric analysis, direct fragmentation and to facilitate separation from residual buffer salts. The permethylated glycans were analysed using FAB mass spectrometry. This approach allows mass spectrometric structural analysis of the glycans to be performed directly on glycoproteins prepared using normal bioanalytical techniques, without requiring the use of separation techniques on a preparative scale. Clearly, this method offers perspectives for the analysis of glycosylated allergens, and in general of glycoproteins present in complex mixtures of biological material or when only minute amounts of sample are available and other separation techniques are not available. The ability to carry out glycan analysis directly on the blotted protein spot avoids the need for scaling-up and carrying out lengthy preparative manipulations that are not necessarily guaranteed to yield the protein corresponding to the spot of interest.

The new approach we have developed was used to determine the structure of the glycans from several isoforms of the horse dander major allergen Equ c 1. These glycans are fully or partially sialylated bi- and triantennary glycans commonly occurring in mammalian N-glycoproteins. It has been reported that IgE from allergic patients are able to recognise a non-glycosylated recombinant form of Equ c 1, suggesting that at least some of the IgE epitopes are present in the protein moiety of the allergen and that the protein structure plays a role in the immunogenicity of a glycoprotein [7]. However, our previous study suggested that the carbohydrate moiety of the glycosylated allergens is also likely to interact, at least partially, with IgE from patient sera [9]. Taken together, our data and those from Gregoire et al. [7] suggest that if the glycan chains of the glycosylated horse allergens play a role in eliciting allergic responses in patients, this is most probably not based on the glycan moiety alone, but on the

overall structure of the glycoprotein. The influence of the glycan on the structure of the glycoprotein has to be further investigated. Isolation of larger quantities of these horse allergens is now the next important step in studying the effect of deglycosylation on allergic responses.

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