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Structural and immunological properties of arabinogalactan polysaccharides from pollen of timothy grass (*Phleum pratense* L.)

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Abstract—Extracts from pollen of timothy grass (*Phleum pratense* L.) contain up to 20% arabinogalactan proteins (AGPs). Separation of the AGP polysaccharide moieties by tryptic digestion, size exclusion chromatography (GPC), and reverse phase HPLC yielded arabinogalactan fractions AG-1 and AG-2 with molecular weights of ~15,000 and ~60,000 Da, respectively. The backbones of both polysaccharides are composed of (1→6)-linked β-D-galactopyranosides with β-D-GlcUA*p* or 4-*O*-Me-β-D-GlcUA*p* at their terminal ends as revealed by chemical analysis, FT-IR, MALDI-MS, and NMR spectroscopy. AG-1 contains a small number of β-L-Ara*f* side chains while AG-2 possesses a variety of (1→3)-linked units, which consist of β-L-Ara*f*-(1→, α-L-Ara*f*-(1→3)-β-L-Ara*f*-(1→, and α-L-Ara*f*-(1→5)-β-L-Ara*f*-(1→ as well as a small number of longer arabinogalactan side chains. In contrast to crude pollen extracts, the immunological properties of the arabinogalactan mixture reveal an IgG4 reactivity instead of IgE reactivity. Structural properties of timothy pollen arabinogalactan might thus influence the immune response.

Keywords: Arabinogalactan structure; Grass pollen; Immunological properties; Allergy; NMR; *Phleum pratense* L.

1. Introduction

Arabinogalactan proteins (AGPs) are macromolecular glycoproteins, which occur in all higher plants.[1–4] They are assumed to play an important role in vegetative growth, cellular development, and programmed cell death and thus are presumably involved in molecular interactions of intercellular signaling.[1,4–7] However, precise biological functions of single AGPs have not yet been determined.[1,2,8] Recently, the polysaccharide moiety of arabinogalactan proteins have gained interest.[2,3] Some arabinogalactans, for example, reveal potent immune-enhancing properties[9–12] and larch arabinogalactans have been discussed as nutraceutical agents in clinical therapy.[11–13] Therefore, arabinogalactans derived from different kinds of plant materials are currently under investigation for their structural and functional properties.[3,9–11,13]

Up to 10% of the population in industrialized countries are suffering from allergy directed against grass pollen. Especially the pollen of timothy grass (*Phleum pratense* L.) have been studied intensively concerning their allergenicity and protein content.[14] These pollen contain up to 20% (w/w) arabinogalactan proteins consisting of 95% polysaccharide and 5% protein.[15] However, the arabinogalactan moiety of these AGPs has not yet been studied in detail. We now report the

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isolation and separation of the arabinogalactan moieties from timothy grass pollen extract and describe several structural details and immunological properties of the two major arabinogalactan fractions, which may be involved in modulation of the immune response.

2. Results

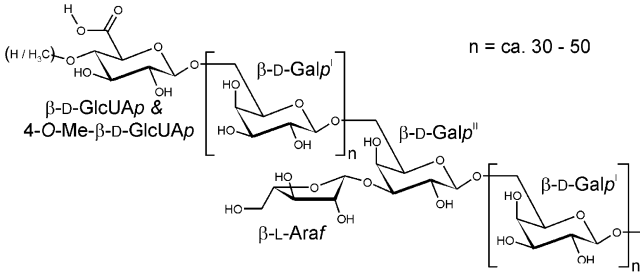
The timothy grass pollen extract investigated here contained 10% arabinogalactans in its dry weight. The isolated arabinogalactans were composed of 68% (w/w) D-galactose and 32% (w/w) L-arabinose as well as small amounts of other carbohydrates. Their monomeric D-Gal and L-Ara units bear diverse substitution patterns, which are shown in Table 1(a). The arabinogalactans were fractionated by HPLC resulting in two main polysaccharides in yields of 56% (w/w) and 44% (w/w) designated as AG-1 and AG-2, respectively. These two fractions contain several not completely separated sub-fractions, likely differing in molecular size and the number of charged groups. Size exclusion chromatography and MALDI-TOF mass spectrometry revealed the molecular weight range of 10,000–20,000 Da for AG-1, whereas AG-2 consists of polysaccharides with ca. 60,000 Da together with shorter ones of about 15,000 Da. Both samples further contain small fragments of about 2000 Da. While AG-1 mainly consists of D-galactose, AG-2 contains D-galactose and L-arabinose in approximately equal amounts. In both arabinogalactans small amounts of glucuronic acid and 4-*O*-methyl-glucoronic acid were also present as identified by wet chemical analysis and FT-IR spectra (C=O carboxylate stretch frequency at 1690 cm^{-1}). Methylation analysis of AG-2 provided quite similar results compared with the investigation of the arabinogalactan mixture described above (Table 1(b)).

NMR spectroscopic analysis indicates that AG-1 is a linear homopolysaccharide with repeating [→6)-β-D-Gal*p*-(1→] units. The downfield shifted signal of C-6, the $^3J_{C,H}$ couplings, and the NOEs between H-1 and H-6a/b indicate (1→6) interglycosidic linkages of all D-galactose units. Chemical shifts of this repeating unit are listed in Table 2 (panel a) and the backbone is shown in Scheme 1. Additionally, AG-1 carries β-L-Ara*f*-(1→ as well as β-D-GlcUA*p*-(1→ and 4-*O*-Me-β-D-GlcUA*p*-(1→ in amounts of about 2–3%. These saccharide units are possibly bound to the polygalactan as side chains and as terminal ends. Proton chemical shifts of these glucuronic acids are listed in Table 2 (panel b) and their structures are shown in Scheme 1.

AG-2 contains a D-galactan backbone, which also consists of (1→6)-linked β-D-galactopyranosides and is quite similar to the one of AG-1. However, 30–50% of the β-D-galactoses in this backbone reveal different chemical shifts, NOEs, and $^3J_{C,H}$ couplings of C-3 and H-3 indicating that side chains are bound to these units in (1→3) linkage. The side chains also influence the adja-

Table 1. Partially methylated alditol acetates derived from AG-1/AG-2 mixture after GPC purification (a) and from AG-2 after HPLC purification (b)

Retention time [min]	Partially methylated alditol acetate	Relative peak area [%]	
		(a)	(b)
10.00	2,3,5-Tri-*O*-methylarabinitol	22	12
12.04	2,5-Di-*O*-methylarabinitol	10	8
12.70	2,3-Di-*O*-methylarabinitol	13	9
16.72	2,3,4-Tri-*O*-methylgalactitol	33	35
18.49	2,4-Di-*O*-methylgalactitol	22	36



Scheme 1. Structural details of AG-1. The galactose backbone is shown with the terminal glucoronic acids and the infrequently occurring single arabinose side chain. Chemical shifts of these groups are listed in Table 2.

Table 2. Proton (δ_H [ppm]) and carbon (δ_C [ppm]) chemical shifts of AG-1. (The corresponding structures are shown in Scheme 1.)

	H-1	H-2	H-3	H-4	H-5	H-6a/b	Me	C-1	C-2	C-3	C-4	C-5	C-6
Panel (a): Backbone saccharides													
β-D-Gal*p*	4.41	3.48	3.62	3.90	3.86	3.98/3.85	—	104.0	71.7	73.4	69.7	74.7	70.4
Panel (b): Terminal end													
β-D-GlcUA*p*	4.48	3.28	3.45	3.21	3.64	—	—	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-*O*-Me-β-D-GlcUA*p*	4.48	3.27	3.46	3.24	3.64	—	3.40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Panel (c): Side chain saccharide													
β-L-Ara*f*	5.18	4.17	3.86	4.06	3.74/3.65	—	—	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

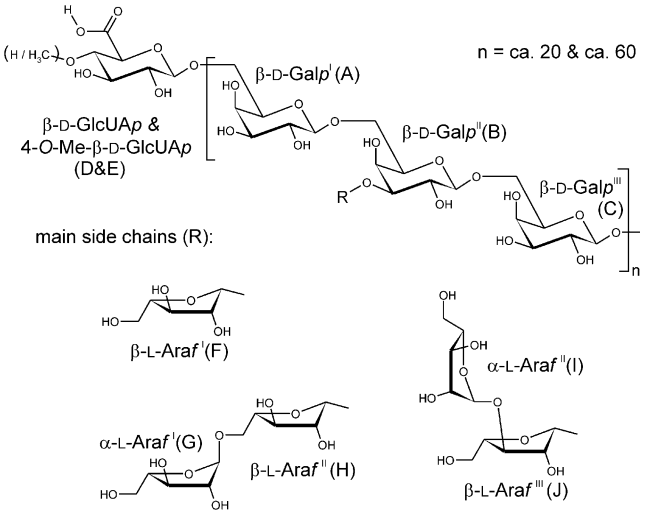
n.d.: not determined due to low signal to noise ratio.

cent galactose units in the backbone, which therefore vary slightly in their chemical shifts. NMR shifts of the different β-D-Gal*p* in the backbone are listed in Table 3 (panel a) and the structure is shown in Scheme 2.

The side chains mainly consist of furanosidic L-arabinoses and vary in length and structure. However, three short side chains occur more frequently. The shortest one consists only of a single β-L-Ara*f*-(1→ and the two others are α-L-Ara*f*-(1→3)-β-L-Ara*f*-(1→ and α-L-Ara*f*-(1→5)-β-L-Ara*f*-(1→. All of these side chains are (1→3) linked to β-D-Gal*p* in the backbone. Chemical shifts of these three short side chains are listed in Table 3 (panel c) and their structures are shown in Scheme 2. Longer side chains containing 5-substituted β-D-Gal*f* are also present albeit in smaller amounts. The positions of the different side chains on the backbone were not further investigated.

β-D-GlcUA*p*-(1→ and 4-*O*-Me-β-D-GlcUA*p*-(1→ are also present in AG-2. Their chemical shifts are listed in Table 3 (panel b). They are both (1→6) linked to a β-D-Gal*p* and located at the terminal end of the backbones. However, the existence of glucuronic acids at the end of some side chains cannot be excluded. Several structural details of the different side chains in AG-2 are summarized in Scheme 2 and Figure 1 shows the corresponding HMQC spectrum.

The immunological properties were analyzed from the desalted fraction AG-2, isolated by anion exchange chromatography. The immunoreactivity was determined by the use of sera from donors with and without grass pollen allergy. No IgE reactivity was detected, however, all sera contained IgG directed against the polysaccharides. There was no significant difference between the two groups regarding total IgG (containing all subclasses; Fig. 2A). However, the sera of the grass pollen allergic group contained considerably less of the 'allergy-protective' IgG4 than those of the control group (Fig. 2B).



Scheme 2. Structural details of AG-2. The galactose backbone is shown with the terminal glucorunic acid and 4-*O*-methyl glucuronic acid. Additionally, the three most abundant side chains are visualized. The letter code of the saccharide units is used according to the code in Figure 1 and Table 3, where chemical shifts of the shown groups are listed.

3. Discussion

Some structural details of the timothy pollen arabinogalactan moieties distinguish them from most arabinogalactans of other plants, which do not belong to the phylogenetical distinct group of the grasses.[1–4,8–10,16,17] The consequent β-(1→6) linkage of D-Gal*p* monomers in the backbones occurs only in a few arabinogalactans from other plants, for example in *Angelica acutiloba* Kitagawa[18] and *Tridax procumbens* Linn.[19] The arabinan side chains of these two heteropolysaccharides are also similar to AG-2. Seldom occurrence of β-D-Gal*f* is another distinguishing feature of AG-2, because furanosidic galactose is rare in plant arabinogalactans,[3] but it

Table 3. Proton (δ_H [ppm]) and carbon (δ_C [ppm]) chemical shifts of AG-2. (The corresponding structures are given in Scheme 2 and the HMQC spectrum is shown in Fig. 1.)

	H-1	H-2	H-3	H-4	H-5 (a/b)	H-6a/b	Me	C-1	C-2	C-3	C-4	C-5	C-6	Me
Panel (a): Backbone saccharides														
β-D-Gal*p*^{I} (A)[a]	4.36	3.47	3.59	3.89	3.84	3.97/3.85	—	104.3	71.5	73.5	69.6	74.7	70.3	—
β-D-Gal*p*^{II} (B)	4.48	3.58	3.68	4.07	3.63	3.97/3.85	—	104.1	70.6	81.0	69.3	75.9	70.3	—
β-D-Gal*p*^{III} (C)	4.41	3.48	3.61	3.90	3.85	3.97/3.85	—	104.3	71.4	73.4	69.5	74.7	70.3	—
Panel (b): Terminal ends														
β-D-GlcUA*p* (D)	4.43	3.29	3.49	3.50	3.76	—	—	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—
4-*O*-Me-β-D-GlcUA*p* (E)	4.48	3.29	3.48	3.24	3.74	—	3.44	103.8	73.8	76.2	83.0	76.3	176.3	60.9
Panel (c): Side chain saccharides														
β-L-Ara*f*^{I} (F)	5.17	4.15	3.86	4.08	3.75/3.65	—	—	110.1	82.1	77.3	84.7	62.1	—	—
α-L-Ara*f*^{I} (G)	5.02	4.05	3.88	4.02	3.75/3.68	—	—	108.2	81.3	77.3	84.8	62.1	—	—
β-L-Ara*f*^{II} (H)	5.18	4.18	3.95	4.15	3.81/3.74	—	—	110.2	83.4	77.5	83.5	67.3	—	—
α-L-Ara*f*^{II} (I)	5.03	4.08	3.99	3.84	3.75/3.67	—	—	102.3	77.3	74.9	82.8	62.1	—	—
β-L-Ara*f*^{III} (J)	5.18	4.36	3.89	4.18	3.75/3.65	—	—	110.2	80.7	84.5	83.2	63.8	—	—

n.d.: not determined due to low signal to noise ratio.

[a] Letters (A)–(J) refer to the same letters given in Scheme 2.

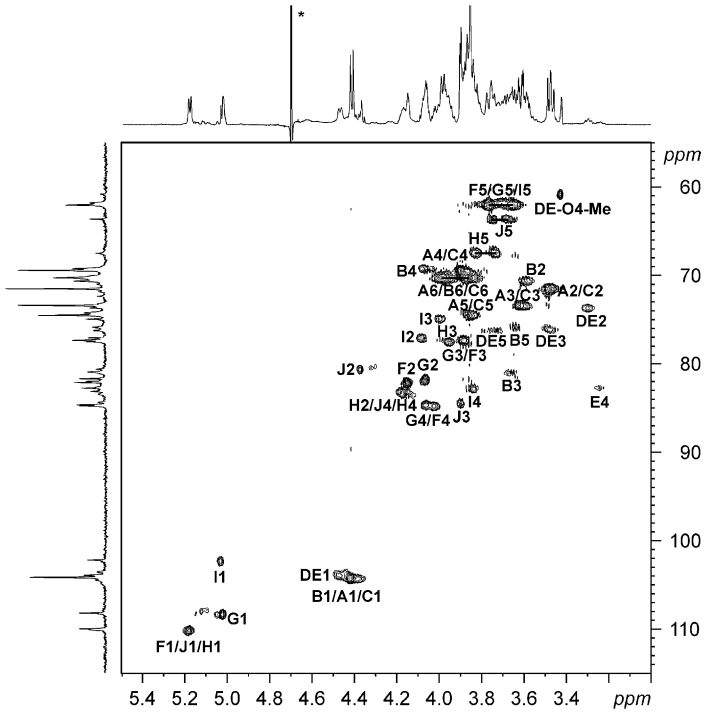


Figure 1. HMQC spectrum of AG-2. Separately recorded one-dimensional ^{1}H and ^{13}C spectra are projected to the f_2 and f_1 axis, respectively. Signals of the monosaccharide units listed in Table 3 are indicated in the spectrum. The letter code of the signals is according to the designation in Scheme 2 and Table 3. The numbers were given according to the common carbohydrate numbering. The signal from HDO is indicated by an asterisk (*).

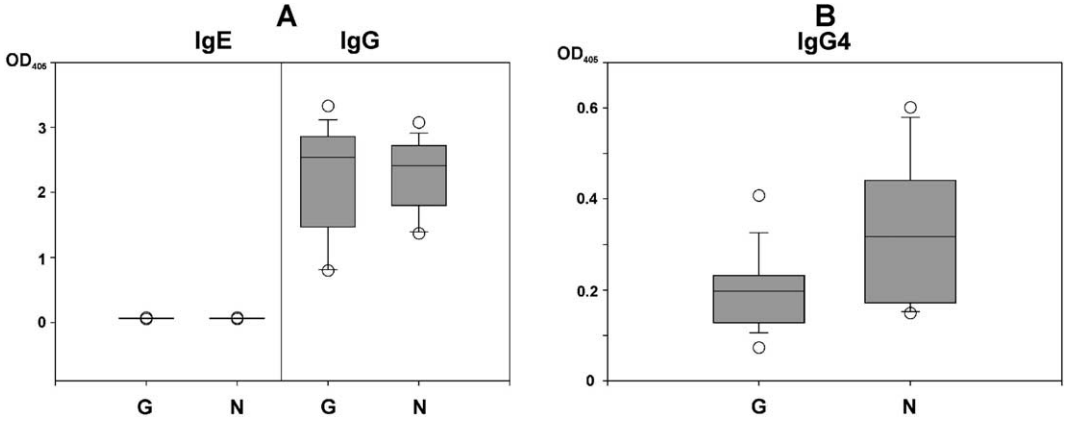


Figure 2. Determination of total IgG and IgG4 reactivity of timothy grass pollen arabinogalactan (AG-2) by ELISA (OD 405 nm). The groups of grass pollen allergic (G) and non-grass pollen allergic (N) individuals are shown with mean value, standard deviation (2s) and extreme values.

is quite common in polysaccharides of *Mycobacteria*.[20,21] Glucuronic acid as well as other hexuronic acids are charged saccharides, which are found in several arabinogalactans.[16,22,23] However, their exact position in the arabinogalactans has only been localized in a few cases.[16]

The natural biological function of timothy grass pollen arabinogalactans and AGPs is still unknown, as well as the functions of all other arabinogalactan proteins from plant materials.[1,2,8] However, the unusual structural properties make timothy grass pollen arabinogalactan an interesting candidate for further immunological investigations. Arabinogalactan from larch has been found to influence innate immunity (e.g., NK cells).[24] The immunological properties of timothy pollen arabinogalactan make it extremely unlikely that they are directly involved in the classical pathomechanism of type I allergy. Additionally, the IgG reactivity for the first time indicates an influence of plant arabinogalactans on the adaptive immune response. The difference in IgG4 levels is especially interesting, as an increase in allergen-specific IgG4 levels appears to be linked to successful desensitization therapy.[25,26] The IgG4 reactivity of the polysaccharides (without a corresponding IgE

reactivity) suggests an immunomodulatory effect. In contrast to the recently described lipid mediators, which appear to have an allergy-promoting influence,[27] this induction of an IgG, especially IgG4 response is a potential antagonistic influence. The experiments described above have been performed using tryptically digested extracts, whereas future immunological tests should include the whole arabinogalactan proteins as the immune system is originally confronted with the latter.

4. Experimental

4.1. Materials

Timothy grass pollen have been obtained from ARTU Biologicals, Lelystad, The Netherlands. All chemicals used were purchased from Sigma–Aldrich Chemical Co., St Louis, USA in the highest available purity and utilized without further purification.

4.2. Isolation of the polysaccharides

Timothy grass pollen (5.2 g) was treated with 160 mL 0.1 M NH_4HCO_3 (30 min, 37.0 °C) on a rotary shaker, centrifuged (30 min, 12,000 U min^{-1}, 4.0 °C), and filtered through a 0.45 μm filter. The filtrate was dialyzed three times against double distilled water (12 h each, 4.0 °C) and lyophilized. The resulting material (~360 mg) was tryptically digested, lyophilized, and fractionated by size exclusion chromatography (GPC) on Sephadex G-50 superfine (Amersham Bioscience, Uppsala, Sweden) in 0.05 M pyridinium acetate buffer (pH 5.0). Fractionation was monitored with a differential refractometer (Knauer, Berlin, Germany). The fraction between 170 and 340 mL contained 34.9 mg arabinogalactan (10%). This material was further separated by HPLC on a Hypersil PEP 300 C4 column (Thermo Hypersil-Keystone, Bellefonte, USA) and alternatively on a Source Q column (Amersham Bioscience, Uppsala, Sweden). Flow rate for the reverse phase column was 1.0 mL min^{-1} and a gradient of CH_3CN (0–80%) containing 0.1% CF_3COOH was applied over 80 min. Flow rate for the Source Q anion exchange column was 2.0 mL min^{-1} and a gradient of 0–0.5 M NaCl in 10 mM Na_2HPO_4 (pH 7.5) for 20 column volumes was used. Both preparations provided two polysaccharide containing fractions (3–5 and 6–10 mL), which were lyophilized. The respective fractions of both separations were identical.

4.3. Sugar analysis

The isolated fractions were hydrolyzed with 2.0 M CF_3COOH (4.0 h, 100 °C) and reduced with $NaBH_4$ (16.0 h, rt). Resulting boronic acid was esterified and removed three times with 20.0 mL 5.0% acetic acid in MeOH at 20.0 °C under evaporation. In order to identify glucuronic acids the isolated fractions were furthermore treated with 2.0 M HCl in CH_3OH (1.0 h, 85 °C). All remaining products were acetylated with Ac_2O in pyridine (1+1 [v/v], 45 min, rt). GC–MS analysis was performed on a HP 5989A (Hewlett–Packard, Palo Alto, USA) equipped with a HP-5 MS column using a temperature gradient of 150 °C (3 min)→320 °C at 5.0 °C min^{-1}. The absolute configurations of the sugars were determined by GLC of acetylated (*R*)-2-octyl glycosides according to Leontein et al.[28]

4.4. Methylation analysis

Methylation of both fractions was performed using CH_3I in DMSO in the presence of solid NaOH powder (60 min, rt).[29] The resulting materials were hydrolyzed with 4.0 M CF_3COOH (2.0 h, 100 °C), reduced with $NaBD_4$ (16.0 h, rt), and acetylated with acetic acid anhydride in pyridine (45 min, RT). GC–MS analysis was performed using the same conditions as described above.

4.5. Molecular mass

The molecular mass was estimated by size exclusion chromatography on a Superdex 75 column (Amersham Bioscience, Uppsala, Sweden), run with a 0.25 M NH_4HCO_3 buffer (pH 7.0). Calibration was performed using the low molecular weight calibration kit for gel filtration (Amersham Bioscience, Uppsala, Sweden).

4.6. Mass spectrometry

The MALDI-TOF mass spectra were performed on a Bruker-Reflex™ II (Bruker-Franzen, Bremen, Germany) in linear arrangement with an acceleration voltage of 28.5 kV. 2,5-Dihydroxybenzoic acid was used as a matrix and spectra were recorded in negative and positive ion mode.

4.7. IR spectroscopy

The IR spectra were measured on a Perkin–Elmer Spectrum One (Perkin–Elmer, Wellesley, MA, USA) in the reflection mode. The sample was applied on a gold layer as sample holder on which the IR beam was reflected to pass the sample twice.

4.8. NMR spectroscopy

The isolated compounds were dissolved in D_2O (~8 mg in 0.7 mL) and transferred into 5 mm high precision

NMR sample tube (Promochem, Wesel, Germany). Sodium 3-trimethylsilylpropanoate-d_4 (TSP) was used as internal standard for proton (δ_H 0.00) and carbon (δ_C 0.00) measurements. 1D proton as well as COSY, TOCSY, ROESY, HMQC, HMBC, and HMQC-TOCSY measurements were recorded at 300 ± 0.1 K and 600.13 MHz on a Bruker DRX-600 AVANCE spectrometer (Bruker, Rheinstetten, Germany). 1D Carbon spectra have been performed on a Bruker DPX-360 spectrometer at 300 ± 0.1 K and 90.6 MHz. All spectra were acquired and processed using the Bruker XWINNMR 3.1 software. The 1D spectra were recorded by acquisition of 16k data points and a relaxation delay of 1.0 s. After zero filling to 32k data points and Fourier transformation spectra were performed with a range of 7200 Hz (^{1}H) and 20,000 Hz (^{13}C), respectively. To determine the 2D spectra 256 experiments with 2048 data points each were recorded. After linear forward prediction to 512 data points in the f_1 dimension and sinusoidal multiplication in both dimensions they were Fourier transformed to 2D spectra with a range of 2400, 20,000, and 30,000 Hz for all proton and carbon (HMQC and HMBC) spectra, respectively.

4.9. Immunological characterization

Sera (12 grass pollen allergic patients and 12 individuals without grass pollen allergy) were kindly provided by Dr. Ute Lepp (Research Center Borstel, Clinical Ambulance, Borstel, Germany). 96-well MultiSorb ELISA plates (Nunc, Roskilde, Denmark) were coated with 2.0 μg of the arabinogalactan (AG-2) in 100 μL PBS, pH 7.0 per well for an incubation period of 2.0 h at room temperature. Excess binding capacity was blocked by incubation with 120 μL of 0.05% Tween 20 in 0.10 M Tris buffered saline, pH 7.4 (TTBS; Merck, Darmstadt, Germany) for 1.0 h at room temperature. The wells were incubated overnight with 100 μL human sera (diluted 1:10 in TTBS) at 4.0 °C. Subsequently the supernatant was removed, the wells washed four times and filled with 100 μL alkaline phosphatase-conjugated (AP) mouse anti-human IgE (diluted 1:2000 in TTBS; Allergopharma, Reinbek, Germany), AP-goat anti-human IgG (diluted 1:10,000; Dianova, Hamburg, Germany) or AP-anti-human IgG4 (diluted 1:1000; Pharmingen, San José, USA). The plates were incubated for 2.0 h at room temperature and after four washing steps (3 times pH 7.4, 1 time pH 9.5) bound immunoglobulin was visualized with 100 μL TTBS, pH 9.5 containing *p*-nitrophenylphosphate (1.0 mg mL^{-1}). The plates were incubated for 2.0 h at 37.0 °C and the reaction was stopped by addition of 20 μL 3.0 M NaOH. Optical densities were measured with a Dynatech MR-7000 ELISA reader (Acterna, Germantown, USA) at 405 nm.

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