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Research paper

Recombinant glycoproteins resembling carbohydrate-specific IgE epitopes from plants, venoms and mites

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ABSTRACT

Background: N-linked glycans present in venoms, pollen and mites are recognized by IgE antibodies from >20% of allergic patients but have low or no allergenic activity.
Objectives: To engineer recombinant glycoproteins resembling carbohydrate-specific IgE epitopes from venoms, pollen and mites which can discriminate carbohydrate-specific IgE from allergenic, peptide-specific IgE.
Methods: One or two N-glycosylation sites were engineered into the N-terminus of the non-allergenic protein horse heart myoglobin (HHM) using synthetic gene technology. HHM 1 and HHM 2 containing one or two N-glycosylation sites were expressed in baculovirus-infected High-Five™ insect cells and a non-glycosylated version (HHM 0) was obtained by mutating the glycosylation motif. Recombinant HHM proteins were analyzed regarding fold and aggregation by circular dichroism and gel filtration, respectively. IgE reactivity was assessed by ELISA, immunoblotting and quantitative ImmunoCAP measurements. IgE inhibition assays were performed to study cross-reactivity with venom, plant and mite-derived carbohydrate IgE epitopes.
Results: HHM-glycovariants were expressed and purified from insect cells as monomeric and folded proteins. The HHM-glycovariants exhibited strictly carbohydrate-specific IgE reactivity, designed to quantify carbohydrate-specific IgE and resembled IgE epitopes of pollen, venom and mite-derived carbohydrates. IgE-reactivity and inhibition experiments established a hierarchy of plant glcyoallergens (nPhl p 4 > nCyn d 1 > nPla a 2 > nJug r 2 > nCup a 1 > nCry j 1) indicating a hitherto unknown heterogeneity of carbohydrate IgE epitopes in plants which were completely represented by HHM 2.
Conclusion: Defined recombinant HHM-glycoproteins resembling carbohydrate-specific IgE epitopes from plants, venoms and mites were engineered which made it possible to discriminate carbohydrate- from peptide-specific IgE reactivity.

1. Introduction

Immunoglobulin E (IgE)-reactive asparagine (N)-linked carbohydrates occur in the form of glycoproteins in a variety of unrelated allergen sources (e.g., plants, venoms, mites) as cross-reactive structures. IgE-reactive glycoproteins had been discovered in plants and venoms as early as 1981 [1] and, due to their sensitivity to periodate treatment and resistance to proteolytic digestion could be discriminated from cross-reactive peptide epitopes [2]. Plant, venom and mite-derived alpha 1,3-core-fucose is not found on human proteins [3] but cross-reactivity had been reported in pollen, fruits and vegetables, [4,5] insect

Abbreviations: RT, Room temperature; CD, Circular dichroism; hIg, Human immunoglobulin; IgE, Immunoglobulin E; ISAC, Immuno solid-phase allergen chip; ISU, Immuno solid-phase allergen chip standardized units; kU/L, Kilo units per liter; kUA/L, Kilo units antigen per liter; N, Natural; OD, Optical density; PBS, Phosphate-buffered saline; R, Recombinant; SDS, Sodium dodecyl sulphate; BSA, Bovine serum albumin; HDM, House dust mite; Alpha-Gal, Galactose-alpha-1,3-galactose; AAL, Aleuria arantia lectin; PNGase, Peptide N-glycosidase.

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Research in context

Evidence before this study

>25% of the world population suffer from immunoglobulin E (IgE)-associated allergy. Those allergens which are capable of inducing severe allergic inflammation in sensitized patients are mainly protein antigens. By contrast, IgE recognition of carbohydrate epitopes almost never induces allergic reactions. Accordingly, IgE antibodies against non-allergenic carbohydrate epitopes may give rise to false positive results in serological tests for allergy. We performed a search in PubMed for studies in human without language restriction with terms "carbohydrate" AND "allergy" AND "epitope" AND "diagnosis" and "IgE" for articles published until October 29, 2018. We found several studies describing the poor allergenic activity of carbohydrate-specific IgE and that these IgE antibodies cross-react with carbohydrate epitopes in a large variety of unrelated allergen sources such as insects and plants. Very few studies reported attempts to provide glycoproteins which could be used to detect carbohydrate-specific IgE antibodies in allergic patients but the origin of IgE sensitization to carbohydrate epitopes was not known.

Added value of this study

Our study is the first report of the recombinant expression of a glycoallergen which contains strictly carbohydrate-specific IgE epitopes of plants, venoms and also mites. Importantly, the recombinant glycoallergen is the first to resemble the cross-reactive carbohydrate epitopes of plants, venoms and mites. By IgE inhibition experiments a hitherto unknown heterogeneity of carbohydrate IgE epitopes is demonstrated which suggests that grass pollen may be the most potent inducers of carbohydrate-specific IgE in carbohydrate sensitized subjects.

Implications of all the available evidence

Our study provides evidence that IgE sensitization to carbohydrate epitopes originates mainly from plant sources, most likely grass pollen. The recombinant glycoproteins can be used for the quantification of carbohydrate-specific IgE and can serve as diagnostic tool for the discrimination of non-allergenic carbohydrate- and allergenic peptide-specific IgE.

venoms [6] and mites [7]. Even glycoproteins form parasites, such as helminths, carry cross-reactive carbohydrate moieties [8,9].

A study analyzing sera from >6000 subjects by IgE serology indicated that >20% of the tested sera showed IgE reactivity to carbohydrate epitopes [10]. However, unlike other IgE-reactive carbohydrates such as galactose-alpha-1,3-galactose, alpha-Gal [11] which trigger severe allergic reactions, asparagine (N)-linked carbohydrate moiety alpha-1,3-core-fucose does not elicit allergic inflammation [12,13]. In fact, several studies demonstrated that IgE antibodies directed against these N-glycan moieties have no or low ability to induce clinically relevant symptoms. For example, it has been shown that the major timothy grass pollen allergen, Phl p 4 which contains these N-glycan IgE epitopes [14] induced only poor responses to skin prick testing [15]. Furthermore, subjects sensitized against this allergen had no symptoms of grass pollen allergy and were negative in basophil activation tests [14,16]. Another elegant study showed by double-blind placebo controlled oral challenge that human lactoferrin which had been expressed in rice and hence contained plant-derived N-linked glycan IgE epitopes did not induce allergic reactions in sensitized subjects [17]. In line with this result, it was found that N-linked glycan epitopes are frequently recognized by subjects sensitized to peanuts but do not induce allergic reactions upon peanut consumption [8,18]. In insect venom allergy, IgE reactivity to cross-reactive carbohydrate epitopes in bee and wasp is a frequent problem which may hamper the identification of the culprit insect and the correct prescription of the appropriate AIT treatment [19,20].

The detection of IgE antibodies specific for N-linked glycans which have little or no clinical relevance and their discrimination from peptide-specific, allergenic IgE responses is therefore a major challenge in clinical allergology [21]. Accordingly there have been several attempts to identify and purify natural glycoproteins containing IgE-reactive carbohydrate- but not peptide-specific IgE epitopes or to obtain carbohydrate markers which made it possible to discriminate carbohydrate- from peptide-specific IgE. However, most of these markers have been obtained by purification from natural sources and the extent of IgE cross-reactivity with carbohydrate epitopes in plants, venoms and mites has not been analyzed in depth. Here we report for the first time the recombinant expression, purification and characterization of glycoproteins which resemble the carbohydrate-specific IgE epitope repertoire of plants, venoms and mites and demonstrate their suitability for the discrimination between carbohydrate- and peptide-specific IgE in sensitized patients. Moreover, our results provide evidence for a hitherto unknown diversity of plant-derived carbohydrate epitopes.

2. Materials and methods

2.1. Expression in baculovirus-infected insect cells and purification of recombinant glycoproteins

The non-allergenic monomeric protein horse heart myoglobin (HHM) was selected as molecular scaffold to engineer recombinant glycoproteins. HHM was converted into two glycoproteins (HHM 1, HHM 2) by adding one or two N-glycosylation sites to its N-terminus, respectively (Fig. 1A). This was achieved in the form of synthetic genes optimized for insect cell expression which encode the amino acid sequences displayed in Fig. 1A. A non-glycosylated control protein (HHM 0) was obtained by changing Asparagine (N) to Glutamine (Q) in the N-glycosylation site (NLT) of HHM 1 (Fig. 1A). The cDNAs coding for the three proteins contained sequences coding for a C-terminal hexahistidine tail and were subcloned into the pTM1 vector [22], harbouring the baculoviral polyhedrin promotor sequence (ATG-biosynthetics, Merzhausen, Germany). HHM 0 was expressed in *Spodoptera frugiperda* (Sf9) insect cell line, whereas HHM 1 and HHM 2 were expressed in *Trichoplusia ni* (High Five) insect cells [23]. Sf9 and High Five cells were obtained from Life Technologies (Carlsbad, CA, US). Recombinant His-tagged proteins were purified from the culture supernatants using a nickel-chelating affinity matrix Ni-NTA agarose (Qiagen, Hilden, Germany). Protein concentrations were determined by BCA assay (Pierce, Rockford, IL, US) and purity was assessed by SDS-PAGE followed by Coomassie blue staining under reducing and non-reducing conditions [24]. A protein molecular weight marker (PageRuler prestained Protein Ladder Plus, Fermentas, St. Leon-Rot, Germany) was used as standard.

3. Immunoblotting

Purified recombinant HHM 0, HHM 1 and HHM 2 were subjected to SDS PAGE (12.5% SDS polyacrylamide gels) and blotted onto nitrocellulose membranes [25] and probed with an α-histidin-tag mouse IgG_1 antibody (Dianova, Hamburg, Germany). Bound IgG_1 was visualized with an alkaline phosphatase labeled anti-mouse IgG_1 antibody (BD, San Jose, CA, US). For the detection of α-(1,3) linked fucose residues nitrocellulose-blotted HHM 0, HHM 1 and HHM 2 were blocked two times for 10 min and one time for 30 min with 3% (*w*/*v*) BSA in PBS/0.05% (*v*/*v*) Tween (PBST) and subsequently incubated with 1 μg/mL

Fig. 1. Construction plans for recombinant horse heart myoglobins (HHM) with and without carbohydrate epitopes. (A) Sequences and (B) schematic overviews of HHM (blue) derivatives with one (HHM1), two (HHM2) N-glycosylation sites (green) or without N-glycosylation sites (HHM 0: N4Q mutation; brown). Spacers are indicated in black. Hexa-histidine tags are indicated in red.

biotinylated *Aleuria arantia* Lectin (AAL) (Vectorlabs, Burlingame, CA, US) for 4 h at room temperature. The membrane was washed three times for 10 min with PBST and then incubated one hour with horseradish peroxidase-labeled Avidin (BD, San Jose, CA, US). After three times washing for 10 min with PBST, the binding of AAL to HHM glycovariants was detected and visualized by chemiluminescence, ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL, US).

To assess the IgE-reactivity of the recombinant HHM 1, HHM 2 and the non-glycosylated control (HHM 0), the nitrocellulose-blotted proteins were incubated with patients´ sera that were 1:10 diluted in gold buffer [50 mM sodium phosphate pH 7.4, 0.5% (*v*/*v*) Tween-20, 0.5% (*w*/*v*) BSA and 0.05% (w/v) sodium azide]. Bound IgE was detected with ^{125}I –labeled anti-human IgE (BSM Diagnostica, Vienna, Austria) and visualized by autoradiography [26]. Serum from a non- allergic patients and buffer without serum served as negative controls.

3.1. PNGase A digestion

The removal of N-glycan branches was performed by PNGase A (NEB, Ipswich, MA, US) digestion following manufacturer's instructions. Two μg HHM 0, HHM 1 and HHM 2 were denatured by adding 1 μL of denaturation buffer and H_2O to a total reaction volume of 10 μL and incubated for 10 min at 100 °C. Afterwards, the proteins were incubated on ice and centrifuged for 10 s. Two μL of Glycobuffer 3 (10×), 2 μL 10% NP-40 and 6 μL H_20 were added. One μL PNGase A were added to the reaction volume, mixed gently and incubated for 1 h at 37 °C. For control purposes 1 μL H_2O was added instead of PNGase A. Protein samples were subjected to SDS PAGE, Coomassie-stained or immunoblotted and tested for IgE-reactivity with patients' sera.

3.2. Biochemical and biophysical analyses of proteins

Size exclusion chromatography was performed on an Äkta Pure (GE Healthcare, Chicago, IL, US) system by injecting 500 μL sample onto a Superdex 200 10/300 (GE) column equilibrated with a 150 mM NaCl, 50 mM Na/K phosphate pH 6.8 buffer with a flow rate of 0.5 mL /min. Bio-Rad Gel Filtration Standard (Bio-Rad Laboratories, Hercules, California, US) was run under the same conditions and the elution volumes of the four protein peaks were used for logarithmic linear regression ($R^2 = 0.9998$) to estimate the analytes´ masses. Far UV circular dichroism (CD) spectra and temperature scans of HHM glycovariants were performed as described previously [27].

3.3. Patients' sera, allergen extracts, allergens

Serum samples of 73 patients who had IgE sensitizations to glycoallergens from plants and insect venoms were studied. The demographic and clinical characterization is shown in Table S1. The molecular sensitization profiles of the patients' sera were analyzed with a customized allergen microarray using ISAC chip technology (Thermo Scientific, Phadia AB, Uppsala, Sweden) as described [28]. The cutoff to be considered positive for IgE sensitization was ≥0.3 immuno solid-phase allergen chip standardized units (ISU). The IgE sensitizations according to IgE reactivities to marker allergens and symptoms of insect venom and/or pollen allergy according to clinical history are indicated in Table S1. The anonymized analysis of the sera was approved by the ethical committee of the Medical University of Vienna (EK1641/2014).

Honey bee (*Apis mellifera*) and wasp venom (mixture of *Vespula germanica* & *Vespula vulgaris*) were purchased from Sigma-Aldrich (St. Louis, Mo, US). rBet v 1 was purchased from Biomay AG (Vienna, Austria). nPhl p 4 was a kind gift from Allergopharma Joachim Ganzer KG (Reinbek, Germany). Timothy grass pollen (*Phleum pratense*) was purchased from Allergon (Thermo Fisher Scientific, Ängelholm, Sweden) and pollen extracts were prepared as described [29]. rApi m 1 was expressed in *Spodoptera frugiperda* (Sf9) insect cells as non-glycosylated and his-tagged protein and purified by Nickel-affinity chromatography as described [23]. The N-glycosylation site (N-X-S/T) (Asparagine-X (any amino acid)-Serine/Threonine) of Api m 1 was mutated by an exchange of Asparagine to Glutamine [30]. rVes v 5 was expressed in *Escherichia coli* BL21 (DE) cells as his-tagged protein and purified [19]. *Alternaria alternata* extract was obtained from Inmunotek (Madrid, Spain). HDM and *Blomia tropicalis* extracts were prepared as described [31].

3.4. Measurement of carbohydrate-specific IgE

Carbohydrate-specific IgE reactivity was measured by ELISA and by quantitative ImmunoCAP measurements. For ELISA, serum samples were diluted 1:10 in PBS containing 0.5% (*w*/*v*) BSA and 0.05% (*v*/v) Tween 20 and tested for IgE-reactivity to HHM 2 and for control purposes HHM 0 and human serum albumin (HSA). Sera from non-allergic patients and buffer were included as controls. Bound IgE was detected [27]. Quantification of carbohydrate-specific IgE levels was performed by ImmunoCAP measurements. Coupling of biotinylated HHM 2 to Streptavidin ImmunoCAPs (o121) was performed as described [31]. In brief, HHM 2 was dialyzed against bicarbonate buffer (0.1 M $NaHCO_3$, 1 M NaCl) and incubated with Biotin-X-X-NHS (Biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester, Sigma, St. Louis, MO, US) for 3 h. Excess of biotin was removed by dialyzing against PBS. Aliquots of 50 μL of the biotinylated HHM 2 (100 μg/ mL) were added onto prewashed Streptavidin ImmunoCAPs and incubated for 30 min at RT und used for the determination of HHM 2 reactive IgE with Phadia®100 (Phadia).

3.5. IgE-Inhibition assays

Immunoblot-inhibition: Patients' sera were diluted 1:10 and pre-incubated with 5 μg/mL of each allergen. Pre-absorbed sera were exposed to nitrocellulose-blotted allergen extracts and bound IgE was detected as described [26].

Inhibition of IgE binding to micro-arrayed allergens: Serum samples were pre-incubated with or without 5 μg HHM 2 or buffer alone over night at 4 °C. IgE-reactivity of non-pre-incubated and pre-incubated serum samples to micro-arrayed allergen components was analyzed with a customized allergen microarray (Thermo Scientific, Phadia AB, Uppsala, Sweden) [28]. The percentage of inhibition was calculated as follows: Percentage inhibition = 100-100×(ISU $IgE^{HHM\ 2}$)/ISU IgE^{buffer}].

3.6. Statistical analysis

Correlation between levels of carbohydrate-specific IgE measured by ImmunoCAP or ELISA was analyzed according to Pearson. Differences in IgE levels among the 6 natural glycosylated allergens were analyzed

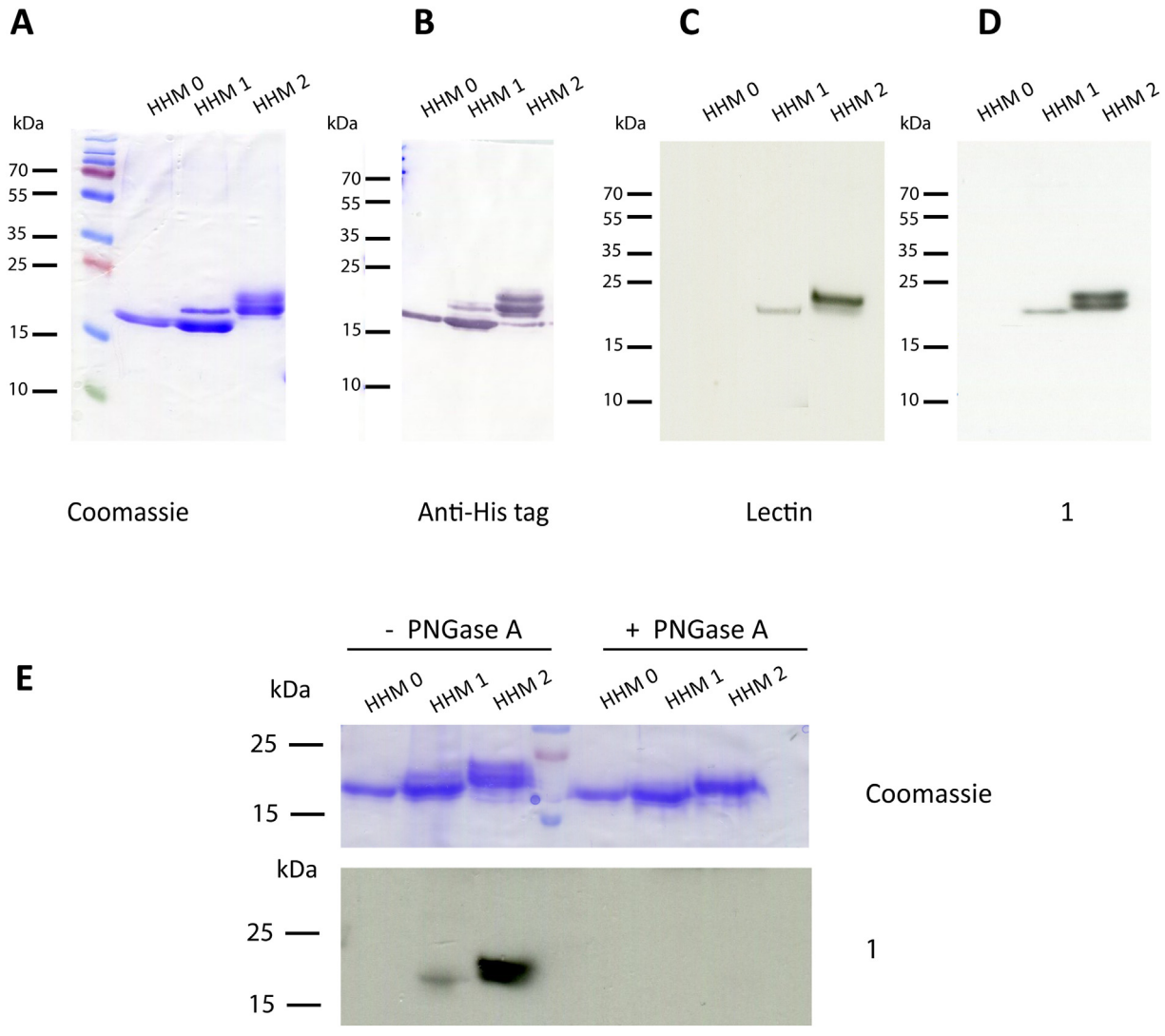

Fig. 2. Purification and IgE reactivity of glycosylated horse heart myoglobin derivatives. (A) Coomassie-blue stained SDS-PAGE containing insect cell-expressed and purified recombinant HHM 0, HHM 1 and HHM 2. Nitrocellulose-blotted purified HHM 0, HHM 1 and HHM 2 detected with anti-His-tag antibodies (B), with biotinylated lectin AAL (C) or serum IgE from a patient (i.e., patient 1) containing carbohydrate-specific IgE (D). (E) Coomassie-stained SDS-PAGE (upper part) and IgE immunoblot (lower part, patient 1) containing HHM 0, HHM 1 and HHM 2 treated with (+) and without (−) PNGase A. Molecular weights are indicated in kDa.

with the Mann-Whitney *U* test. Results with a *p*-value ≤.05 were considered significant.

4. Results

4.1. Design of recombinant glycoproteins

In order to generate recombinant monomeric glycoproteins which allow to detect and measure IgE antibodies specific for carbohydrate structures which do not occur in mammals the following strategy was chosen. We grafted one or two N-glycosylation sites onto a non-allergenic protein, horse heart myoglobin (HHM) which per se occurs as a strictly monomeric protein in solution. Synthetic genes coding for HHM with no (HHM 0), one (HHM 1) or two (HHM 2) N-glycosylation sites at the N-terminal part of HHM and a C-terminal hexahistidine tail were designed (Fig. 1A). The N-terminal amino acid sequences of HHM 1 and of HHM 2 include besides the one or two glycosylation sites (Fig. 1, NLT: green, underlined) three N-terminal Glycines and small flexible spacers consisting mainly of Glycines (Fig. 1, black), which should enhance the probability of glycosylation as predicted by NetNGlyc 1.0 Server to >80%. In the non-glycosylated control protein (HHM 0), the Asparagine in the glycosylation site of HHM 1 was exchanged by a Glutamine. The synthetic genes were codon-optimized for expression in baculovirus-infected insect cells as eukaryotic host to yield glycosylated proteins.

4.2. Expression and purification of recombinant glycoproteins with strictly carbohydrate-specific IgE reactivity

HHM 0, HHM 1 and HHM 2 were expressed in baculovirus-infected insect cells and purified via Nickel affinity chromatography to homogeneity. Typically 2 mg of each of the proteins was achieved per 30 mL of culture. The analysis by SDS-PAGE and subsequent Coomassie staining revealed one band at approximately 17 kDa for the non-glycosylated HHM 0 and two bands at approximately 17 and 21 kDa for HHM 1. For HHM 2 bands at approximately 21 and 23 kDa were observed (Fig. 2A). Purified and immunoblotted HMM 0, HHM 1 and HHM 2 were then detected with an anti-His-tag antibody (Fig. 2B), which reacted with each of the bands stained by Coomassie and an additional faint protein band at around 17 kDa, in the HHM 2 preparation. Next, nitrocellulose-blotted HHM 0, HHM 1 and HHM 2 were tested with α-(1,3)-fucose specific *Aleuria aurantia* lectin (AAL) revealing a single 21 kDa band in the HHM 1 preparation, whereas two bands of approximately 21 and 23 kDa were stained in the HHM 2 preparation (Fig. 2C). In the non-glycosylated HHM 0 preparation no α-(1,3)-fucose was detected.

A first IgE immunoblot experiment performed with serum from a patient which had shown IgE-reactivity to a panel of natural glycosylated allergens (e.g. nPhl p 4, nJug r 2, nPla a 2, nCry j 1, nCyn d 1, nCup a 1). HHM 1 and HHM 2 but not HHM 0 bound the patients' IgE, which was detected by an anti-human IgE antibody (Fig. 2D). The specificity of IgE reactivity for carbohydrate epitopes on HHM 1 and HHM 2 was confirmed by IgE immunoblotting using serum samples from 16 patients with and 4 patients without IgE reactivity to natural glycoproteins. In addition, buffer without addition of serum was tested as a negative control. We found that all 17 sera which had reacted with natural glycoproteins also showed IgE-reactivity to the glycosylated HHM 1 and HHM 2 but not to HHM 0 (Fig. 3). Neither the four sera without carbohydrate-specific IgE nor buffer showed any reactivity to HHM 1, HHM 2 or HHM 0 (Fig. 3). The specificity of patients' IgE for carbohydrate epitopes was confirmed in an additional experiment. HHM 0, HHM 1 and HHM 2 were digested with PNGase A, a glycosidase which selectively cleavesN–linked glycans with or without α (1,3)-core fucose residues [32]. We found a shift of the molecular mass of HHM 1 and 2, but not of HHM 0 by SDS-PAGE and Coomassie staining after 1 h of digestion (Fig. 2E). Furthermore, PNGase A-digested HHM 1 and HHM 2 completely lost their IgE reactivity (Fig. 2E). Since HHM 1 and HHM 2 showed identical IgE reactivity further IgE reactivity experiments were performed mainly with HHM 2.

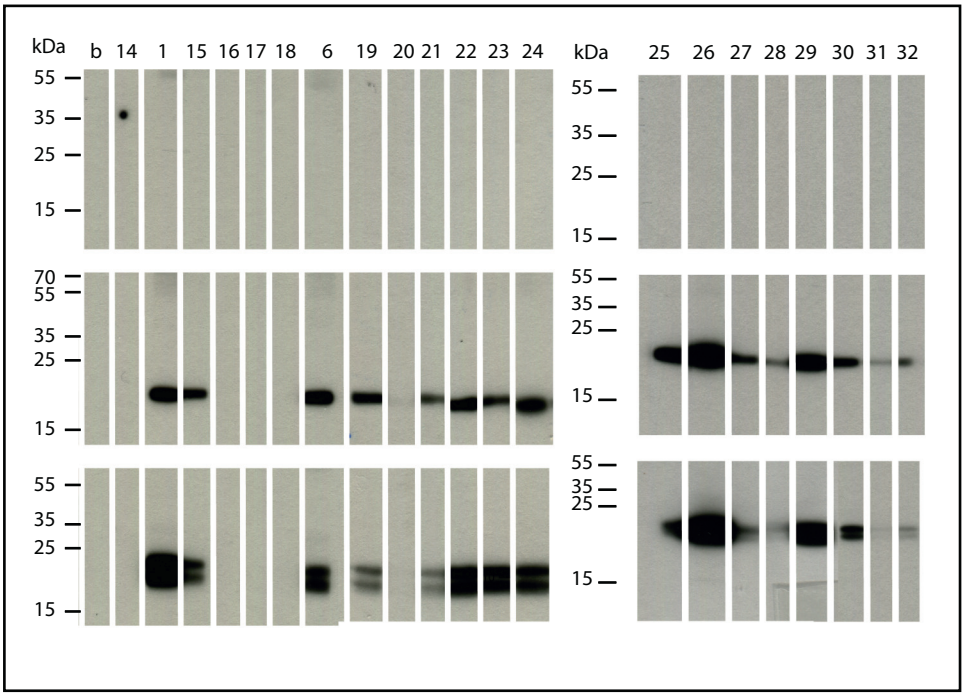

Fig. 3. Nitrocellulose-blotted HHM 0, HHM 1 and HHM 2 were incubated with sera from allergic patients or buffer without serum (b). Bound IgE was detected with ^{125}Iodine-labeled anti-human IgE and visualized by autoradiography. Molecular weights are indicated in kDa.

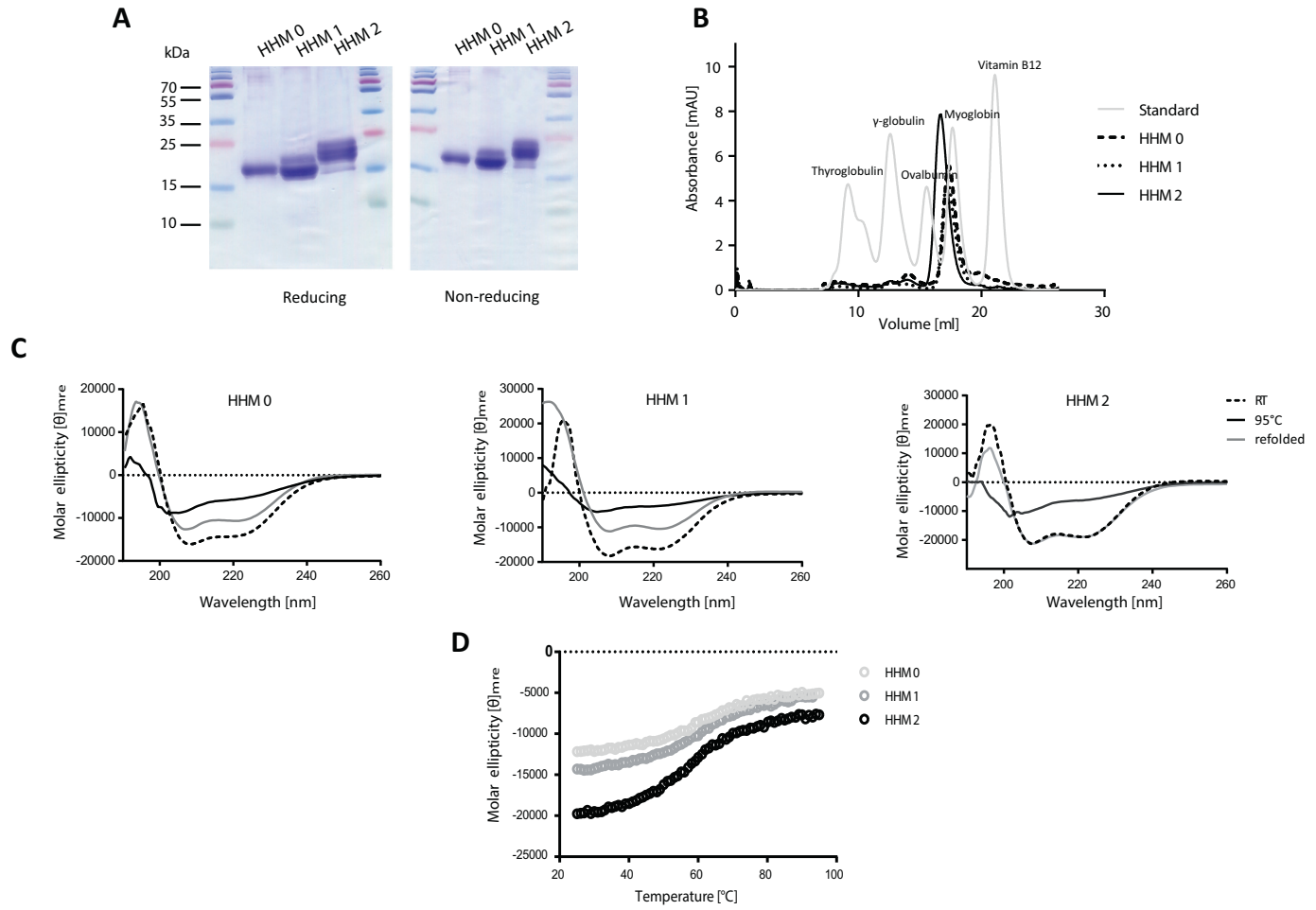

Fig. 4. Biochemical and biophysical characterization of HHM variants. (A) Coomassie-stained SDS-PAGE with HHM derivatives separated under reducing and non-reducing conditions. Molecular weights are indicated in kilo Dalton (kDa). (B) Size exclusion analysis of HHM variants using a Bio-Rad Gel Filtration Standard. Elution volumes (x-axis: mL) and absorbance units (y-axis) are shown. (C) Circular dichroism analysis of HHM variants at room temperature (RT) (dashed line), 95 °C (black line) and after cooling back to RT (gray line). Molecular ellipticities (y-axes) at different wavelengths (x-axes) are shown. (D) Thermal denaturation curves (y-axis: molecular ellipticities; x-axis: temperature) of HHM variants measured at 220 nm.

4.3. Biochemical, biophysical and structural characterization of HHM 0, HHM 1 and HHM 2

In a first set of experiments we investigated if the proteins are monomeric or form aggregates. Purified HHM 0, HHM 1 and HHM 2 migrated as defined bands of expected molecular weights, when separated by SDS PAGE under reducing and non-reducing conditions without presenting evidence for aggregation (Fig. 4A). This was confirmed by size exclusion chromatography which showed that HHM 0, HHM 1, HHM 2 are in a monomeric state with apparent molecular masses (i.e., their hydrodynamic radii) that coincided with the degree of glycosylation (Fig. 2B). According to the size exclusion profile HHM 1 appeared 0.9 kDa larger than HHM 0 and HHM 2 was 5.8 kDa larger than HHM 1.

Far UV CD spectra of HHM 0, HHM 1 and HHM 2 revealed minima at 222 nm and 208 nm as well as a maximum at 193 nm (Fig. 4C). These features are characteristic for proteins with predominant alpha-helical fold [33]. The HHM glycovariants showed thermal stability, since they almost completely refolded after heating to 95 °C and cooling back to RT (Fig. 4C). The melting point, defined as the midpoint of transition, shifts slightly from HHM 0, HHM 1 and HHM 2 from 59.1 to 61.1 °C, indicating that the attached glycan-branches have a slight influence on the melting point of the proteins (Fig. 4D).

Table 1
IgE levels to HHM 2 quantified by ImmunoCAP. Shown are carbohydrate-specific IgE levels determined by ImmunoCAP (kUA/L), by ELISA (OD values) and total IgE levels (kU/L) for 10 patients with IgE reactivity to carbohydrates.

Patient ID	HHM 2 CAP [kUA/L]	Total IgE [kU/L]	HHM 2 ELISA [OD 405 nm]
1	13.9	> 8000	1.79
3	2.5	99.2	0.43
4	1.4	653	0.65
6	3.8	n.d.	0.91
7	3.4	252	0.51
9	4.6	145	1.24
12	9.6	638	1.36
33	9.0	562	1.45
34	2.4	61.5	0.45
35	9.9	65.8	0.87

4.4. Recombinant HHM 2 allows quantification of carbohydrate-specific IgE by ImmunoCAP measurements

By coupling biotinylated HHM 2 to Streptavidin ImmunoCAPs we were able to determine the carbohydrate-specific IgE levels in sera from ten patients who had shown IgE reactivity to glycosylated allergens (Tables S1). The specific IgE levels ranged from 1.4 to 13.9 kUA/L (Table S2) with an average of 3.6% of total IgE (min. 0.2% to max. 15%). Sera from the same patients were also tested for HHM 2-specific IgE reactivity by ELISA (Table 1). In fact, carbohydrate-specific IgE levels measured by ELISA and by ImmunoCAP were significantly correlated (Pearson $r = 0.84$, $p = .0022$) (Fig. 5).

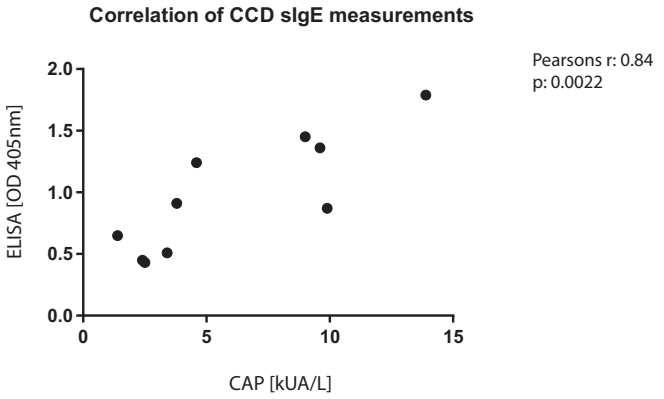

Fig. 5. Correlation of CCD-specific IgE levels measured by ELISA (y-axis: OD values) and ImmunoCAP (x-axis: kUA/L). Pearson's correlation coefficient and p value is indicated. Pearsons $r = 0.84$, $p = .0022$.

4.5. Recombinant HHM 2 resembles carbohydrate-specific IgE epitopes from plants, insects and mites

In a next set of experiments we investigated if the carbohydrates displayed on HHM 2 cross-react with carbohydrates found on glycoallergens from different allergen sources. Insect venoms as well as grass pollen are important and completely unrelated sources of glycoallergens. We therefore tested if HHM 2 can inhibit IgE binding to glycoallergens present in bee and wasp venom as well as in timothy grass pollen extracts (Fig. 6A,B). Three serum samples (2, 3, 4) from patients with bee and/or wasp sensitization were tested in IgE immunoblot-inhibition experiments (Fig. 6A). Preincubation of the sera with HHM 2 inhibited IgE reactivity to Api m 2 (i.e., hyaluronidase, 44 kDa), a glycosylated allergen in bee venom extracts. Complete inhibition of IgE binding to Api m 1 was observed for those patients with IgE reactivity to the carbohydrate moieties of Api m 1 (i.e., patients 3 and 4) but not for patient 2 who reacted also with the protein core of Api m 1 as demonstrated by inhibition of IgE binding to Api m 1 with a non-glycosylated Api m 1 (Fig. 6A,upper part). In wasp venom (Fig. 6A,lower part), HHM 2 inhibited IgE binding to the glycoallergen Ves v 2 (38 kDa) and a 70 kDa IgE reactive band but not to the non-glycosylated allergens Ves v 1 (34 kDa) and Ves v 5 (23 kDa) (Fig. 6A,lower part). The unrelated non-glycosylated birch pollen allergen, Bet v 1, did not inhibit IgE binding to any IgE-reactive bands in bee and wasp venom, Ves v 5 inhibited IgE binding only to Ves v 5 in wasp extract but not to any IgE-reactive band in bee venom.

One of the most important glycoallergens in grass pollen is Phl p 4 which contains IgE-reactive carbohydrates as well as protein epitopes. HHM 2 inhibited IgE binding to Phl p 4 almost completely in two patients who preferentially recognized the carbohydrate epitopes of Phl p 4 (i.e., patients' 6 and 7, Fig. 6B) but not for the patient reacting mainly with the protein epitopes (i.e., patient 5, Fig. 6B). The unrelated birch pollen allergen, Bet v 1 did not inhibit IgE binding to any of the IgE-reactive bands in grass pollen (Fig. 6B).

While insect venoms and plants are known as rich sources for glycoallergens, much less is known about their occurrence in mites. It

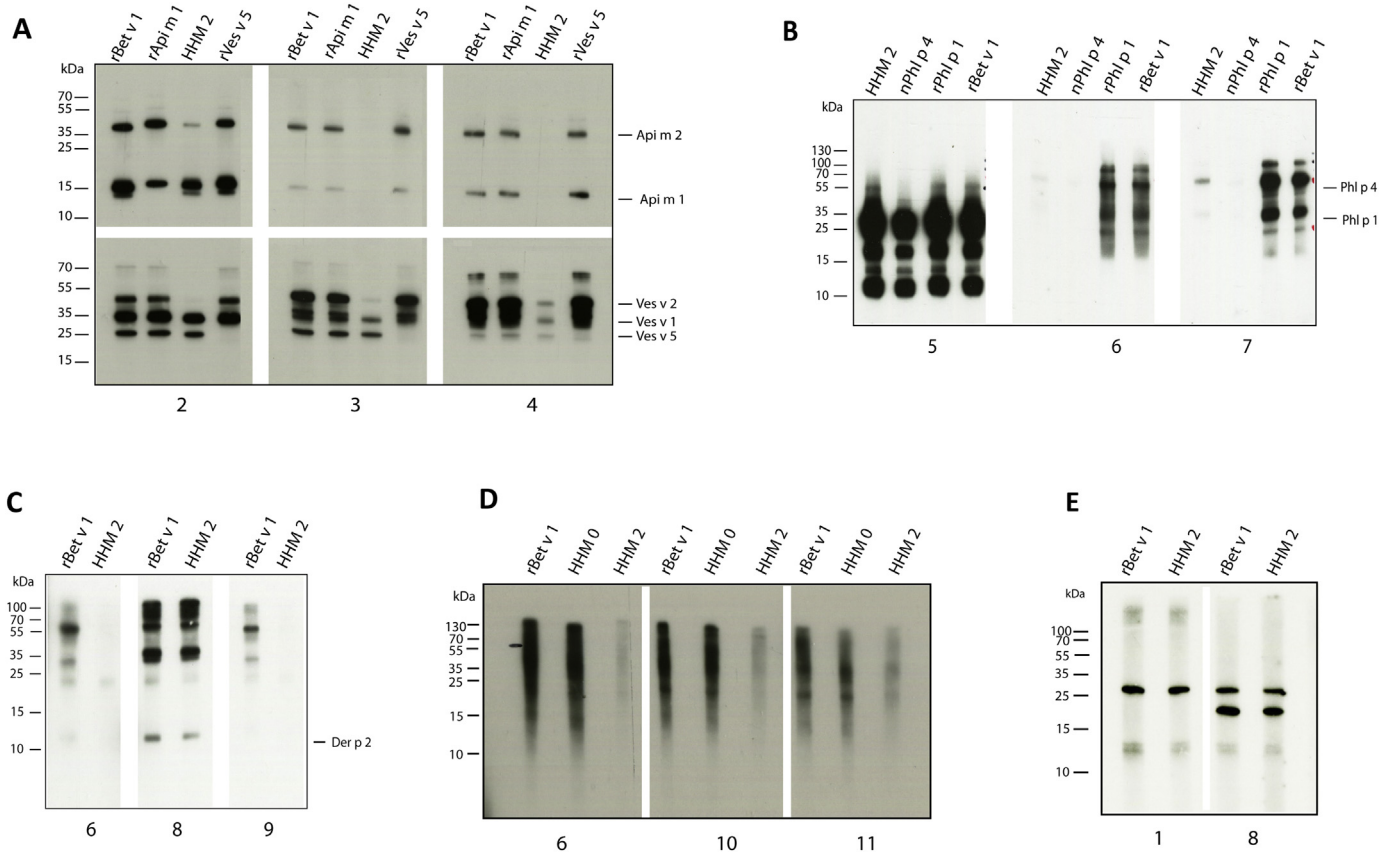

Fig. 6. HHM 2 inhibits allergic patients IgE-binding to carbohydrate epitopes in different allergen sources. (A) Sera from three bee and wasp sensitized patients (2, 3, 4) were pre-incubated with rBet v 1, non-glycosylated rApi m 1, HHM 2 or rVes v 5 and tested for IgE-reactivity to blotted bee (upper part) and wasp venom (lower part) extracts. (B) Sera from three grass pollen sensitized patients (5, 6, 7) were pre-incubated with HHM 2, nPhl p 4, rPhl p 1 or rBet v 1 and tested for IgE-reactivity to blotted grass pollen extract. (C) Sera from three HDM sensitized patients (6, 8, 9) were pre-incubated with HHM 2 or rBet v 1 and tested for IgE-reactivity to blotted house dust mite extract. (D) Sera from *Blomia tropicalis*-sensitized patients (6, 10, 11) were pre-incubated with rBet v 1, HHM 0 or HHM2 and tested for IgE-reactivity to blotted *Blomia tropicalis* extract. (E) Sera from two Alternaria-sensitized patients (1, 8) were pre-incubated with HHM 2 or Bet v 1 and tested for IgE-reactivity to blotted *Alternaria alternata* extract. Molecular weight is indicated in kDa (Table 1).

is also not known if mite-derived carobohydrates cross-react with IgE antibodies reacting with plant- and insect-derived carbohydrates. Interestingly, we found that HHM 2 strongly inhibited IgE binding to high-molecular weight proteins in a *Dermatophagoides pteronyssinus* extract in two (patients' 6 and 9) of three HDM allergic patients with IgE reactivity to high molecular weight HDM proteins (Fig. 6C). We also observed an inhibition of IgE binding by HHM 2 to high molecular weight glycoproteins in *Blomia tropicalis* mite extract (Fig. 6D) indicating that HHM 2 carbohydrate epitopes resemble carbohydrate-specific IgE epitopes in insects, plants and mites. In a first pilot experiment we also searched for cross-reactive carbohydrate IgE epitopes in *Alternaria* but did not obtain any inhibition of IgE binding with HHM 2 (Fig. 6E).

4.6. Inhibition of IgE binding to glycoallergens in different plants with HHM 2 indicates heterogeneity of IgE-reactive plant carbohydrate epitopes

Next we investigated if HHM 2 can inhibit IgE reactivity to well-defined glycoallergens from different plants. For this purpose we performed IgE inhibition experiments using a panel of >170 micro-arrayed allergens which include important major plant glycoallergens (i.e., nPhl p 4: Timothy grass pollen; nCyn d 1: Bermuda grass pollen; nPla a 2: Plane tree; nJug r 2: Walnut; nCup a 1: Cypress; nCry j 1: Cedar). Fig. 7A shows a screen-shot of the IgE reactivity profile of serum from a patient with or without pre-adsorption with HHM 2. HHM 2 completely blocked IgE binding to the glycoallergens nCyn d 1, nCup a 1, nPhl p 4, nPla a 2, nJug r 2 but not to the non-glycosylated venom allergens rApi m 1, rVes v 5, rPol d 5 (Fig. 7A). We then pre-adsorbed sera from 47 patients (Tables S1) with HHM 2 and determined the percentage of inhibition of IgE binding to the aforementioned 6 major plant glycoallergens (Table 2). The average inhibitions of IgE binding were as follows: nPhl p 4: 93.4%; nCyn d 1: 85.2%; nPla a 2: 99%; nJug r 2: 97.6%; nCup a 1: 89.7%; nCry j 1: 98.3%. Only in a few patients (i.e., patients 2, 39, 43, 64, 65, 67, 70, 72, 73) who seemed to contain protein-specific IgE the inhibition of IgE binding obtained with HHM 2 was <80%. Fig. 7B, left panel shows the allergen-specific IgE levels measured for the six major plant glycoallergens for those patients for whom a >90% inhibition of IgE binding was observed by pre-incubation with HHM 2 and who accordingly reacted almost exclusively with carbohydrate epitopes on these allergens. Similar results were obtained for patients who were mainly sensitized to venom allergens (Fig. 7B, middle panel) as well as for those who were sensitized to venom allergens and respiratory and/or food allergens (Fig. 7B, right

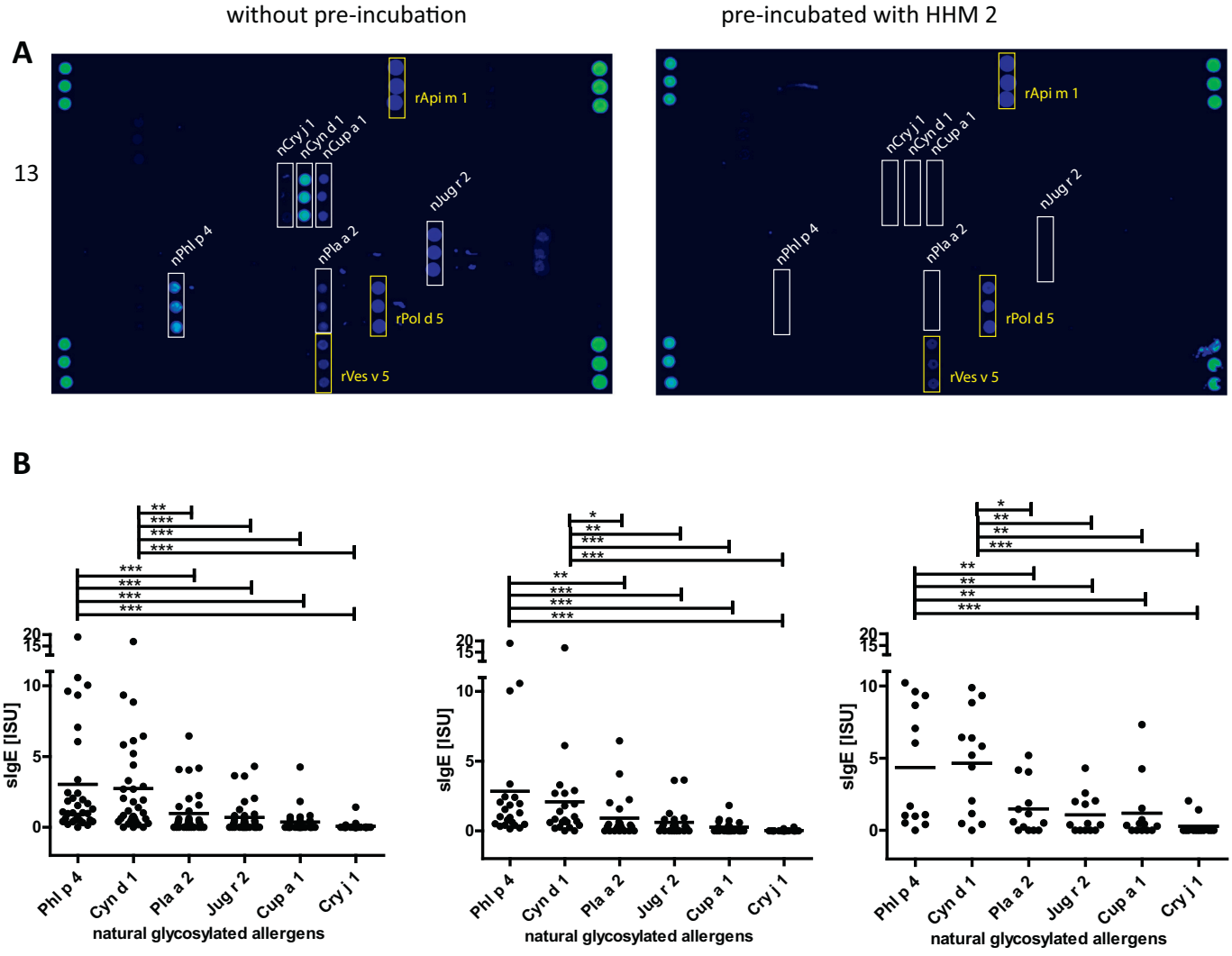

Fig. 7. HHM 2 selectively inhibits patients' IgE binding to natural glycosylated micro-arrayed allergens. (A) Screen shot of IgE reactivities of a serum (patient #13, Table E1) with (right) and without (left) preincubation with HHM 2. Natural glycosylated allergens are indicated by white boxes, recombinant non-glycosylated insect venom allergens by yellow boxes. (B) Hierarchy of carbohydrate-specific IgE reactivities (y-axis: ISU IgE; mean values indicated) to natural glycosylated micro-arrayed plant allergens for all patients from Table 2 for which a >90% inhibition with HHM 2 was obtained (left panel), those with venom sensitization only (middle panel) and those with sensitization to venoms and respiratory and/or food allergens (right panel) (x-axis). Significant differences are shown. $^{**}P < .01$ and $^{***}P < .001$.

Table 2
HHM 2 inhibits patients' IgE binding to micro-arrayed glycosylated plant allergens. Shown are patients IgE levels (ISU IgE) to glycosylated plant allergens (nPhl p 4, nCyn d 1, nPla a 2, nJug r 2, nCup a 1, nCry j 1) without and with HHM 2 preincubation as well as the percentage of inhibition of IgE binding obtained with HHM 2 preincubation. Colour code: dark orange: >15 ISU IgE; orange: 3–15 ISU IgE; light orange: 0.3–2.9 ISU IgE.

	Phl p 4 [ISU]			Cyn d 1 [ISU]			Pla a 2 [ISU]			Jug r 2 [ISU]			Cup a 1 [ISU]			Cry j 1 [ISU]		
Patient ID	- HHM2	+HHM 2	%	- HHM 2	+HHM 2	%	- HHM 2	+HHM 2	%	- HHM 2	+HHM 2	%	- HHM 2	+HHM 2	%	- HHM 2	+HHM 2	%
2	6.15	0.5	91.9	10.84	7.33	32.4	1.32	0	100	1.99	0	100	2.39	0	100	0	0	
3	2.41	0	100.	2.69	0.11	95.9	0.62	0	100	0.92	0	100	0.82	0	100	0	0	
4	10.05	0	100	3.29	0	100	2.24	0	100	1.25	0	100	0	0		0	0	
7	4.51	0.2	95.6	4.33	0.47	89.1	3.18	0	100	1.47	0	100	6.46	0.15	97.7	1.23	0	100
9	13.44	0.47	96.5	11.5	1.41	87.7	1.98	0	100	2.96	0.23	92.2	0.31	0	100	0	0	
12	9.34	0	100	9.34	0	100	4.17	0	100	4.3	0	100	4.25	0	100	1.42	0	100
13	19.01	0.25	98.7	16.93	0.31	98.2	4.08	0	100	3.61	0	100	1.82	0	100	0.27	0	100
34	6.05	0	100	5.2	0.13	97.5	1.14	0	100	0	0		0	0		0	0	
35	0.4	0	100	0.18	0	100	0	0		0	0		0.24	0	100	0	0	
36	2.77	0.44	84.1	2.11	0.16	92.4	0.48	0	100	0	0		0.56	0	100	0	0.16	
37	10.58	0.12	98.9	6.11	0.16	97.4	1.57	0	100	3.64	0	100	0	0		0	0	
38	8.67	0.28	96.8	6.4	0.57	91.1	1.88	0.63	66.5	2.57	0.23	91.1	1.52	0	100	0	0	
39	2.41	1.2	50.2	1.34	0.77	42.5	0.52	0	100	0.89	0.37	58.4	1.4	0.47	66.4	0.27	0	100
40	1.53	0	100	1.01	0	100	0.29	0	100	0.32	0	100	0.68	0	100	0	0	
41	2.45	0	100	2.88	0.14	95.1	2.01	0	100	0.82	0	100	0	0		0	0	
42	1.68	0	100	2.04	0	100	0.51	0	100	2.04	0	100	0	0		0	0	
43	1.05	0.35	66.7	1.22	0	100	0.31	0	100	0.33	0	100	0.68	0.61	10.3	0	0.21	
44	0.39	0	100	0.59	0	100	0.37	0	100	0	0		0.38	0	100	0	0	
45	3.36	0	100	1.79	0	100	0	0		0	0		0	0		0	0	
46	0.61	0	100	0.62	0	100	0.37	0	100	0.3	0	100	0	0		0	0	
47	1.01	0	100	0.62	0	100	0	0		0.24	0	100	0	0		0	0	
48	2.06	0	100	2.69	0	100	0.47	0	100	0.82	0	100	0	0		0	0	
49	1.85	0	100	1.39	0	100	0.18	0	100	0.45	0	100	0	0		0	0	
50	0.97	0	100	1.15	0	100	0.23	0	100	0.37	0	100	0.43	0	100	0	0	
51	0.96	0	100	0.83	0	100	0	0		0	0		0.28	0.3	0.0	0	0	
52	0.21	0	100	0.23	0	100	0	0		0	0		0	0		0	0	
53	0.78	0	100	0.77	0	100	0.28	0	100	0.22	0	100	0.58	0	100	0	0	
54	1.95	0	100	1.92	0	100	0.52	0	100	0.55	0	100	0.73	0	100	0.16	0	100
55	0.14	0	100	0	0		0	0		0	0		0	0		0	0	
56	1.28	0	100	0.68	0	100	0.42	0	100	0.32	0	100	0	0		0	0	
57	0.51	0	100	0.41	0	100	0	0		0	0		0	0		0	0	
58	0.4	0	100	0.46	0	100	4.05	0	100	0	0		0.33	0	100	0	0	
59	0.51	0	100	0	0		6.45	0	100	0	0		0	0		0	0	
60	0.32	0	100	0.27	0	100	0.21	0	100	0	0		0	0		0	0	
61	0	0		0	0		0	0		0	0		0	0		0	0	
62	0.46	0	100	0.4	0	100	0	0		0	0		0	0		0	0	
63	1.02	0	100	4.4	4.45	0.0	0	0		0	0		0.46	0	100	0	0	
64	1.86	0.22	88.2	2.7	1.42	47.4	0.3	0	100	0.3	0	100	0	0		0	0	
65	14.53	3.91	73.1	6.55	1.89	71.1	0.75	0	100	0.98	0	100	0	0		0	0	
66	9.61	0.64	93.3	8.85	0.83	90.6	1.45	0	100	1.8	0	100	0.29	0	100	0	0	
67	18.63	5.58	70.0	21.76	9.8	55.0	1.06	0	100	0.77	0	100	0.75	0	100	0	0	
68	10.22	0.23	97.7	9.89	0.74	92.5	5.19	0	100	1.99	0.29	85.4	7.33	0.87	88.1	2.04	0.21	89.7
69	7.06	0	100	5.83	0	100	0.58	0	100	0.37	0	100	0.73	0	100	0	0	
70	36.24	18.31	49.5	18.27	14.94	18.2	0.23	0	100	0	0		0	0		0	0	
71	1.08	0	100	6.44	12.87	0.0	0	0		0.46	0	100	0	0		0	0	
72	0.8	0.17	78.8	0.72	0.32	55.6	0	0		0	0		0	0		0	0	
73	6.8	2.32	65.9	1.19	0	100	0.39	0	100	0.24	0	100	0	0		0	0	
Mean inhibition rates:			**93.4**			**85.2**			**99.0**			**97.6**			**89.7**			**98.3**

panel). Thus there seems to be an interesting hierarchy of IgE binding to carbohydrate epitopes on the plant allergens in terms of specific IgE levels and numbers of reactive patients as follows: nPhl p 4 > nCyn d 1 > nPla a 2 > nJug r 2 > nCup a 1 > nCry j 1. This hierarchy is derived from the observation that 100% of patients for whom a >90% inhibition was observed reacted with nPhl p 4, 90% with nCyn d 1, 66% with nPla a 2, 57% with nJug r 2, 42% with nCup a 1 and 9% with nCry j 1. We found it even more interesting that there were patients whose IgE binding to the different plant glycoallergens was completely inhibited by the carbohydrates displayed on HHM 2 but showed a selective binding to only certain of the plant glycoallergens. For example, IgE binding of patient 4 to the plant glycoallergens was completely inhibited by HHM 2 but the patient reacted only with Phl p 4, Cyn d 1 Pla a 2 and Jug r 2 but not with the carbohydrates of Cup a 1 and Cry j 1. Likewise, patient 59 reacted only with the carbohydrates on Pla a 2 and Phl p 4 but not with those on Cyn d 1, Cup a 1, Jug r 2 and Cry j 1.

5. Discussion

IgE-reactive N-linked glycans occur in the form of glycoproteins in a large variety of unrelated allergen sources including plants, insects and mites. In contrast to peptide IgE epitopes, carbohydrates show no or low allergenic activity. Often patients show IgE reactivity to the glycan as well as to the protein portion of a glycoallergen and may exhibit varying allergenic sensitivity to the same glycoallergen as has been shown for example for the major grass pollen allergen Phl p 4 [14] and the venom allergen Api m 2 [34]. It is therefore important to discriminate between carbohydrate-reactive and peptide-reactive IgE antibodies in serological tests to interpret the clinical relevance of serological IgE reactivity data.

We describe the engineering, expression and characterization of recombinant glycoproteins, HHM 1 and HHM 2 which contain strictly carbohydrate-specific IgE epitopes. The strict specificity of IgE reactivity to the carbohydrate epitopes on HHM 1 and HHM 2 is shown by the fact that none of the tested sera reacted with the protein backbone alone, HHM 0, which was expressed as a non-glycosylated variant by mutation of the N-glycosylation site. It is further underlined by the fact that treatment with PNGase A, a glycosidase which selectively cleaves N-linked glycans, completely abolished IgE reactivity to HHM 1 and HHM 2. In a set of IgE cross-reactivity experiments carried out with HHM 2 we found that HHM 2 is the first recombinant carbohydrate marker which resembles the IgE epitope spectrum of N-linked glycans of insect venoms, plants and even mites. This is most likely due to the fact that HHM 2 was produced by recombinant expression in insect cells and therefore may show a broader IgE cross-reactivity than plant-derived carbohydrate markers. For example, the plant-derived bromelain carrying MUXF3 glycan structures [35] as well as a semisynthetic CCD marker based on bromelain carbohydrates [10] did not show IgE cross-reactivity with mite-derived carbohydrates whereas HHM 2 inhibited IgE binding to carbohydrate epitopes in house dust and tropical mites. HHM 2 therefore appears to resemble carbohydrate-specific IgE epitopes of a wide variety of unrelated allergen sources, including mites. To investigate in detail IgE reactivity to plant-derived carbohydrate epitopes we tested a panel of important plant glycoallergens, i.e., nPhl p 4 from timothy grass pollen, nCyn d 1 from Bermuda grass pollen, nPla a 2 from Plane tree, nJug r 2 from walnut, nCup a 1 from Cypress and nCry j 1 from Cedar for IgE cross-reactivity. We found that HHM 2 almost completely inhibited IgE reactivity to each of the tested glycoallergens in most of the tested patients with mean IgE inhibitions >85%. Incomplete inhibitions of <80% were only observed in patients for Phl p 4 and Cyn d 1 who were grass pollen allergic and hence reacted also with peptide epitopes. The IgE inhibition experiments revealed also another interesting aspect. In fact, the analysis of those patients whose IgE binding to the panel of plant glycoallergens was almost completely inhibited with HHM 2, demonstrated that certain of these patients showed selective IgE recognition of carbohydrates on certain plant allergens indicating a hitherto unknown fine-specificity of plant carbohydrate epitopes which may be due to differences in the glycosylation patterns. In those patients who mainly reacted with carbohydrate epitopes based on the IgE inhibition experiments done with HHM 2 it appeared that there is a hierarchy of carbohydrate recognition as follows: nPhl p 4 > nCyn d 1 > nPla a 2 > nJug r 2 > nCup a 1 > nCry j 1. This hierarchy was observed for patients who were mainly sensitized to venom allergens as well as for those who were sensitized to venom allergens and in addition to respiratory and/or food allergens. We have observed a similar hierarchy of IgE recognition of plant glycoallergens earlier when testing allergic subjects from Africa [18] and from the Asia [16]. This would argue for the fact that this hierarchy of IgE reactivity to plant-derived carbohydrates is not confined to Europe but may be broadly applicable and could indicate that the primary sensitization to carbohydrates is due to primary sensitization to grass pollen-derived carbohydrates.

Limitations of our study are that we have not performed a detailed biochemical characterization of the carbohydrate moieties on HHM 2 and that we compared it only with one known CCD marker (i.e., MUXF3).

However, the strength of our study is that we demonstrate that the recombinantly produced glycoprotein HHM 2 resembles carbohydrate-specific IgE epitopes from a broad variety of unrelated allergen sources. Furthermore, it can be used for the exact quantification of carbohydrate-specific IgE levels as demonstrated by ImmunoCAP experiments. When using HHM 2 in IgE inhibition experiments it is possible to discriminate between carbohydrate- and peptide-specific IgE which should be very useful in clinical practice.

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Conflict of interest statement

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Author contributions

PG: Designed and performed experiments, analyzed data, wrote manuscript, read manuscript; CL, GH: Performed experiments, analyzed data, wrote manuscript, read manuscript; USB, PK, WK, CK, NN: Provided samples and clinical data, analyzed data, read manuscript; IM, RV: Designed and supervized experiments, analyzed data, wrote manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.12.002.

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