

Development of electron spin resonance radical immunoassay for measurement of airborne orchard grass (*Dactylis glomerata*) pollen antigens

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Received: 6 August 2007 / Accepted: 20 November 2007
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Abstract We have developed a highly sensitive method for the measurement of airborne orchard grass (*Dactylis glomerata*: Dac g) pollen antigens using an electron spin resonance (ESR) radical immunoassay. In this immunoassay, the lowest detectable level of Dac g antigen in a sample is 0.1 arbitrary unit; the amount of Dac g antigen in single pollen grains was found to be as 1.84 units. Thus, Dac g antigens can be detected in amounts of 1/20th of that contained in the grain. This immunoassay enables early detection of grass pollen antigens. Such

information may be useful for patients with grass pollinosis, especially for those who show symptoms when only low levels of the pollen antigens are present in air. In this study, minor amounts of Dac g antigen (cross-reactive antigens) were detected in late March, after which the levels gradually increased. The levels were detected to be 10 units/m³ until the middle of May and then increased after blooming of orchard grass. High levels were maintained until the middle of June. Some patients who suffer from grass pollinosis show symptoms in late April and early May, when the airborne Dac g antigen levels were found to be 5–10 units/m³.

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Keywords Airborne pollen · Allergen ·
ESR radical immunoassay · Orchard grass ·
Japanese cedar

Abbreviations

BCA	Bicinchoninic acid
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BSA	Bovine serum albumin
CNBr	Cyanogen bromide
Dac g	<i>Dactylis glomerata</i>
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal bovine serum
ESR	Electron spin resonance
HRP	Horseradish peroxidase
MONALISA	Monitoring Network of Allergen by Immuno-Sampling

NHS	N-hydroxysuccinimide
PBS	Phosphate-buffered saline
RSI	Relative signal intensity
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPT	Skin prick test

1 Introduction

The number of airborne pollen grains is typically counted morphologically using microscopy. Pollen information is derived from these data. However, because grass pollen shares similar morphological characteristics, it is difficult to discriminate between species of pollen grains using light-microscopic pollen counts. It is known that species of grass pollen antigenicity differ with each species of grass pollen, and the pollen antigens of some grasses are not cross-reactive with those of other grass species (Esch 1999; Martin et al. 1985; Weber and Nelson 1985). Fortunately, most grasses associated with pollinosis in our region are cross-reactive species belonging Poöideae. In addition, submicron-size particles bearing pollen antigens such as starch granules (Spieksma et al. 1991) exist other than as antigens from the pollen itself. Such submicron-size particles have been isolated from pollen grains under wet conditions (Suphioglu et al. 1992) and are also considered to be the causative agents of pollinosis.

To obtain more information about the pollen, it is desirable to measure antigens that are cross-reactive with grass pollen. We applied an immunochemical technique for the quantification of airborne grass pollen antigens. A major problem in this context is that only small amounts of these antigens are present in air, and a highly sensitive analytical technique is required for such measurements. Recently, an electron spin resonance (ESR) radical immunoassay was developed as a highly sensitive method for detecting hepatitis B surface antigen (Matsuo et al. 1998; Aoki et al. 2002). In our previous study, we have shown that the sensitivity of this immunoassay is 10- to 100-fold higher than that of the conventional enzyme-linked immunosorbent assay (ELISA) (Aoyama and Takahashi 2004; Takahashi et al. 2007).

In this study, we used the ESR radical immunoassay to quantify airborne grass pollen antigen.

Orchard grass pollen antigen was chosen as the target, because this species is universally distributed throughout our region and is considered to be the most common species contributing to total regional airborne grass pollen antigens.

2 Materials and methods

2.1 Sampling and antigen extract

A cyclone sampler, CM 90 (Burkard Manufacturing, Rickmansworth, UK) was installed at Iwanami in Yamagata City, and airborne pollen antigens were collected in 1-ml tubes. No large community of grasses is present in any direction within 500 m of the sampling site. The tubes were replaced and samples were collected at 6:00 daily. The antigens in the airborne samples were extracted with 100 µl of 0.125 M ammonium bicarbonate in 0.1% bovine serum albumin (BSA) for 2 h at room temperature. The scratch extract of *Dactylis glomerata* pollen (B3SFV2) (Torii Pharmaceutical, Tokyo, Japan) was used for the standard solution, and we defined the concentration of the scratch extract as 100,000 arbitrary units/ml. Dac g content in single pollen grains was determined by extraction of a reference pollen provided from International Biological (Piedmont, OK) with 300 µl of 0.125 M ammonium bicarbonate overnight at 4°C. After centrifugation, the aliquot was used to measure Dac g antigen, and the pellets were used to count the number of Dac g pollen grains.

2.2 Preparation of horseradish peroxidase (HRP)-conjugated antibody

Antibodies against Dac g antigens were prepared with Japanese white rabbits. Two rabbits were immunized subcutaneously with 400 µg (200 µg each rabbit) Dac g antigen from Greer Laboratories (Lenoir, NC) mixed with Freund's complete adjuvant. Boosts were given at 4, 8 and 12 weeks after the first injection and small bleeds were taken a week after each boost to check the antibody response. A large bleed was taken a week after the final boost (Kane and Banks 2000). Antisera were precipitated with 35% saturated ammonium sulfate, and they were dialyzed with 0.125 M NaCl and 0.05 M phosphate-buffered saline (PBS) at

pH 7.0. The ammonium precipitates were then passed through protein A column to purify IgG fraction. A portion of the IgG fraction was further purified with the antigen-combined column, and the purified samples were eluted with 10 mM glycine-hydrochloric acid at pH 2.0. The eluate was immediately replaced with PBS (pH 7.0). The final protein concentration of the refined antibody against Dac g was 0.5 mg/ml as measured using the BCA protein assay kit (Pierce, Rockford, IL). The antigen-combined column was made using Dac g antigen (Funakoshi, Tokyo, Japan) reacted with CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). The refined antibody was conjugated with horseradish peroxidase using peroxidase labeling Kit-SH (Dojindo Molecular Technologies, Tokyo, Japan).

2.3 Electron spin resonance (ESR) radical immunoassay for Dac g antigen

Dac g antigen for SPT extract (Torii Pharmaceutical) was diluted 2,000-fold with PBS. The protein concentration of the Dac g antigens measured with BCA protein assay kit (Pierce) was 2.75 mg/ml. One hundred μ l of the diluted antigen was put in a 96-well plate (Nunc, Kamstrupvej, Denmark), and the samples were reacted for 6 h at 4°C. After three washes with ultra pure water, 370 μ l Stabilguard (SurModics, Eden Prairie, MN) was placed in each well, and the reaction was blocked overnight at 4°C. The plate were washed with ultra-pure water, dried in a desiccator, and kept at 4°C until use. Measurements were carried out as follows. Seven different concentrations of Dac g standard solution (5, 10, 20, 40, 100, 200, and 400 units/ml) were prepared prior for each measurement. One hundred μ l of the standard solution or 100 μ l of PBS containing 2% BSA was placed in each well of Dac g antigen-coated plate, and then 0.125 M ammonium bicarbonate (30 μ l) or an airborne sample (30 μ l) was added to each well. Then, 50 μ l HRP-conjugated antibody against Dac g diluted with 10% fetal bovine serum (FBS) and 0.1 M PBS was added to each well, and the solutions were mixed thoroughly. Each sample was usually put in a single well, and in some noticeable cases, samples were measured in duplicate. The seven standard solutions were measured each time. The plate was left for 2 h at room temperature or left overnight at 4°C. After

several washes in washing solution, 150 μ l of 4 mM *p*-acetamidophenol and 0.34 mM 1-hydroxy-2,2,5,5-tetramethyl-3-imidazoline 3-oxide and 0.01% hydrogen peroxide were added to each well and reacted for 1 h at 37°C. The enzyme reaction was stopped with 50 μ l sodium azide (100 mM). The amount of nitroxide radical (stable radical) produced as a result of the enzyme reaction was measured with an ESR device (FR30, JEOL, Tokyo, Japan) at a center field of 336.1 ± 5 mT. Details of the ESR measurement technique have been reported elsewhere (Matsuo et al. 1998; Aoki et al. 2002).

2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The pollen protein samples were analyzed by SDS-PAGE (12.5% acryl amide concentration; Bio-Rad, Hercules, CA). Prestained protein molecular weight standards-high (Life Technologies, Tokyo, Japan) was used as a molecular weight standard. Electrophoresis was performed at a constant voltage of 12 V for 1 h. After electrophoresis, the gel was placed on a nitrocellulose membrane, and the antigens were transferred onto the membrane. The gels were stained with Coomassie-blue G-250. The membrane was blocked with PBS in 5% BSA overnight at 4°C, then the samples were reacted with 500-fold diluted biotinylated antibody against Dac g for 2 h. Antibody against Dac g was biotinized with long-arm NHS biotin (Vector Laboratories, Burlingame, CA) (Abdul-Ahad and Brett 2000). After three washes with PBS, the samples were reacted with 500-fold diluted alkaline phosphatase conjugated streptavidine (Vector Laboratories). Finally, the Dac g antigen bands were visualized with BCIP/NBT substrate (KPL, Washington, DC).

3 Results

3.1 Dac g reacts with the antibody against Dac g

The Dac g antigen from Greer laboratory that was used for immunization showed five clear bands in SDS-PAGE, with approximate molecular weights of

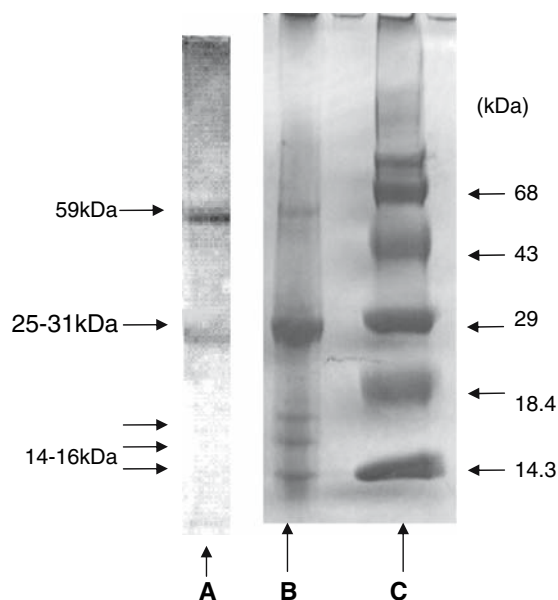


Fig. 1 Determination of molecular components of Dac g extract analyzed by SDS-PAGE and antibody against Dac g specificity by Western blotting. A: Antibody against Dac g analyzed by Western blotting. B: Molecular components of the Dac g extract analyzed by SDS-PAGE. C: Molecular weight markers: lysozyme (14.3 kDa), β -lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), and albumin (68 kDa)

59, 25–31, 16, 15, and 14 kDa. The 25–31 kDa band was broad and dense. The 59 and 25–31 kDa bands reacted with the antibody against Dac g. Three bands of low molecular weights, 14, 15, and 16 kDa, did not react with the antibody against Dac g (Fig. 1). Therefore, the antibody against Dac g contained antibodies against 59 and 25–31 kDa antigens.

3.2 Standard curve of ESR radical immunoassay

A standard curve was obtained using ESR radical immunoassay, and the results are shown in Fig. 2. The vertical axis indicates the relative signal intensity (RSI) (%); the horizontal axis indicates Dac g concentration expressed as unit/ml. The RSI (%) was calculated from the following equation.

$$\text{RSI}(\%) = \frac{\text{Signal intensity of a standard Dac g}}{\text{Signal intensity of a zero standard (without Dac g)}} \times 100$$

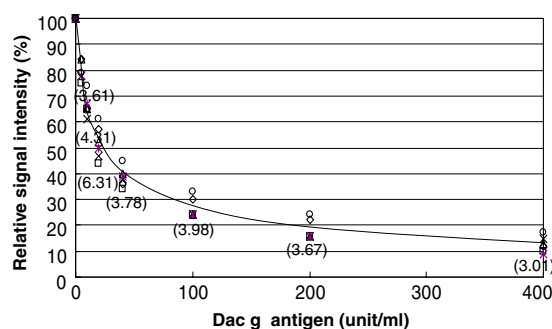


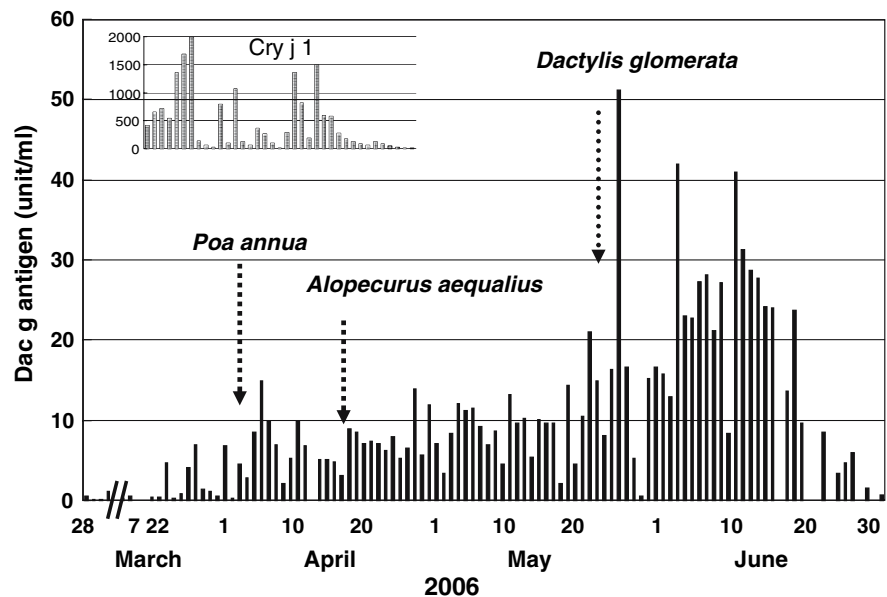
Fig. 2 Relationship between Dac g levels and relative signal intensity determined by ESR radical immunoassay. Seven concentrations (5, 10, 20, 40, 100, 200, and 400 units/ml) of Dac g were measured five times, and the RSI (%) was plotted on the vertical axis. A regression curve is derived from the mean value of the five measurements. Standard errors are shown in parentheses

The standard error of RSI (%) was in the range of 3.0–6.3% (mean 4.1%, $n = 6$) (Fig. 2), and therefore we defined a 90% intercept value of RSI to the zero standard signal intensity as the detection limit in this study. A zero standard means maximum binding capacity of the system in this case. The 90% intercept value of the signal intensity was approximately 3.5 units/ml. On the other hand, the amount of Dac g in single pollen grains was found to be 1.84 units/grain (1.38–2.24 units/grain, $n = 8$) based on a reference pollen obtained commercially.

3.3 Measurement of airborne Dac g antigen

The level of airborne Dac g antigens was examined during the period of 22 March and 30 June in 2006 (Fig. 3). Minor amounts of cross-reactive antigens to Dac g were already detected in late March, and the levels gradually increased thereafter. The levels were found to be 10 units/m³ until the middle of May. The Dac g levels increased after the blooming of orchard grass, and a high level of Dac g antigen was maintained until the middle of June. Symptoms of some grass pollinosis patients had already begun in

Fig. 3 Daily fluctuation of airborne Dac g measured by ESR radical immunoassay during the period from 7 March to 30 June 2006. Arrows indicate the flowering time of relevant grasses near the sampling site. Daily fluctuation of airborne Cry j 1 level is shown. The horizontal scale is adjusted for comparison



late April, when the airborne Dac g levels were fluctuating between 5 and 10 units/m³.

The relationship between the daily amount of airborne Cry j 1 (a major pollen allergen from Japanese cedar pollen) and that of Dac g was examined, and no relationship between the two values was observed (between 22 March and 10 May, $r = -0.1828$, $n = 50$).

4 Discussion

We have developed a highly sensitive method for measuring airborne Dac g antigens using ESR radical immunoassay. The 90% intercept value was calculated as 3.5 units/ml as described in Sect. 3. Each measurement needs 30 μ l extract; therefore, a level of Dac g in excess of 0.1 unit in the sample is detectable. The Dac g content in single pollen grains of *D. glomerata* was determined as 1.84 units. Thus, the amount of Dac g that could be detected was as low as 1/20th of that contained in single grains. Using this method, the Dac g antigen was detectable during the early stage of the grass pollen season. This approach is expected to provide useful information for grass pollinosis patients, especially for those who show symptoms at times when only a low level of antigen is present in air. Data are available 2½ h after sampling, namely, 30 min extraction (Takahashi

et al. 2001), 60 min antibody reaction and 60 min radical reaction. We could use the above treatment times without sensitivity loss on the occasion of the pollen allergen information. It is possible to supply the pollen allergen information to local residence through mass media until evening time on the same day.

Also, we have been providing information about Cry j 1 antigen in airborne pollen during the pollen season since 2005. It is well known that some patients displayed symptoms several weeks before airborne Japanese cedar pollen has been detected by microscopy. No airborne Cry j 1 has been detected using the conventional ELISA during this times. The development of the ESR radical immunoassay has now made it possible to conduct such measurements, and it has been reported that some patients show their symptoms during a period in which airborne Cry j 1 levels fluctuating between 1 and 3 pg/m³. Such information could be useful for the patients whose symptoms begin early in the flowering season (Takahashi et al. 2007). Now we have been supplying airborne Cry j 1 information through internet and local TV at the pollen season.

Low levels of Dac g were present during the latter half of the Japanese cedar pollen season. Japanese cedar pollinosis is the most common in Japan, and more than 10% of Japanese suffer from it. Some patients who suffer from the pollinosis during this

season think that Japanese cedar pollen is their causative pollen, but it is clarified from this study that Dac g pollen was also detected in air at that time. It has been reported that some grass pollinosis patients show symptoms in late April and early May in our region (Takahashi et al. 1987), and this time corresponds to the period when early flowering species of grasses start to bloom. It is likely that the symptoms of these patients are provoked not only by Japanese cedar pollen, but also by grass pollen. Interestingly, no positive correlation was observed between the amounts of Cry j 1 and Dac g antigen in air, samples of which were collected during Japanese cedar pollen season. Dac g and Cry j 1 do not have any cross-reactive antigens. The comparison was made because we want to examine whether they appear in air simultaneously or independently controlled under different meteorological conditions, and further whether there are more patient symptoms in connection with the appearance of these antigens.

Airborne samples were taken using Burkard Cyclone sampler in this research. The manufacturer of the sampler announced that the collection efficiency of the sampler is 90% in 1- μ l range particles; that is to say, allergens existed as minute particles under 1 μ l, and gas-shaped particles could not be sampled. In Europe, the MONALISA (Monitoring Network of Allergen by Immuno-Sampling) project was initiated in 2005. The aim of the project is to characterize pollen allergens such as Poaceae, *Betula*, *Ambrosia*, *Artemisia*, *Cupressus*, *Parietaria*, and *Olea* pollens for the benefit of pollinosis patients. Several sampling methods were examined in the MONALISA project. According to Rantio-Lehtimäki of Turk University, samplings into the liquid were diluted, and ELISA results were not reliable (personal communication). The advantage of the Cyclone sampler is that allergens in large volumes of air could be collected in a very small quantity of extraction medium (at least 50 μ l). Therefore, we chose the cyclone sampler for the study.

In this research, we used an antibody against Dac g antigen, because *D. glomerata* is the most widely distributed species in our region and is considered to be the main species contributing to the total airborne grass pollen antigens in this region. A number of studies have been conducted to analyze *D. glomerata* pollen antigens (Esch and Klapper 1989; Cuerin-Marchand et al. 1996; Roberts et al. 1993;

Leduc-Brodard et al. 1996; van Oort et al. 2001). The extent of cross-antigenicity among grass antigens remains difficult to estimate. The antibody used in this study recognized antigens with molecular weights of 59 and 27–35 kDa. It is likely that 59 kDa antigen is Dac g 4, and 27–35 kDa antigen is Dac g 1 and/or Dac g 5. According to Esch (1999), Dac g 5 has a wide molecular weight distribution and is cross-reactive within pollen antigens from Poöideae. Therefore, Dac g 5 was considered to be one of the suitably defined antigens for our purpose.

As for antibody, several categories of antibodies are available for the airborne antigen measurements, e.g., antibodies created from crude antigen extracts, antibodies created from defined antigens, monoclonal antibodies, and polyclonal antibodies. There are no commercial products for antibodies against grass pollen antigens in Japan. First of all, we must prepare antibodies against grass pollen antigens. Monoclonal antibodies are easy to manage concerning quality control as indicated by the example that the MONALISA project has selected them. However, we prepared polyclonal antibodies because we want to use antibodies that have wide specificities related to local grass pollinosis, and it can be made easily. There is, certainly, a problem with quality control among preparations. However, we do not worry about this matter concerning the standpoint of pollen allergen information, because outstanding sensitivity of the ESR method made it possible to dilute the HRP-conjugated antibody to 10,000-fold. So, we can use the same preparation for many years. Addition to this, we think suitable antibody will differ from place to place as discussed in the next paragraph.

Some patients show symptoms before the flowering of *D. glomerata*. The amounts of airborne allergens causing grass pollinosis can be roughly estimated to quantify the airborne Dac g levels, because the antibody used in the present study reacts not only to Dac g pollen antigens, but also to grass pollen antigens cross-reactive with Dac g from other plants of the Poöideae subfamily. Thus, the airborne grass pollen antigens observed before *D. glomerata* flowering season that bloom until late May (Takahashi et al. 1993) may not have been Dac g itself, but may have been other species having cross-reactive antigens with Dac g antigen. Two early blooming species of grasses in our region are *Alopecurus aequalis* and *Poa annua*. However, early blooming species are not

limited to the above-mentioned species; for example, *Anthoxanthum odoratum* blooms prior to the major grass pollen season in some areas in Japan (Sudo et al. 2005). It would be desirable to investigate antibodies with a broad range of specificity against major allergens related to local grass pollinosis. Moreover, it would be helpful to examine antibodies in immunization with a mixture of grass pollen antigens from different species. Antibodies suitable for such studies will be those against the most important species in a particular region, and we think the suitable antibody will differ from place to place. Further research is still needed to clarify the differences among applied antibodies and to identify the appropriate antibodies for the measurement.

Acknowledgement We would like to thank to Dr. Takao Akatsuka, Professor of Emeritus of Yamagata University and President of Yamagata College of Industry and Technology for his helpful comments.

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