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Original Article

Evidence of a novel allergenic protein Narcin in the bulbs of *Narcissus tazetta*

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Abstract: Several plant-derived allergens have been identified which result in the formation of immunoglobulin E antibodies. Primarily, these allergens belong to the protein families including seed storage proteins, structural proteins and pathogenesis-related proteins. Several allergens are also reported from flower bulbs which cause contact dermatitis. Such symptoms are highly common with the bulb growers handling different species of *Narcissus*. *Narcissus* toxicity is also reported if the bulbs are consumed accidentally. The present study aimed to characterize the protein from the bulbs of *Narcissus tazetta* responsible for its allergenic response. A 13 kDa novel allergenic protein, Narcin was isolated from the bulbs of *Narcissus tazetta*. The protein was extracted using ammonium sulfate fractionation. The protein was further purified by anion exchange chromatography followed by gel filtration chromatography. The N-terminal sequence of the first 15 amino-acid residues was determined using Edman degradation. The allergenicity of the protein was measured by cytokine production using flow cytometry in peripheral blood mononuclear cells. Further estimation of total IgE was performed by ELISA method. This novel protein was found to induce pro-inflammatory cytokines and thus induce allergy by elevating total IgE level. The novel protein, Narcin isolated from *Narcissus tazetta* was found to exhibit allergenic properties.

Keywords: Allergen, *Narcissus tazetta*, cytokines, IgE, ELISA, flow cytometry

Introduction

Plants are a source of some of the major allergens which act as small antigens and result in allergenic response by production of specific antibody immunoglobulin E (IgE) antibodies [1-3]. Such antigens normally enter the body at very low doses by diffusion across mucosal surfaces and trigger allergenic responses [4] namely allergic rhinitis, rhinoconjunctivitis, allergic asthma, contact dermatitis etc. [5-8]. The most widespread groups of plant allergens that are reported belong to the superfamilies of prolamin, cupin and plant defense system [9-12]. Hemagglutinins and lectins are also known to have allergenic effects through their interaction with IgE and histamine release [13-15]. Apart from these, several allergens from plant flower bulbs are also responsible for toxicity (type I hypersensitivity) and contact dermatitis (type IV hypersensitivity) [16-18]. Development of tulip fingers are due to the allergen tulipalin A [19]. IgE-mediated asthma, rhinoconjunctivitis, and contact urticaria are reported from tulip and Easter lily [18].

Narcissus, from the family of *Amaryllidaceae*, has been also known to cause contact dermatitis (lily rash) in many individuals [20, 21]. In addition to this, strong animal and human toxicity including diarrhea, vomiting and cough have been observed when the bulbs were mistakenly ingested [22-24]. The cause of allergenicity in these plants has been poorly characterized. So far, alkaloids, masonin and homolycorin have been shown to be responsible for some irritant properties [21]. IgE mediated allergy has been reported from flowers of *Amaryllidaceae* family including *Narcissus* [25].

In the present study we have isolated, purified and characterized Narcin, a 13 kDa protein from *Narcissus tazetta* with potent allergenic properties. The N-terminal sequence of the pro-

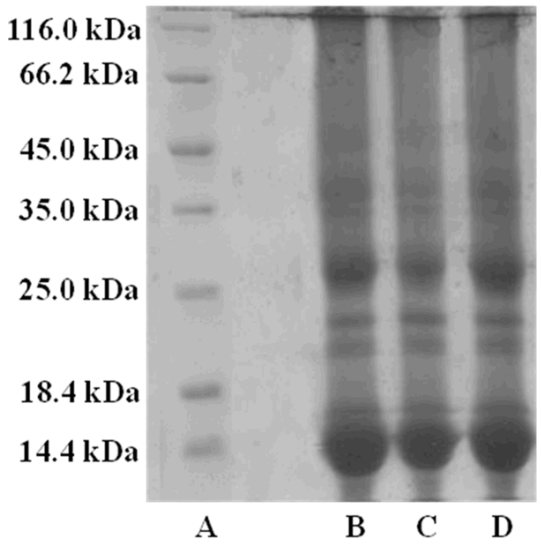

Figure 1. SDS-PAGE showing proteins present in extracts of *Narcissus tazetta* after ammonium sulphate precipitation. Lane A is protein molecular weight marker and Lane B, C, and D are showing protein bands in extract from *Narcissus*.

tein was determined. Our studies using flow cytometry and ELISA have shown this protein to induce production of cytokines and IgE in peripheral blood mononuclear (PBMC) cells respectively.

Materials and methods

Purification of Narcin

Samples of the underground bulbs of *Narcissus tazetta* were obtained from local nurseries. The bulbs were cut into small pieces and pulverized in the presence of liquid nitrogen in a ventilated hood. The pulverized samples were mixed in extraction solution containing 0.2 M sodium chloride and 50 mM sodium phosphate buffer pH 7.2 and stirred for 24 h at 277 K. 2.5 g polyvinylpyrolidine (PVP) was mixed with 100 ml of the above solution and was further homogenized. The homogenate obtained was centrifuged at 277 K at 5000 g for 30 min. The protein was precipitated with 80% saturated ammonium sulfate. The precipitated protein was mixed in 50 mM sodium phosphate buffer pH 7.2. The proteins present in the solution were examined in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (**Figure 1**). The protein solution was loaded onto a DEAE-Sephadex A-50 column (50 × 2 cm) equilibrated with 50 mM sodium phosphate buffer pH 7.2. The proteins were eluted using a continuous gradient of 0.0-0.5 M NaCl in the same buffer in which two peaks were collected and pooled separately. The second peak containing the low molecular weight protein was loaded onto a Sephadex G-50 column (150 × 1 cm) equilibrated with 25 mM Tris-HCl, pH 8.0 and proteins were eluted using the same buffer. The fractions corresponding to a molecular mass of 13 kDa were pooled, collected, dialyzed and lyophilized. The purity of the sample was checked by SDS-PAGE.

N-terminal sequencing

The protein was further used for N-terminal protein sequencing using PPSQ21A automatic protein sequencer (Shimadzu, Japan) in order to identify the protein.

Allergenicity test

In this study, a total of peripheral blood samples from 5 healthy donors with no history of atopy were analyzed. All peripheral blood samples were collected in heparinized vial and immediately processed for the analysis of cytokine production in response to Narcin. All samples were obtained with the approval of the local Ethical Committee after informed consent had been given by the donor.

Isolation of peripheral blood mononuclear cells from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll Hypaque gradient centrifugation and suspended in complete RPMI-1640 (Caisson Laboratories, Logan, UT) supplemented with 2 mM Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% heat inactivated fetal calf serum as described previously [26]. The viability of cells was measured by trypan blue dye exclusion test and was greater than 97%. These cells were used for in vitro culture.

Cell culture and flow cytometry

We performed in vitro stimulation of freshly isolated PBMCs (0.5×10^6 /ml) to measure cytokine production with or without the protein (10 µg/ml) in presence of 10 µg/ml of Brefeldin A, a Golgi transport inhibitor (Sigma-

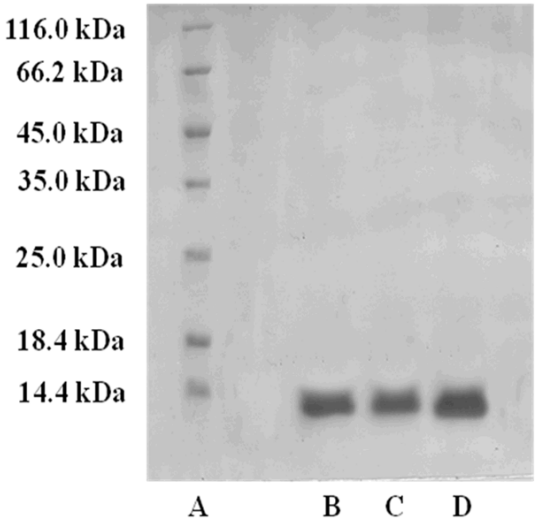

Figure 2. SDS-PAGE showing purified 13 kDa protein from *Narcissus tazetta*. Lane A is protein molecular weight marker and Lane B, C and D are showing Narcin, a 13 kDa purified protein from *Narcissus* after gel filtration chromatography.

Aldrich, St Louis, MO, USA) for cytokine analysis and without Brefeldin A for IgE estimation. After 24 hrs, culture cells were washed and surface stained with anti-CD4 (Biolegend, San Diego, California, USA) followed by intracellular staining for by interferon-γ (IFN-γ), interleukins, IL-10, IL-4 and IL-13 (BD Bioscience). In all cases, negative control samples were incubated with irrelevant isotype-matched mAbs (BD Bioscience) in parallel with experimental samples. Stained cells were run in BD FACS Calibur (BD Biosciences) and subsequently analyzed by FlowJo software (Tree Star Inc., Ashland, OR).

Total IgE estimation

Total IgE levels in the supernatants of stimulated cells (PBMCs, 0.5 × 10^6/ml) were measured with an ELISA as described previously [27, 28]. Total IgE estimation was carried out in culture supernatant of each normal control by enzyme-linked immunosorbent assay (ELISA) using human IgE ELISA Quantitation Set (Bethyl Laboratories, Inc, UK). Monoclonal anti-IgE was coated into wells as per manufacturer's instruction. After washing, culture supernatant diluted 1:2 (v/v) with the zero buffer was incubated for 30 min at 25°C. Followed by washing, horse radish peroxidase conjugated anti-human IgE was added and incubated for 60 min. The color was developed by adding tetramethyl benzidene (TMB). The enzyme reaction was stopped with 100 µl of 0.18 M sulphuric acid. Absorbance was measured at 450 nm using Dynatech ELISA reader.

Statistical analysis

Statistical significance of results was determined using Prism 5 software (GraphPad, La Jolla, CA). Data are expressed as mean ± S.D. Differences were considered significant at $P < 0.05$.

Results

Purification and identification of Narcin

The protein, Narcin was purified using ammonium sulfate precipitation followed by anion-exchange chromatography in accordance with the acidic nature of the protein. A single band corresponding to an approximate molecular weight of 13 kDa was obtained after the final SDS-PAGE analysis of the protein samples after gel filtration chromatography (**Figure 2**). The sequence of the 15 N-terminal amino-acid residues was determined to be 1 Ala-Asn-Ile-Leu-Asn-Ser-Ile-Leu-Pro-Ala-Tyr-Asn-Leu-Pro-Phe 15. Since this sequence did not show exact identity to any known protein, Narcin was characterized as a novel plant protein.

Allergenicity test

To obtain the best conditions for stimulation in freshly isolated PBMCs derived from peripheral bloods of healthy donor in our study, we performed a dose and kinetic response of our novel protein for cytokines (IFN-γ) secretion by flow cytometry. We found 24 hour of culture and 10 µg/ml dose of Narcin were optimal for the cytokine response. PBMCs (0.5 × 10^6 /ml) were cultured for 24 hours with Golgi bodies transport blocker in presence Narcin (10 µg/ml) and with media alone (complete RPMI and Golgi bodies transport blocker (10 µg/ml)). Isotype controls have been used to confirm the specificity of primary antibody binding and to avoid any nonspecific antibody binding. Cultured cells were washed and surface stained with anti-CD4 and followed by IFN-γ, IL-10, IL-4 and IL-13 intracellular staining.

We first examined frequency of IFN-γ and IL-10 production by CD4+ T cells in response to stimulation by Narcin (**Figure 3A**). Upon Narcin stim-

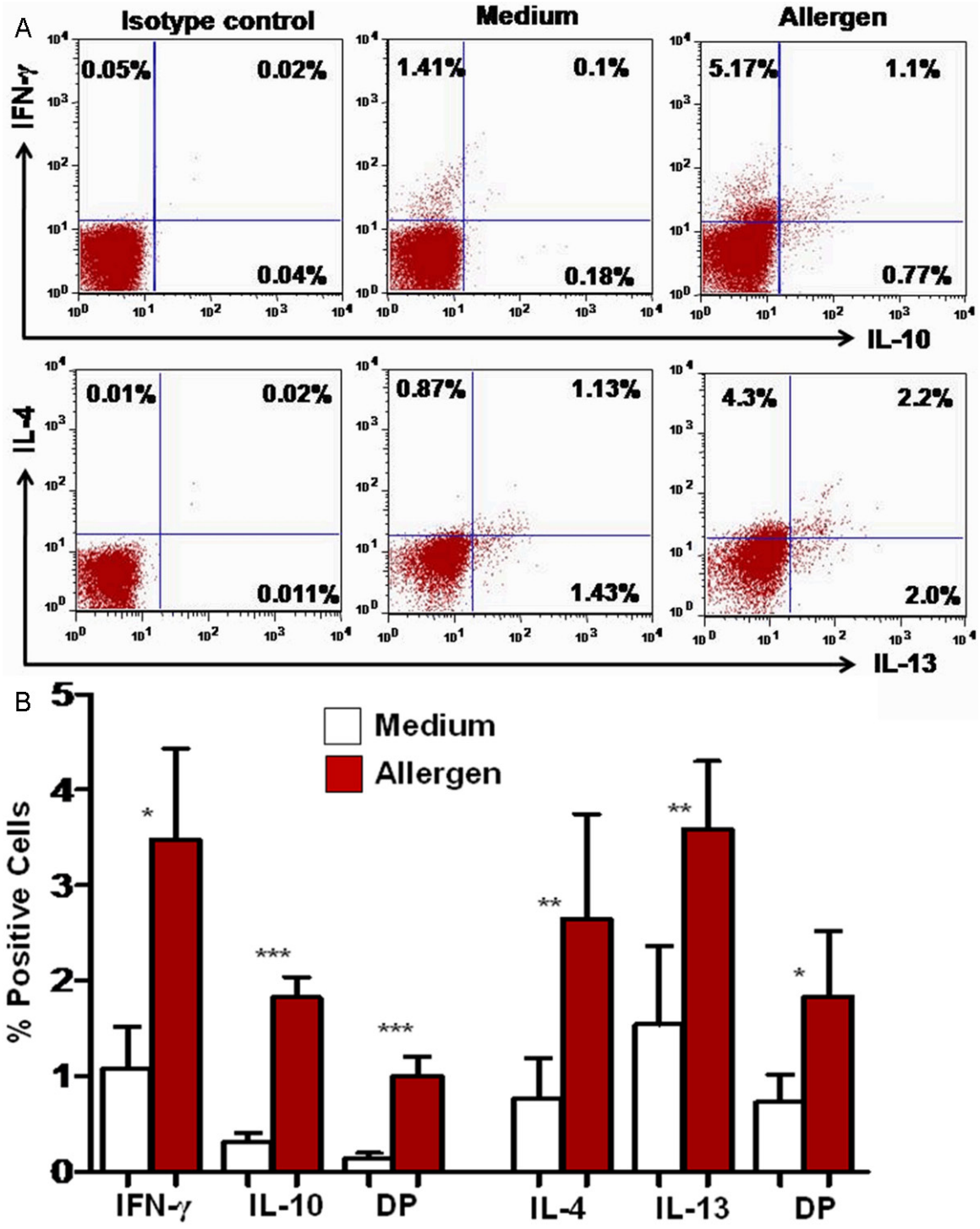

Figure 3. Effect of Narcin on cytokine production. A: Representative FACS plot showing percentage production of IFN-γ, IL-10 (upper panel) and IL-4 and IL-13 (lower panel) on gated CD4+ T cells. B: Data presents the mean ± S.D. for five individual experiments (n = 5). *Represents $P \leq 0.05$, **represents $P \leq 0.005$ and ***represents $P \leq 0.0005$. DP is double positive (Both IFN-γ and IL-10 or IL-4 and IL-13) CD4 T cells.

ulation, the frequency of IFN-γ was 3.4 ± 1.9 (p = 0.043) compared to un-stimulated cells producing IFN-γ at a frequency of 1.1 ± 0.43 (**Figure 3B**). The percentage of IL-10 positive cells was also found to be 1.8 ± 0.21 (p = 0.0001) when stimulated by the protein in con-

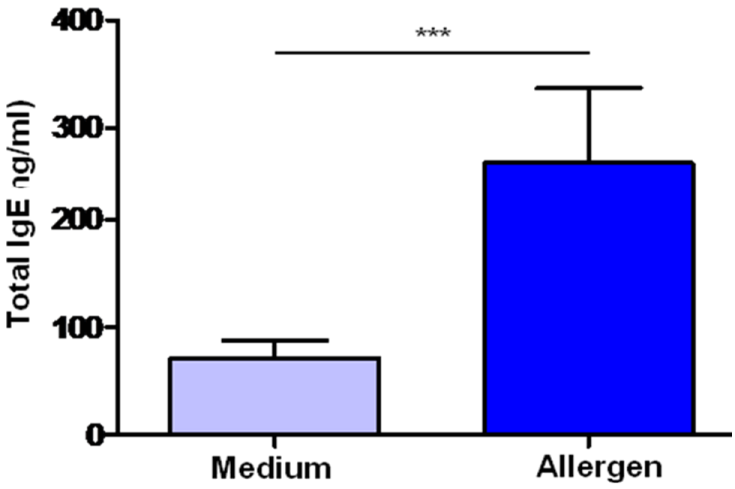

Figure 4. Effect of Narcin on total IgE levels. Addition of protein (10 μg/ml) significantly increased the level of IgE. Data shown is mean ± S.D. for 5 individual experiments in triplicate (n = 5). Statistical analysis was based on non parametric Wilcoxon rank sum test for paired samples. *Represents P ≤ 0.05, **represents P ≤ 0.005 and ***represents P ≤ 0.0005.

trast to 0.31 ± 0.08 in un-induced cells (**Figure 3B**). We also observed a profound increase in the frequency of dual cytokines (DP = both IFN-γ and IL-10) producing CD4+ T cells in response to our novel allergen.

Since CD4+ T cells are the major sources of IL-4 and IL-13 in allergenic disease developments, we analyzed Narcin induced production of IL-4 and IL-13 cytokines (**Figure 3A**). A considerably high percentage of IL-4 positive cells of about 2.6 ± 1.2 (p = 0.0043) and IL-13 of about 3.5 ± 1.4, (p = 0.0047) producing CD4+ T cells was observed in cells stimulated with Narcin (**Figure 3B**). The respective values in un-stimulated cells were found to be 0.71 ± 0.41 for IL-4 and 1.51 ± 0.51 (p = 0.001) for IL-13 (**Figure 3B**). In addition to this, the frequency of IL-4 and IL-10 dual cytokine producing CD4+ T cells were also increased significantly after stimulation with Narcin.

Since increased allergen induced IL-13 secretion is most strongly associated with increased levels of IgE, we also measured the total IgE levels in cultured supernatant of healthy control PBMCs by ELISA. We found a substantial increase in the levels of total IgE in response to this allergen (10 μg/ml) (**Figure 4**). The entire study subject showed an increase in IgE levels in Narcin stimulated cells by about 3.7 folds (p = 0.0001) compared to un-stimulated cells.

Discussion

We have identified the allergenic properties of Narcin, a novel plant protein, isolated from the species of *Narcissus tazetta*. While *Narcissus* poisoning is common when ingested [20], the exact cause of the allergenicity has not been clearly identified. Narcin, isolated and purified from the bulb of this plant, could be a potent allergen. Our results indicate that this novel plant-derivative induces pro-inflammatory cytokines and also elevates the total IgE level. Our data supports that IgE synthesis is controlled by cytokines, particularly by IL-4 and IL-13 [29].

Allergen specific T cells produce IL-4 and IL-13 and both have been shown to be potent switch factors for IgE synthesis in human B cells [30]. The specific IgE produced in response to the allergen binds to the high affinity receptor for IgE on mast cells, basophils and activated eosinophils [31]. The tendency of IgE over-production is influenced by genetic and environmental factors. Once IgE is produced in response to an allergen, exposure to the allergen triggers an allergenic response [32]. Thus our data suggest a role of this novel protein isolated from *Narcissus tazetta* in evoking allergenic response. The proteins explaining contact allergy by Narcin (type IV hypersensitivity) probably still waits to be studied.

Commonly, pharmacotherapy like use of antihistamines and topical corticosteroids that control the symptoms of allergenic conditions can only target the clinical symptoms but not the underlying immune mechanism [33, 34]. Specific immunotherapy vaccination and development of new anti-allergenic drugs are some of the potential approaches towards the treatment [35, 36]. However, these extracts consisting of allergenic and non-allergenic components can themselves induce severe anaphylactic side-effects upon therapeutic administration [37]. Thus, there is an urgent need to develop novel drugs against these allergenic proteins to control their allergenic response. So it is necessary to characterize these allergens in order

to develop potential drugs against these proteins.

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Disclosure of conflict of interest

None.

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