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Impact of Urbanization on the Proteome of Birch Pollen and Its Chemotactic Activity on Human Granulocytes

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Key Words
Allergy · Birch pollen · Difference gel electrophoresis · Pollution · Two-dimensional polyacrylamide electrophoresis

Abstract
Background: Epidemiologic studies reveal a dramatic increase in allergies in the last decades. Air pollution is considered to be one of the factors responsible for this augmentation. The aim of this study was to analyze the impact of urbanization on birch pollen. The birch pollen proteome was investigated in order to identify differences in protein abundance between pollen from rural and urban areas. The allergenicity of birch pollen from both areas was evaluated by assessing its chemotactic potency as well as its protein and allergen contents. ***Methods:*** Difference gel electrophoresis (DIGE) was used to analyze the pollen proteome. The chemotactic activity of aqueous pollen extracts was determined by migration assays of human neutrophils. ***Results:*** DIGE revealed 26 differences in protein spot intensity between pollen from urban and rural areas. One of these proteins was identified by de novo sequencing as the 14-3-3 protein, which resembles a stress-induced factor in other plant species. Furthermore, extracts from pollen collected in urban areas had higher chemotactic activity on human neutrophils compared to pollen from rural sites. ***Conclusions:*** The present study points to an impact of air pollution on allergen carrier proteome and release of chemotactic substances. The increment in proinflammatory substances such as pollen-associated lipid mediators might contribute to the described urban-rural gradient of allergy prevalence. Furthermore, our study suggests that allergenicity is determined by more than the sole allergen content.

Introduction

In recent decades, increasing sensitization and prevalence rates of allergic diseases attracted worldwide attention and concern [1]. Several studies have reported a low incidence of allergic diseases among people from rural areas [2–4]. A traditional way of life and the role of parasite infections were examined as possible protective factors.

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In order to explain observed differences in allergy prevalence rates, epidemiological studies have demonstrated an association between pollution such as traffic emissions and an increased prevalence of allergic symptoms [5, 6]. Traffic emissions such as diesel exhaust particles have an impact on the immune system and promote a Th2-dominated immune response [7]. Furthermore, it was shown that diesel exhaust particles can prime the immune system to develop a neoantigen proallergenic immune response with high levels of allergen-specific IgE [8, 9].

A yet unanswered question is whether air pollution has a direct impact on the allergen carrier, i.e. on pollen physiology, changing its allergenicity, leading (in part) to higher sensitization rates or enhanced symptom severity in humans.

The bioavailability of pollen allergens is influenced by environmental factors such as light intensity, temperature and humidity [10–12]. Several in vitro studies have shown that the bioavailability of allergens is directly influenced by pollution [13–17]. In addition, traffic-related pollutants such as NO_2 and O_3 facilitate the release of allergen-rich cytoplasmic granules from pollen and therefore increase the quantity of allergens in the respirable submicronic fraction [16]. Finally, relevant atmospheric concentrations of pollutants such as NO_2 and O_3 may cause nitration of airborne allergens like Bet v 1 [18], leading to the formation of new allergenic epitopes [19].

However, other factors aside from the allergen itself and its intrinsic function [20] influence the allergenicity of an allergen carrier [21, 22]. Recent data point to adjuvant, nonallergenic components released from the allergen carrier together with the allergen. It has been shown that pollen grains release proinflammatory (NADPH) oxidases [23, 24], oxylipins [25, 26] and immunomodulatory substances [27–29] which contribute to the allergenicity of pollen grains.

The aim of this study was to analyze the influence of anthropogenic factors on pollen grains from trees grown in urban and rural areas. By presenting for the first time a partial proteome reference map for birch pollen, we evaluated protein abundance changes between rural and urban pollen using difference gel electrophoresis (DIGE). Seven of the 26 differentially expressed proteins were de novo sequenced. The identified 14-3-3 protein was significantly upregulated in urban pollen. Furthermore, urban pollen extracts had enhanced chemotactic activity on human neutrophils compared to rural pollen. This supports the concept that pollutants influence pollen grains. This in turn may influence the allergenicity of pollen grains and thus favor allergic sensitization.

Materials and Methods

Blood Samples

Healthy controls (n = 11) without a history of allergic diseases were tested by RAST and skin prick tests for 15 common allergens including birch and grass pollen. All subjects tested negative and total IgE was <20 IU/ml. Volunteers did not take any medication for at least 15 days before blood sampling. The ethical committee of the Technical University of Munich approved the study, and volunteers were enrolled after written informed consent.

Collection of Birch Pollen

Birch pollen was collected in the spring of 2003 during the flowering season in the city center of Munich, Germany (urban), and in the area north of Munich (rural). Catkins were sampled on a sunny day (temperature at 20°C, SD ± 3°C) from 8 trees at 2 m above the ground. Pollen was extracted by sieving catkins with a 100-μm sieve followed by a 72-μm sieve and stored at –78°C until further analysis.

Genetic Analysis of Birch Trees

Multilocus molecular data from amplified fragment length polymorphisms (AFLPs) were used for genetic analysis of the *Betula* species in order to investigate whether trees were genetically different or identical (commercial clones are genetically identical). For DNA extraction, a leaf (1 cm^2) was disrupted with a tissue lyser (Qiagen, Heidelberg, Germany), followed by DNA extraction with the DNeasy plant DNA extraction kit (Qiagen). The AFLP assay [30] was performed according to Arens et al. [31] and Schenk et al. [32]. We used three selective primer combinations according to Bonin et al. [33] on 18 samples. Amplified fragments were separated and analyzed on a LI-COR 4300 DNA analyzer (LI-COR Biosciences, Lincoln, Nebr., USA).

For data analysis, Tiff images were imported into Quantar software (Keygene, Wageningen, The Netherlands). Presence (1) or absence (0) of each polymorphic AFLP band was scored for all genotypes. Neighbor-joining analysis was carried out based on the similarity matrix of Jaccard distances among trees using NTSYSpc 2.10j (Applied Biostatistics Inc., USA). All accessions that display more than 98.5% similarity potentially represent clones [31]. Accessions were subsequently clustered according to their similarity by constructing a dendrogram using neighbor-joining analysis. In such a dendrogram, identical genotypes, if present, will cluster. Moreover, genotypes from different species will cluster in separate groups in the dendrogram. A series of 60 reference trees from botanical gardens [32] were included to enable species identification. This reference set included several commonly grown species *(B. albosinensis, B. alleghaniensis, B. chichibuensis, B. costata, B. davurica, B. ermanii, B. grossa, B. humilis, B. lenta, B. maximowicziana, B. medwediewii, B. nana, B. nigra, B. papyrifera, B. pendula, B. platyphylla, B. populifolia, B. pubescens, B. pumila, B. schmidtii,* and *B. utilis)* and several commercially available hybrids. The similarity values and subsequent clustering between the genotypes that were sampled in this study and trees from this reference set yield information on the taxonomic identity of the sampled trees.

Characterization of the Tree Site

The area around the trees was characterized by calculating a traffic score for traffic pollution by ranking the distance from the

tree to the nearest streets. The distance to the nearest street was split into three categories: 0 to ≤10 m (3 points), >10–50 m (2 points), >50 m (1 point). Furthermore, the distance to the nearest major road was characterized by the categories 0 to ≤10 m (3 points), >10–50 m (2 points), >50–150 m (1 point), >150 m (0 points). Transit traffic (3 points), rush hour traffic (2 points) and traffic from residential areas (1 point) were considered as well. Points were given for major road (3 points), minor road without speed limit (2 points) and minor road with speed limit 30 km/h (1 point). The points from each location were summed, leading to the traffic score. The higher the score, the greater the estimated impact of traffic pollution on the site. The nitrogen dioxide concentration was measured at the sites according to Palmes' principle [34]. In brief, the passive sampler depends on the transfer of NO_2 by diffusion to triethanolamine-coated meshes/collector at the sealed end of a tube; the open end of the tube is exposed to the environment. After 7 days' exposure, the passive sampler is analyzed for NO_2 adsorption.

Climate and weather at the study sites were characterized by meteorological data (daily mean, minimum and maximum air temperature, precipitation from October 2002 to June 2003) from two climate stations: (1) the urban climate station 'München-Stadt' (48°16′N, 11°54′E) of the German Meteorological Service, situated in central Munich, and (2) the forest-monitoring site 'Freising' (48°25′N, 11°40′E) of the level II plots (ICP Forests), situated 1.2–5.5 km north of the studied rural birch trees.

Sample Preparation and Two-Dimensional Electrophoresis

100 mg pollen were disrupted and dissolved in 1 ml lysis buffer (7 M urea, 2 M thiourea and 4% CHAPS (w/v); pH 8.5) and sonicated (2 × 30 s, 20% power 60 W, 9 cycles) on ice. After 45 min of centrifugation at 14,000 *g*, the supernatant was collected and adjusted to pH 8.5 by adding HCl. For DIGE, 100 μg of protein from each sample was labeled with CyDyes™ (GE Healthcare Bio-Sciences AB, Uppsala, Sweden): rural samples were labeled with Cy3, urban samples with Cy5, and the internal standard with Cy2 according to the manufacturer's instructions. The internal standard contained equal amounts of all 8 samples. Four gels were run in total, each containing 1 urban and 1 rural sample, and the internal standard. Two-dimensional (2D) electrophoresis was performed according to Görg et al. [35, 36].

Image Acquisition and Statistical Analysis

After electrophoretic separation, the gels were scanned on a Typhoon 9400 scanner (GE Healthcare Bio-Sciences AB). The comparative analysis was performed with the software package DeCyder (GE Healthcare Bio-Sciences AB) according to the manufacturer's instructions. Two groups with 4 members were compared by using the internal standard and by applying Student's t test. Results with $p \leq 0.05$ were considered significant.

De novo Sequencing

The expression levels of spots No. 505, 544, 557, 1341, 1370, 1636 and 1667 (fig. 1) in pollen samples from rural and urban areas are significantly different ($p \leq 0.05$, Student's t test). Protein abundance of these spots was sufficient to perform de novo sequencing. Protein spots were excised from Coomassie-stained gels [37], digested with trypsin and analyzed by Consorzio Proteomics (Sassari, Italy) with a nano-ESI quadrupole time-of-flight tandem MS (Q-TOF MS/MS, Biolynx petide synthesizer; Biochrom, Cambridge, UK), and identified by comparing the deduced peptide sequences with the NCBI nonredundant database using a BLAST search [38].

Pollen Extracts for Bet v 1 Assay

Birch protein was extracted by diluting 5 mg pollen/ml with 0.1 M NH_4HCO_3, pH 8.1 in 15-ml polypropylene tubes (Greiner, Frickenhausen, Germany) according to Buters et al. [39]. Bovine serum albumin was added to 400 μl of each extract, resulting in 0.1% w/v extracts. Samples were lyophilized and stored at 4°C until further analysis. Bet v 1 levels were determined using a sandwich ELISA with two Bet v 1-specific antibodies (MAK 3B4F11D6 and 2E10G6G7, Allergopharma Joachim Ganzer KG, Reinbek/Hamburg, Germany) in a 96-well maxisorb microtiter plate (Nunc, Wiesbaden, Germany) with 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma, Deisenhofen, Germany) as substrate. A standard curve with affinity-purified Bet v 1 (Allergopharma Joachim Ganzer KG) and a positive control were run in duplicate with each. Only values within the linear part of the calibration curve were reported.

Migration Assay

Aqueous pollen extracts were prepared as described previously [25]. The chemotactic activity of aqueous pollen extracts from rural and urban sites was evaluated by measuring neutrophil migration through a 5-μm pore polycarbonate filter in 24-well transwell chambers (Corning Costar, Cambridge, Mass., USA). Briefly, neutrophils of nonatopic donors were added to the top chamber and suspended in complete RPMI plus 0.5% BSA at 1×10^6 cells/ml. After 1 h of incubation at 37°C with 5% CO_2, cells that had transmigrated into the lower chamber were recovered and counted with a FACSCalibur (Becton Dickinson, Heidelberg, Germany) for 60 s at a flow rate of 60 μl/min. Results are displayed as the migration index, which represents the ratio between cells migrated to the lower chamber in the presence of the chemotactic substance and cells migrated to the medium alone. In order to equalize interindividual differences, all migration indices were standardized to the positive control leukotriene B_4 1×10^{-9} M. Student's t test was performed to evaluate significant ($p < 0.05$) differences between the chemotactic activity of rural and urban pollen extracts.

Results

Genetic Analysis of the Birch Trees

Multilocus molecular data from AFLPs were used to investigate the presence of genetically identical trees (clones) in the dataset. The three AFLP primer combinations produced 191 characters (polymorphic bands). The topology of the neighbor-joining tree indicated the presence of two major groups: one containing reference *B. pendula* trees, and one containing *B. pubescens* reference trees. Based on the location in the neighbor-joining tree relative to the reference samples, we concluded that 1 tree of the urban site was from the species *B. pubescens* while

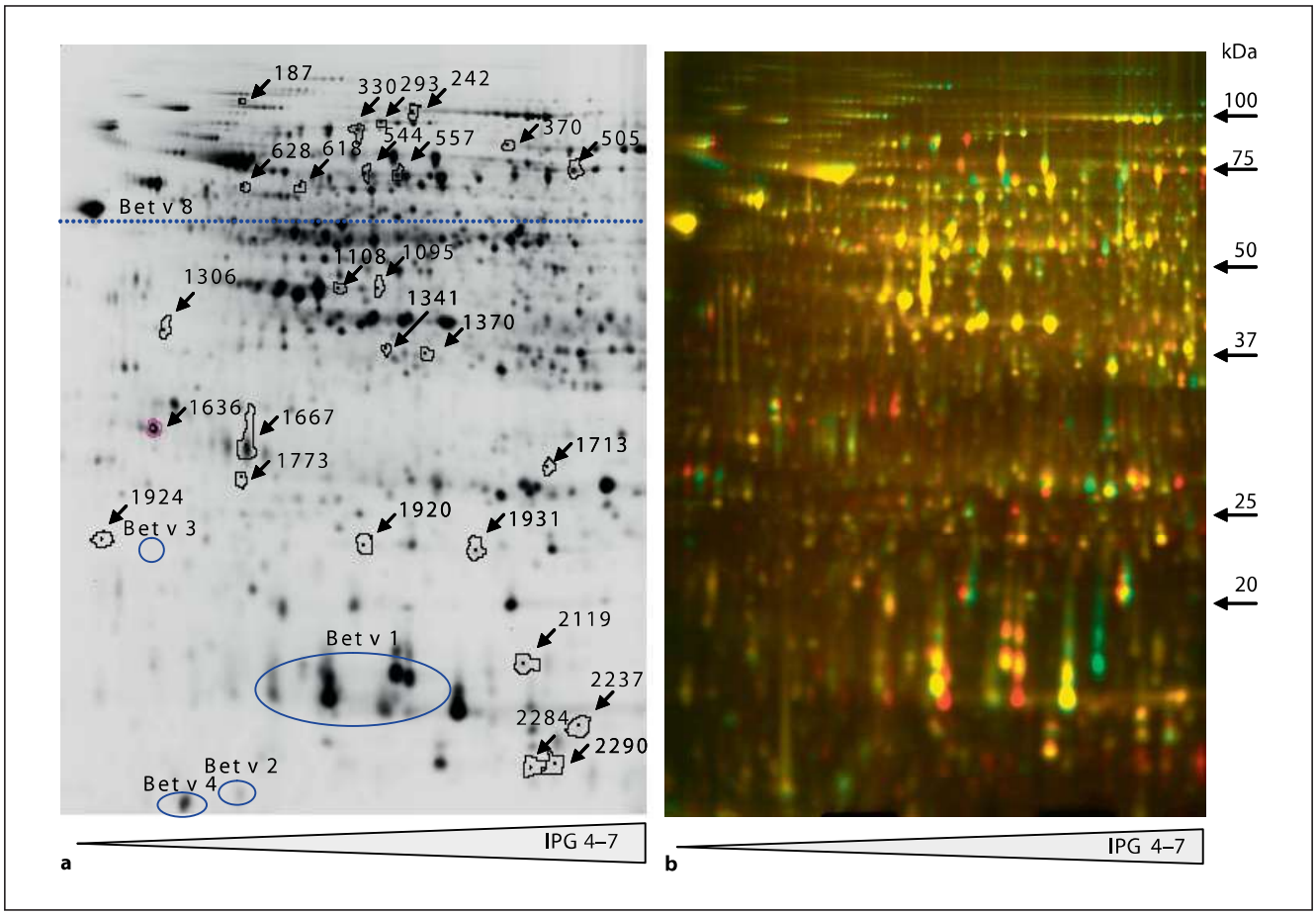

Fig. 1. Analysis of differential protein expression in rural and urban birch pollen extracts by 2D DIGE and DeCyder gel analysis. **a** Numbered protein spots show significant differences in their abundance at the $p \leq 0.05$ level (Student's t test) between pollen from rural and urban areas (n = 4). The regions in which the protein spots for the birch allergens Bet v 1, Bet v 2, Bet v 3, Bet v 4, and Bet v 8 are expected (according to p*I* and MW by the p*I*/M_r tool of ExPASY) are enclosed by blue circles. **b** 2D image of an overlay from a urban sample (red) versus a rural sample (green). Yellow spots are equally abundant in both samples.

all others were *B. pendula* trees. None of the trees were genetically identical (clones), and therefore all trees were included in the analysis. Genetically identical clones in one group could have been considered as a confounding factor because a genetically evolutionary adaptation could not be excluded. Since all the trees had different genetic backgrounds and none of the pollen samples showed very low or high values in all studied parameters, we considered all to qualify as suitable samples.

Characterization of the Birch Sites

The analysis of emission levels using passive samplers revealed that the rural areas had significantly lower nitrogen dioxide concentrations compared to the urban areas (rural: mean 7.46 mg/m^3 ± 0.73, n = 4; urban: mean 23.17 mg/m^3 ± 1.21, n = 4). The traffic score was high in urban areas (mean 11.25 ± 0.96) and low in rural areas (mean 2 ± 2). The significant positive correlation (r = +0.94, p = 0.001) between nitrogen dioxide and traffic scores confirms the validity of the traffic score.

In order to exclude the influence of climate conditions during the preceding winter and early spring prior to birch flowering and pollen harvest (mid and second half of April 2003), we compared the weather conditions at the end of 2002 and at the beginning of 2003 at the urban and rural sites. Daily mean, minimum and maximum

air temperatures were highly correlated between the two sites (r_{mean} = 0.995, r_{min} = 0.989, r_{max} = 0.989, all $p < 0.0001$), although the sums of temperatures (summation above a 0°C threshold from Jan. 1 to May 31), especially for minimum temperatures (e.g. 1,680.7 vs. 1,584.2°C T_{max}, 567.3 vs. 421.4°C T_{min}), were higher at the urban site. Mean monthly temperatures of February and March differed by 1.0/1.8°C (T_{mean}), 0.1/0.9°C (T_{max}), and 2.1/2.0°C (T_{min}). The amount of precipitation measured at the two sites correlated well (r_{prec} = 0.655, all $p < 0.0001$); in early spring, the rural site received slightly less precipitation (e.g. February 34.1 vs. 29.9 mm).

Differentially Expressed Proteins in Pollen from Urban and Rural Sites

The introduction of 2D DIGE technology simplified comparative proteome studies and enabled highest reproducibility [40]. More than 2,000 protein spots of the birch pollen proteome were detected in each gel image; out of these, 1,226 spots were matched in all 12 images, providing the first partial proteome map for birch pollen. A significance level of $p \leq 0.05$ for Student's t tests for spot comparisons with DeCyder software revealed 26 differences in protein spot intensity between rural and urban samples (fig. 1, table 1). Twelve out of 26 protein spots were more intense in the urban pollen extracts (spots No. 330, 505, 1095, 1108, 1306, 1341, 1636, 1931, 2119, 2237, 2284, 2290, with average ratios ranging from 1.14 to 7.83; fig. 2, table 1), while 14 protein spots were less intense (spots No. 187, 242, 293, 370, 544, 557, 618, 628, 1370, 1667, 1713, 1773, 1920, 1924, with average ratios ranging from –1.29 to –6.96; fig. 2, table 1). Figure 2a gives graphic examples for de novo sequenced spots. The expression levels of spots No. 505, 544, 557, 1341, 1370, 1636 and 1667 were significantly different ($p \leq 0.05$, Student's t test) in pollen samples from rural and urban areas. All spots having an average ratio of >2 or <2 are depicted in figure 2b, c. Interestingly, the allergens Bet v 1 (a steroid carrier protein), Bet v 2 (a profilin), Bet v 3 (a calcium-binding protein) and Bet v 4 (a procalcin), whose positions in the 2D gel were obtained by calculating their theoretical isoelectric points (p*I*) and molecular weights (MWs) (ExPASY), showed no differences in protein expression in our study (fig. 1a; Bet v 1 p*I* = 5.39, MW = 17.44 kDa; Bet v 2 p*I* = 5.02, MW = 14.25 kDa, Bet v 3 p*I* = 4.6, MW = 23.14 kDa; Bet v 4 p*I* = 4.76, MW = 9.45 kDa). The minor allergens Bet v 6 (an isoflavone reductase-like-protein) and Bet v 7 (a cyclophilin) with their theoretical p*I*s of 7.8 and 8.7 focus beyond the pH range employed in our study. For Bet v 8 (a pectin esterase), no amino acid sequence has been published so far. However, the size of Bet v 8 was reported as 65 kDa and at that MW no regulated protein spots are situated on our gels (fig. 1a). The major allergen Bet v 1 has many different isoforms, and therefore the encircled region is larger than for the other allergens. To confirm the data on the major birch allergen Bet v 1, its allergen content was established by ELISA according to Buters et al. [39]. The analysis of pollen samples collected in rural areas compared to urban areas revealed no differences in Bet v 1 content ($p = 0.15$, Student's t test; mean of urban samples, Bet v 1: 4,578.8 ± 2,653.6 ng/10 mg pollen, n = 4; mean of rural samples, Bet v 1: 2,087.2 ± 1,466.5 ng/10 mg pollen, n = 4).

Protein Identification

Seven protein spots with differential expression levels between rural and urban sites ($p \leq 0.05$, Student's t test) and high protein abundance were de novo sequenced. Out of these, 3 proteins were upregulated [spots No. 505 (average ratio 1.67), No. 1341 (4.47) and No. 1636 (1.14)], while the other 4 were downregulated in pollen from urban sites [spots No. 544 (average ratio –4.11), No. 557 (–6.96), No. 1370 (–4.69), and No. 1667 (–1.29)]. Mass-spectrometric analysis provided internal peptide sequences of the proteins in these spots (table 1). The homology search using BLAST against SwissProt database/NCBI-nr identified spot No. 1636 as a 14-3-3 protein (accession No. P93208) with a probability-based Mowse score of 157 (scores greater than 28 are significant at $p \leq 0.05$). For the other 6 selected protein spots (spots No. 505, 544, 557, 1341, 1370 and 1667), internal peptide sequences could be obtained (table 1), but the homologies of the deduced peptides to known proteins were not sufficient for confident protein identification. Spots No. 544 and 557 had similar sizes and peptide sequences, and might represent isoforms of the same protein.

Chemotactic Activity of Pollen Extracts on Human Neutrophil Granulocytes

Aqueous pollen extracts are known to induce chemotaxis in human granulocytes [25, 26]. In order to investigate whether urbanization impacts on the pollen's chemotactic activity, aqueous pollen extracts were tested in a neutrophil-migration assay. In this assay, pollen from urban areas exhibit a significantly higher chemotactic activity compared to pollen from rural areas ($p = 0.027$ at 3 mg pollen/ml; fig. 3).

Table 1. Summary of the comparative study of rural and urban pollen extracts by 2D DIGE and DeCyder gel analysis

Spot No.[1]	Peptide sequences	p*I*/MW, kDa (experimental)	p (t test)	Average ratio urban/rural
187	n.d.	5.07/110	0.0048	–1.35
242	n.d.	5.83/100	0.043	–1.38
293	n.d.	5.69/97.92	0.046	–1.40
330	n.d.	5.58/93.75	0.015	7.83
370	n.d.	6.26/85.42	0.0046	–1.82
505	WANFTLEEQAK VLDVAEDVCAR LDKLDYA	6.58/75.0	0.00036	1.67
544	WVGSGAGS SVMNDREFLGQNDTLYAHSLR	5.62/73.3	0.0055	–4.11
557	WENTNDASLFGK CGNVEALHNSEYR NVVFLDGGMKG WVGSGAGSGSGAGFR FLGNQDTLYAHSLR	5.77/72.73	0.014	–6.96
618	n.d.	5.32/69.89	0.0087	–1.90
628	n.d.	5.07/69.89	0.0099	–1.36
1095	n.d.	5.67/46.75	0.012	1.45
1108	n.d.	5.5/46.10	0.048	1.25
1306	n.d.	4.69/40.25	0.015	1.24
1341	VATVSLPR PLVGEVGLDQTK	5.71/38.3	0.0038	4.47
1370	IRLEEK LGMAPTIPGARQLVNHR PFPYK	5.89/37.0	0.0096	–4.69
1636 14-3-3 protein	EENVYMAK IISSIEQKEESR AAQDIANTELAPTHPIR	4.65/31.33 theoretical 4.72/28.88	0.011	1.14
1667	NFAYEKATETLR ATGDL	5.08/30	0.018	–1.29
1713	n.d.	6.45/25.37	0.018	–1.70
1773	n.d.	5.07/25.3	0.014	–1.42
1920	n.d.	5.62/23.5	0.0055	–2.64
1924	n.d.	4.31/23.5	0.012	–3.20
1931	n.d.	6.14/23	0.0082	1.20
2119	n.d.	6.36/18.59	0.032	1.38
2237	n.d.	6.61/16.48	0.047	1.26
2284	n.d.	6.39/15	0.046	2.50
2290	n.d.	6.5/15	0.025	3.01

The results show spot number of master gel, peptide sequences of de novo sequenced spots deduced by MS/MS and their experimental, observed p*I*s and MWs (ExPASY), Student's t test (significance $p \leq 0.05$), average ratio urban/rural.

[1] Positions on master gel are shown in figure 1a. n.d. = Not determined.

Discussion

We analyzed the influence of urbanization on birch pollen grains while specifically focusing on the impact of traffic emission on allergen carriers. To investigate the direct effect of traffic emissions on allergen carriers, we analyzed the allergenicity of birch pollen in two ways, namely by looking at the proteome and at the proinflammatory potential of aqueous pollen extracts.

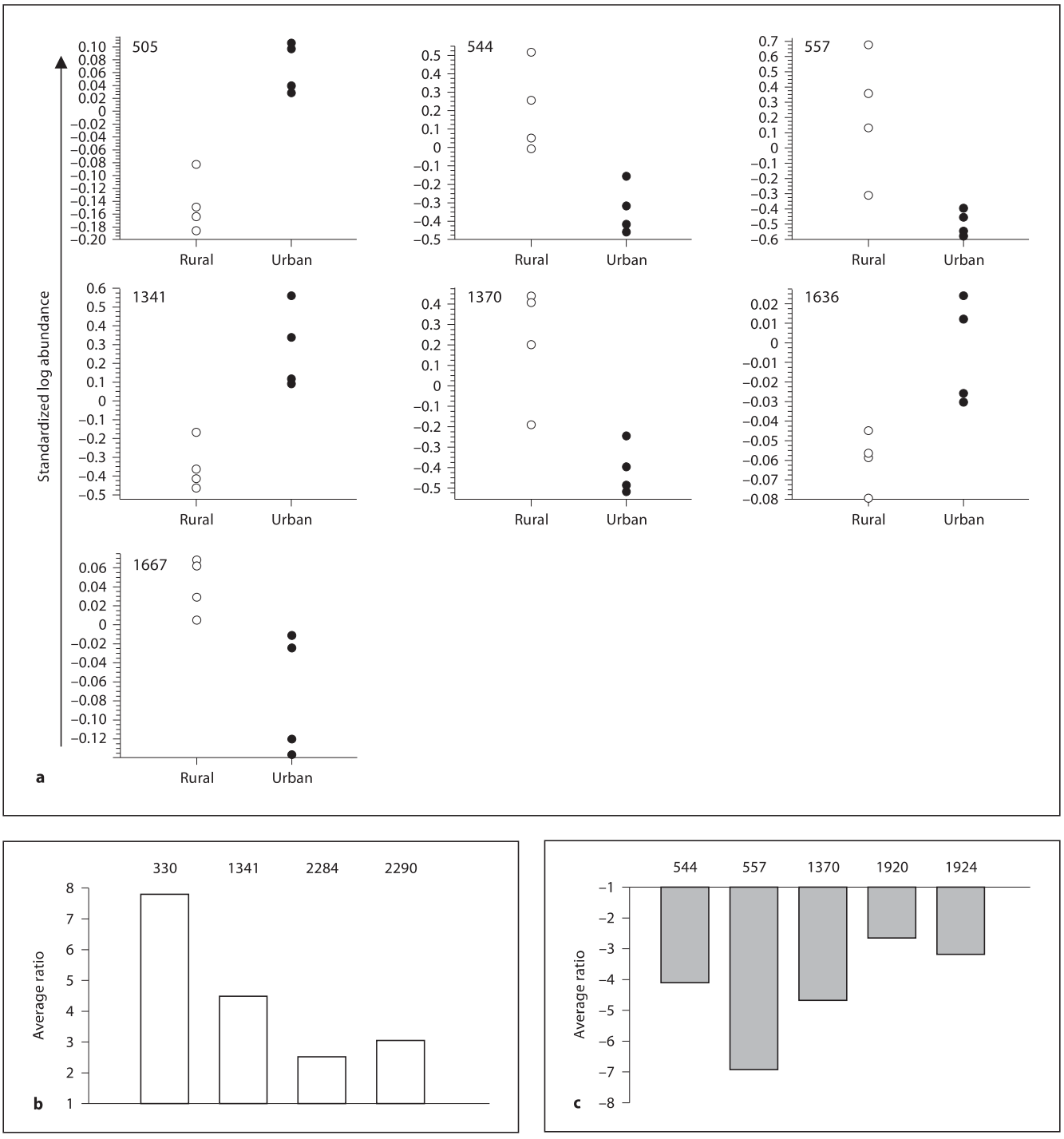

Fig. 2. Detailed spot analyis. **a** De novo sequenced spots – protein expression levels of pollen collected from rural areas compared to pollen collected from urban areas. The differences between the two groups are significant at the 0.05 level (Student's t test) (y-axis optimal display for each spot). **b** All upregulated spots in pollen collected from urban areas with an average ratio >2. **c** All downregulated spots in pollen collected from urban areas with an average ratio <2.

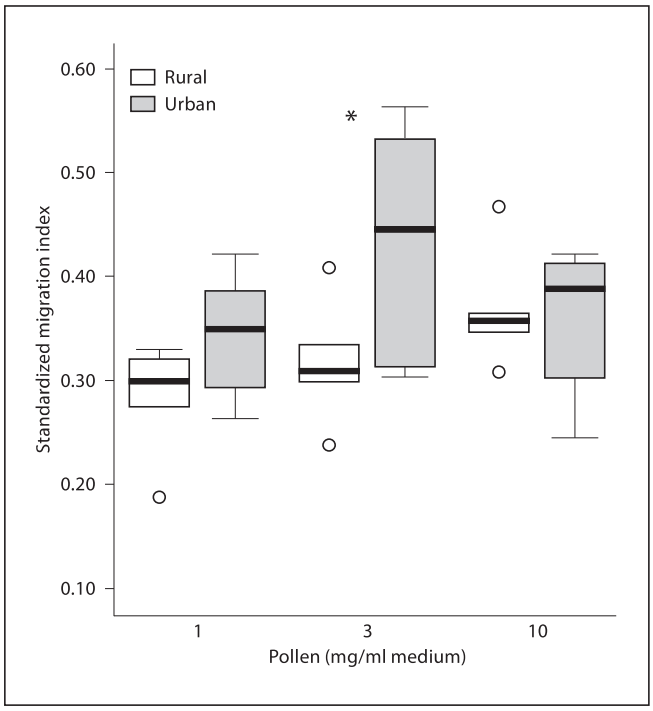

Fig. 3. Migratory response of human neutrophil granolucytes to birch pollen extracts. Comparison of the chemotactic activity of pollen extracts from urban areas (gray, n = 8) compared to pollen from rural sites (white, n = 6). Box and whiskers plot: 50% of all values are displayed in the box. The bottom line of the box is the 25% percentile the upper line of the box is the 75% percentile. The bold line is the median. The whiskers represent the highest or lowest value, which are not declared as extreme values. O = Extreme value which exceeded the length of the box by a factor of 1.5 or more, * significant at the 0.05 significance level.

The introduction of 2D DIGE technology simplified comparative proteome studies and enabled highest reproducibility, resulting in unprecedented statistical confidence [40]. Therefore, this technology could also be a useful tool for allergen extract standardization. With this method, we identified 26 birch pollen proteins that were significantly down- or upregulated when comparing the pollen proteome from urban and rural areas. However, common allergens (Bet v 1, 2, 3 and 4) were not expressed differentially. Notably, pollen from urban areas released significantly more chemotactic factors in the aqueous phase than pollen from rural sites as shown by migration assays with human neutrophils.

The exact identification of all 1,200 birch pollen protein spots is not possible to date due to the limited availability of gene and protein data from birch. Therefore, we concentrated on 7 proteins with high expression levels that also showed significant differences in regulation between urban and rural samples. One significantly regulated protein was identified as a 14-3-3 protein – upregulated in pollen derived from urban areas. For the other 6 proteins that were chosen for sequence analysis, data are depicted in table 1. However, we did not detect any significant matches in the available protein data bases (Mascot Search). We used 2D DIGE, which is able to detect slight but significant differences of protein abundance [40]. This enabled us to detect protein spots (e.g. spots No. 1636 and 1667) with less than a twofold change in protein abundance, but also with more than sixfold differences between the rural and urban samples (e.g. protein spot No. 557). Spots No. 1636 and 1667 in figure 2a show much smaller differences in protein level changes than the other 5 sequenced spots (No. 505, 544, 557, 1341, 1370), but since the differences are statistically significant at the $p \leq 0.05$ level, and moreover all spots from the rural samples were lower in No. 1636 than in the urban samples and vice versa in No. 1667, the protein spots were further analyzed.

To date, allergens are the most intensively studied proteins from allergen carriers. In our study, the protein spots in the potential allergen regions for Bet v 1, 2, 3, and 4 on DIGE analysis, which were deduced by calculating their theoretical p*I*s and MWs (ExPASY), do not show any differences in protein expression. The Bet v 1 content, as determined by ELISA, showed no differences between the rural and the urban groups ($p = 0.15$, Student's t test). Earlier studies that have compared the bioavailability of allergens from pollen grown in polluted or nonpolluted areas provided no conclusive results. The amount of Lol p 5 in *Lolium* pollen was higher in urban compared to rural areas [41]. Cortegano et al. [42] also showed that *Cupressus arizonica* pollen collected in close proximity to roads contained higher amounts of the allergen Cup 3 compared to pollen collected from gardens, which was confirmed by a study from Suárez-Cervera et al. [43]. Similar results were reported by Majd et al. [44] for *Canna indica* pollen. On the contrary, Behrendt et al. [45] showed that Phl p 5 was more abundant in pollen collected from rural areas compared to urban areas. In the same line, Chehregani et al. [46] showed that *Zinnia elegans* extracts from pollen collected in polluted areas exhibited lower protein contents while inducing more severe skin prick test reactions in guinea pigs. This supports the hypothesis that the higher allergenicity of pollen grains from urban areas is not only due to changes in abundance of allergens, but is also influenced by other pollen components.

Nonallergenic proinflammatory and immunomodulatory substances from pollen such as pollen-associated lipid mediators (PALMs) and NADPH oxidases are discussed as adjuvant factors that promote allergic sensitization and exacerbation [21, 23, 25, 27]. In order to investigate the allergenicity of pollen grains also with respect to proinflammatory factors, we performed migration studies with human neutrophils. Notably, aqueous pollen extracts from urban pollen led to a significantly higher induction of migration compared to pollen extracts from rural sites. This in turn implies a higher proinflammatory potential of urban pollen with a higher release of neutrotactic factors from pollen harvested in urban sites. As demonstrated before, proinflammatory LTB_4 like PALMs are generated from linolenic and linoic acid both by autoxidation and enzyme activity of lipoxygenases [27]. Notably, the 14-3-3 protein, which was upregulated in urban pollen, interacts with lipoxygenases in other species [47, 48]. In general 14-3-3 proteins are 30-kDa, phosphoserine/phosphothreonine-binding proteins. They regulate the activities of a wide array of target proteins and are involved in signal transduction and in primary metabolic pathways, via direct protein-protein interactions. Given that under natural exposure conditions PALMs act as adjuvants in allergic reactions, our findings of a higher release of neutrotactic factors from pollen at urban sites could contribute to explain the higher prevalence rates of allergy in urban areas. Further studies are needed to establish the relationship between the upregulation of the 14-3-3 protein in pollen exposed to high pollution levels, the activity of 13-lipoxygenase and the release of PALMs.

In conclusion, the reported connection between urbanization and allergy appears to be a complex puzzle, and there is currently no simple explanation or unique hypothesis that would be applicable. However, the present study suggests a direct impact of air pollution on allergen carriers, which might contribute to the urban-rural gradient of allergy prevalence, and gives evidence that allergenicity is determined by more than the sole allergen content.

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