

RESEARCH ARTICLE

Class A dioscorins of various yam species suppress ovalbumin-induced allergic reactions

Ching-Chi Yang and Kuo-Chih Lin

Department of Life Science & Institute of Biotechnology, National Dong Hwa University, Hualien County, Taiwan, R.O.C

Abstract

Context: Dioscorins, the primary storage proteins in yam tubers, of different species exhibited varying immunomodulatory activities in mice. We inferred that this might be attributed to the various isoforms in the yam tubers.

Objective: We aimed to investigate the antiallergic potential of the Class A dioscorins of various yam species using the ovalbumin (OVA)-induced murine allergy model.

Materials and methods: We purified the recombinant Class A dioscorins (rDioscorins) of various yam species from *Escherichia coli* and evaluated their antiallergic potential by enzyme-linked immunosorbent assay.

Results: The Class A rDioscorins of various yam species suppressed allergic reactions by significantly decreasing the serum IgE and histamine levels. The serum IFN- γ and IgG2a levels significantly increased in all rDioscorin-treated mice. The splenocytes of the rDioscorin-treated mice also exhibited upregulated IFN- γ secretion in response to ConA stimulation. By contrast, the serum IL-5 levels decreased to basal levels in mice treated with Class A rDioscorins and the amount of IL-5 produced by splenocytes decreased in response to ConA stimulation.

Discussion: The Class A rDioscorins suppress allergic reactions, possibly through modulating an imbalanced Th1/Th2 immune response to OVA by promoting Th1 cell responses. Furthermore, the Class A rDioscorins of various yam species exhibited similar immunomodulatory activities in OVA-sensitized mice, which were different from the activities demonstrated by native dioscorins, suggesting that distinct immunomodulatory effects of native dioscorins on mice were attributed to the various isoforms in the yam tubers.

Conclusion: The Class A dioscorins of various yam species exhibit antiallergic activity and are potential immunotherapeutic agents for treating IgE-mediated hypersensitivity.

Keywords

Allergy, herbal medicine, histamine, interferon-gamma, Th1/Th2 imbalance

History

Received 27 February 2014

Revised 2 April 2014

Accepted 27 April 2014

Published online 21 May 2014

Introduction

The allergic diseases caused by type I hypersensitivity reactions include atopic disease, allergic rhinitis and bronchial asthma, comprising the most common allergies in industrialized countries. Type I allergic responses involving IgE-dependent mast-cell degranulation and eosinophil accumulation at inflamed sites are attributed to the development and activation of Th2 cells^{1–3}. The differentiation of allergen-specific Th2 cells leads to the production of IL-4 and IL-13, inducing immunoglobulin class switching to IgE production in B lymphocytes, and producing IL-5, which promotes the growth and differentiation of eosinophils, and IL-9, which enhances mast-cell differentiation^{2–4}. Allergen-binding to the mast cell- and basophil cell-bound IgE leads to the degranulation of vasoactive amines (primarily histamine), lipid

mediators (prostaglandin D, platelet-activating factors, leukotriene (LT)C₄, LTD₄ and LTE₄), chemokines (CXC-chemokine ligand 8 (CXCL8), CXCL10, CC-chemokine ligand 2 (CCL2), CCL4 and CCL5) and cytokines (IL-4, IL-5 and IL-13), causing the immediate symptoms of allergic responses^{1,2,5}.

During allergy suppression, Th1 cells produce IFN- γ and IL-2 cytokines, which suppress the production of IL-4 and IL-5 by inhibiting the proliferation and activation of Th2 cells, production of eosinophils and secretion of IgE and histamine^{1,6}. IFN- γ also suppresses the production of IgG1 and increases the production of IgG2 to suppress allergic reactions^{1,7}. Therefore, allergen-induced Th1/Th2 cell imbalance is considered responsible for the inflammation caused by allergic responses⁸.

Yam (*Dioscorea* spp.) tubers are highly nutritious and widely consumed throughout Africa and Asia. Dried slices of yam tubers are used in traditional Chinese medicine to strengthen the functions of the spleen, kidney, liver and stomach, reduce phlegm and heal fatigue, chronic diarrhea and diabetes⁹. Yam tubers contain approximately 1%–3% proteins on a dry-weight basis and dioscorins, the primary

Address for correspondence: Kuo-Chih Lin, Department of Life Science & Institute of Biotechnology, National Dong Hwa University, Hualien County, Taiwan, R.O.C. Tel: 886-3-8633643. Fax: 886-3-8633630. E-mail: kclin@mail.ndhu.edu.tw

storage proteins in yam tubers, account for approximately 85% of their total soluble protein content¹⁰. Dioscorins exhibit immunomodulatory activities. Recent studies have demonstrated that dioscorins from *D. alata* (designated as Da-dioscorins) activated Toll-like receptor-4 signaling pathway, stimulating the expression of pro-inflammatory cytokines such as TNF α , IL-1 β and IL-6 in the RAW264.7 macrophage and murine bone marrow cells^{11,12}. Moreover, Da-dioscorins enhanced the phagocytic activity of the RAW264.7 macrophage against *Escherichia coli* and stimulated the proliferation of murine splenocytes^{11,13}. Oral administering Da-dioscorins to BALB/c mice stimulated splenocytes to secrete IFN- γ , IL-4 and IL-10, increased the subpopulation in NK and B cells and the number of Peyer's patches¹⁴. Da-dioscorins and dioscorins from *Dioscorea japonica* (designated as Dj-dioscorins) were reported to protect the expression of tight junction proteins from dust mite damage in human airway epithelial cells¹⁵.

Dioscorins of various yam species contain 2 Class A and B isoforms¹⁶. Class A dioscorin is a monomer, whereas Class B dioscorin is a dimer¹⁷. Although the heterogeneity of dioscorin isoforms has been reported, the molecular nature of the heterogeneity of dioscorin isoforms in various yam tubers has not been completely elucidated until recently. Tsai et al. (2013) reported that each of six cDNA clones corresponding to the dioscorin gene family of *D. japonica* and *Dioscorea pseudojaponica* were cloned from yam tubers¹⁸. The protein sequence identity among the Class A or B dioscorins of different yam species is 87%–99%, whereas the protein sequence identity between Class A and Class B is 67%–75%. In addition, one dioscorin gene (*Da-dioA3*) encoding a Class A dioscorin of *D. alata* was isolated using RT-PCR. The results of applying 2-D gel electrophoreses followed by MALDI-TOF-MS and the peptide mass fingerprinting of peptide spots revealed that the Da-dioscorins isolated from the yam tuber of *D. alata* consisted of Class A dioscorins and the Dj-dioscorins isolated from *D. japonica* comprised primarily Class A and a small number of Class B dioscorins. By contrast, the dioscorins isolated from *D. pseudojaponica* (designated as Dp-dioscorins) consisted of a mixture of Class A and B dioscorins. Jheng et al. (2012) recently reported that the recombinant Class A dioscorins (rDioscorins) of *D. japonica* and *D. pseudojaponica* exhibit conformational structures similar to those of the corresponding native dioscorins, and thus retain their antioxidant activities and demonstrate similar levels of immunomodulatory activities; therefore, the rDioscorins purified from *E. coli* can be applied to investigate the biomedical functions of native dioscorins¹⁹.

We previously reported that the native dioscorins of different species exhibited varying immunomodulatory activities in mice¹³. Da-dioscorins stimulated more phagocytic activity in lymphoid cells than did Dj-dioscorins; however, Dj-dioscorins enhanced the proliferation of lymphoid cells more than the Da-dioscorins did. We recently reported that the native dioscorins of various yam species suppressed ovalbumin (OVA)-induced allergic reactions, possibly by modulating an imbalanced Th1/Th2 immune response; however, the Dp-dioscorins exhibited different immunomodulatory effects on OVA-sensitized mice²⁰. All the native

dioscorins suppressed the allergic reactions in OVA-sensitized BALB/c mice by decreasing the serum IgE and histamine levels. The IL-5 and IgG1 levels in Da-dioscorin- and Dj-dioscorin-treated mice were either lower or comparable to those of the positive control mice, whereas both the IL-5 and IgG1 levels in the sera of Dp-dioscorin-treated mice increased significantly. We inferred that the various immunomodulatory effects of Dp-dioscorins on mice might be attributed to the higher amount of Class B dioscorins in Dp-dioscorins compared with that in the other native dioscorins¹⁸.

In this study, we used the Class A rDioscorins of Da-dioscorins, Dj-dioscorins and Dp-dioscorins and the well-characterized OVA sensitization murine model to evaluate how Class A dioscorins suppressed allergic reactions in the OVA sensitization murine model, and examined the *in vivo* effects of Class A dioscorins on the production of IgE, histamine, the Th1 and Th2 cytokines, and the IgG2a and IgG1 antibodies. A discussion on the different immunomodulatory effects of native and recombinant Dp-dioscorins on OVA-sensitized mice was included.

Materials and methods

Experimental animals

Seven-week-old, inbred and certified, male BALB/c mice (approximately 20–25 g) were purchased from BioLASCO (Taipei, Taiwan). Mice were allowed to acclimatize for seven days upon arrival and allocated randomly into treatment groups. Mice were maintained on a 12/12 h light/dark cycles at 22 $\pm$ 2 °C and provided with food (PMI Nutrition International, St. Louis, MO) and water at the Graduate Institute of Biotechnology, National Dong Hwa University, Taiwan. All mice were housed and cared according to the “Guide for the Care and Use of Laboratory Animals” of National Dong Hwa University.

Cloning, expression and purification of rDioscorins

The *Da-dioA3*, *Dj-dioA3* and *Dp-dioA2* cDNA isolated from *Dioscorea alata*, *D. japonica* and *D. pseudojaponica* cDNA libraries, respectively, were used as templates for PCR cloning¹⁰. We designed Da-dioa forward primer (5'-GCTAGCGGCGAGGATGAGTTTAGC-3') with a *NheI* site (underlined) and Da-diob reverse primer (5'-CTCGAGTTATCATATGACACCAAGCTTCG-3') with an *XhoI* site (underlined) to amplify the *Da-dioA3* cDNA (GenBank accession no. AJ253590) encoding the mature protein rDa-dioscorinA3. We designed Dio34a forward primer (5'-GGAGCTCGTAGAGGATGAGTTTAGCTA-3') with a *SacI* site (underlined) and Dio34b reverse primer (5'-GGCTCGAGTTATATGACACCAAGCTTCG-3') with an *XhoI* site (underlined) to amplify the *Dj-dioA3* cDNA (GenBank accession no. AM849820) encoding the mature protein rDj-dioscorinA3. We designed Ecoli96a forward primer (5'-CGAGCTCGATGAGGATGAGTTTAGC-3') with a *SacI* site (underlined) and Ecoli96b reverse primer (5'-TGCTCGAGTCATATGACACCAAGCTT-3') with an *XhoI* site (underlined) to amplify the *Dp-dioA2* cDNA (GenBank accession no. GQ246172) encoding the mature

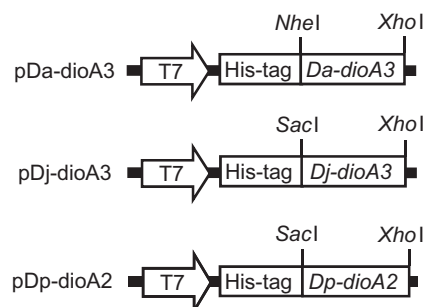


Figure 1. Constructs of pDa-dioA3, pDj-dioA3 and pDp-dioA2 expressing rDa-dioscorinA3, rDj-dioscorinA3 and rDp-dioscorinA2, respectively, as fusion with N-terminal 6xHis tag.

protein rDp-dioscorinA2. The PCR products were digested with restriction enzymes, ligated to pET-28a expression vector (Novagen, Darmstadt, Germany) and then transformed into *E. coli* BL21 StarTM (DE3). The plasmids expressing rDa-dioscorinA3, rDj-dioscorinA3 and rDp-dioscorinA2 proteins were designated as pDa-dioA3, pDj-dioA3 and pDp-dioA2, respectively (Figure 1). DNA sequences of the rDioscorin cDNA and its fusion to the N-terminal 6His-tag were confirmed by DNA sequencing analyses.

To produce large quantity of rDioscorins, overnight cultures of *E. coli* BL21 were transferred to fresh LB media (100 × dilution) containing 50 µg/mL kanamycin and cultured at 25 °C until OD₆₀₀ reached 0.4–0.6. The cultures were then induced with 0.25 mM–0.5 mM IPTG for 4 h. The cells of *E. coli* were resuspended in cold LEW buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by French press (Constant Systems, Darvenry, UK) with 13.1 kpsi. Soluble fraction was separated from cellular debris by centrifugation. Supernatants were applied to a column packed with Protino[®] Ni-IDA resin (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol to bind polyhistidine-tagged proteins. After washing the column with LEW buffer, the rDioscorins were eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The buffer of the eluate was exchanged with 10 mL phosphate-buffered saline (PBS, pH 7.4) by Vivaspin (Amersham, Buckinghamshire, UK) for four times according to the manufacturer's instructions. Endotoxin was removed by ToxinEraserTM endotoxin removal resin (GenScript, Piscataway, NJ) according to the manufacturer's protocol, which resulted in levels of 0.097–0.181 EU/mL as determined by ToxinSensorTM Chromogenic Limulus Amebocyte Lysate endotoxin assay kit (GenScript). Concentrations of the rDioscorins were determined by Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA) and confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loaded with standard concentrations of bovine serum albumin (BSA) (Sigma, St. Louis, MO).

OVA sensitization and rDioscorin injection procedures

Eight-week-old mice were sensitized with 20 µg and 50 µg OVA (Sigma, St. Louis, MO) by intraperitoneal (i.p.) injection on days 1 and 14, respectively, according to the vaccination scheme described by Hsu et al. (2013)²⁰. The OVA was mixed with 2 mg of aluminum hydroxide

(Sigma) in a total volume of 0.2 mL of PBS. Negative control mice were given i.p. injection of 0.2 mL PBS and referred as the unimmunized group. The OVA-sensitized mice were intraperitoneally injected with 2 mg/kg of rDa-dioscorinA3, rDj-dioscorinA3 or rDp-dioscorinA2 on days 7, 15, 18, 21 and 24. The OVA-sensitized mice received PBS instead of rDioscorins were served as positive controls to maintain hypersensitivity. On day 27, the mice were sacrificed by cervical spine dislocation. The body weight of the mice was 25.5 ± 1.2 g for PBS group, 25.3 ± 1.4 g for OVA group, 25.7 ± 1.1 g for rDa-dioscorinA3 group, 25.8 ± 1.7 g for rDj-dioscorinA3 group and 24.8 ± 1.2 g for rDp-dioscorinA2 group. Mice were bled from the retro-orbital venous plexus, and sera were stored at –20 °C until determination of indicated parameters.

Isolation of splenocytes from mice

After sacrifice of the mice, the spleen was immediately removed, cleaned of fat and connective tissue and placed in PBS. The spleens were individually cut into small pieces and were forced through the nylon mesh (mesh size 80 mesh) to obtain cell suspension. The splenocyte suspension was washed with PBS buffer for three times and resuspended with RPMI-1640 medium containing 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. The cell numbers isolated from the tissues were counted with hemocytometer. The cells were used for analyzing cytokine production and cell proliferation.

Measurement of serum histamine levels

The levels of serum histamine were determined by competitive direct enzyme-linked immunosorbent assay (ELISA) (Oxford Biomedical Research, Inc., Rochester, MI) following the manufacturer's protocol. Briefly, the microwells in the assay kit were pre-coated with a monoclonal antibody to histamine. The sample (1:1000 dilution) or standard solutions were first added to the antibody-coated microplate. Horseradish peroxidase-labeled secondary anti-histamine antibody was then added to the microplate, and the mixture was shaken and incubated at room temperature for 45 min. After washing the wells with 300 µL wash buffer for three times, the wells were added with 100 µL of tetramethylbenzidine (TMB) substrate. After incubating the wells for 30 min, 100 µL of stop solution (2 M H₂SO₄) was added into each well to stop the reaction. Absorbance at 450 nm was recorded with an ELISA plate reader (SpectraMax 190, Nihon Molecular Devices, Tokyo, Japan), and the actual amount of sample antibodies in sera was determined by quantifying the amount according to the reference sera.

Determination of total serum IgE, IgG1 and IgG2a levels

Blood obtained on day 27 was centrifuged at 3000 × g for 15 min. The sera were collected and stored at –80 °C for future assay. The titers of the total IgE, IgG1 and IgG2a were determined by sandwich ELISAs according to the manufacture's instruction (Bethyl Laboratories, Inc., Montgomery, TX). Briefly, 96-well plates were coated with

100 μ L purified mouse anti-IgE, anti-IgG1 or anti-IgG2a antibodies (10 μ g/mL in 0.05 M carbonate-bicarbonate, pH 9.6) for 1 h at room temperature, and the wells were washed with 200 μ L wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) for three times. The wells were then blocked with 200 μ L of blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) for 30 min and washed with 200 μ L wash buffer for three times. Reference or diluted sample sera (1:10 dilution for IgE and 1:5000 dilution for IgG1 and IgG2a) were added to the wells and were cultured for 1 h. After washing the wells with wash buffer for three times, the wells were added with 100 μ L of diluted HRP detection antibody (1:50 000 dilution for IgE, 1:100 000 dilution for IgG1 and 1:10 000 dilution for IgG2a) and incubated for 1 h. After incubation, the wells were washed with wash buffer for three times, and 100 μ L TMB substrate was added to the wells. After incubating the wells for 30 min, 100 μ L of stop solution (2 M H_2SO_4) was added into each well to stop the reaction. Absorbance at 450 nm was recorded with an ELISA plate reader (SpectraMax 190, Nihon Molecular Devices), and the actual amount of sample antibodies in sera was determined by quantifying the amount according to the reference sera.

Quantification of IL-5 and IFN- γ levels in sera or tissues

For determining the serum IL-5 and IFN- γ levels, blood obtained on day 27 was centrifuged at $3000 \times g$ for 15 min. The sera were collected and stored at -80°C for future assay. For quantifying the IL-5 and IFN- γ production in tissues, approximately 2×10^5 splenocytes/well were seeded in a 96-well plate and treated with 100 μ L RPMI-1640 medium containing 50 μ g OVA or ConA for 48 h. After incubation, the supernatants were collected and stored at -80°C for future assay. Quantification of cytokine levels were performed by using sandwich ELISA kit purchased from BioLegend (San Diego, CA) for IFN- γ and IL-5. Briefly, the plate was coated with 100 μ L (100 ng) of anti-cytokine antibody (1:199 dilution). After incubation for overnight at 4°C , the wells were washed with 200 μ L wash buffer for three times. Each well was added with 200 μ L of blocking solution and incubated for 1 h. After washing the wells for three times, the wells were cultured with 100 μ L of standard or sample (1:4 dilution) for 2 h at room temperature. After washing, the wells were added with 100 μ L of detection antibody (1 μ g/mL) and incubated for 1 h. After washing the wells, the wells were added with 100 μ L of streptavidin-HRP and incubated for 1 h. After washing the wells, the wells were added with 100 μ L TMB substrate and incubated for 30 min. The enzyme reaction was stopped by adding 100 μ L of stop solution into each well. Absorbance at 450 nm was recorded with an ELISA plate reader (SpectraMax 190, Nihon Molecular Devices), and the actual amount of cytokines was determined by quantifying the amount according to the standard.

Statistic analyses

Results of three separate experiments performed in triplicates for the *in vitro* studies or five mice ($n=5$) for each group of the *in vivo* studies were expressed as mean $\pm$ SD. The results

were analyzed using Student's *t*-test, and the statistical analyses were performed by the statistical software of GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). Differences between the positive control (OVA-sensitized mice) and other groups of rDioscorin-treated mice were considered statistically significant if $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

Results

Expression and production of rDioscorins

The *Da-dioA3*, *Dj-dioA3* and *Dp-dioA2* dioscorin genes encode the Class A dioscorins in *D. alata*, *D. japonica* and *D. pseudojaponica*, respectively¹⁸. The protein sequence identity among these genes was 92%–97%. Our previous study demonstrated that the Class A rDioscorins, rDj-dioscorinA3 (encoded by *Dj-dioA3*) and rDp-dioscorinA2 (encoded by *Dp-dioA2*), isolated from *E. coli* exhibited similar conformational structures and antioxidant and immunomodulatory activities as their corresponding native dioscorins¹⁹. To obtain Class A rDioscorins for various yam species, we cloned the *Da-dioA3* gene from *D. alata* into the expression vector pET-28a and expressed it as rDa-dioscorinA3 with an N-terminal 6xHis-tag in *E. coli* (Figure 1). After the Class A rDioscorins were purified with nickel resin and the endotoxins were removed using endotoxin removal resin, the amount and purity of the rDioscorins was assessed using SDS-PAGE (Figure 2). We routinely obtained 2–3 mg of rDa-dioscorinA3, 15–30 mg of rDj-dioscorinA3 and 4–8 mg of rDp-dioscorinA2 per liter of *E. coli* culture. We also cloned the genes encoding the recombinant Class B dioscorins of *D. japonica* and *D. pseudojaponica* into the expression vector pET-28a in *E. coli*; however, the recombinant Class B dioscorins did not form dimers in *E. coli* as expected (data not shown). Therefore, we did not use the recombinant Class B dioscorins for the following experiments.

Effects of Class A rDioscorins on the suppression of IgE and histamine production

To elucidate whether the Class A rDioscorins could suppress allergic reactions by regulating IgE production and histamine

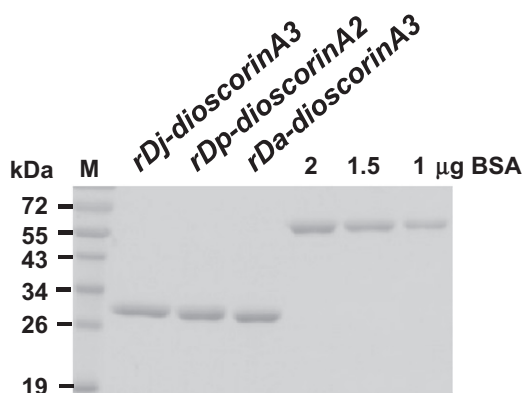


Figure 2. SDS-PAGE of the various rDioscorins purified from *E. coli*. Approximately 1.7 μ g/well of various rDioscorins were subjected to SDS-PAGE.

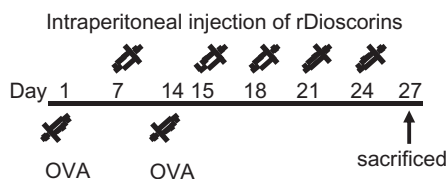


Figure 3. Experimental timeline of treatment regimes.

release, we sensitized BALB/c mice to OVA, followed by i.p. injections of various Class A rDioscorins for 27 days (Figure 3). The healthy negative mice were administered i.p. injections of PBS, whereas OVA-sensitized mice were given i.p. injections of PBS to maintain hypersensitivity and serve as the positive controls. We collected the sera from the mice 27 days after OVA immunization followed by i.p. injections with the Class A rDioscorins; the sera IgE and histamine levels were evaluated to determine whether the Class A rDioscorins suppressed OVA-induced allergic reactions. The results indicated that the IgE levels of the OVA-sensitized mice (positive controls) were 438 ± 23 ng/mL on day 27, approximately 3-fold higher compared with those of the negative control mice (132 ± 44 ng/mL), indicating that the OVA antigen induced an allergic reaction in the murine OVA-allergy model (Figure 4A). Compared with the positive controls, the OVA-sensitized mice exhibited significantly decreased serum IgE levels (224–298 ng/mL) after 27 days of i.p. injections of various Class A rDioscorins.

We also monitored the serum histamine levels of the OVA-sensitized mice injected with the Class A rDioscorins. Figure 4(B) shows that the histamine levels of the positive control mice significantly increased to 1034 ± 68 ng/mL, 3-fold higher compared with those of the negative control mice (344 ± 136 ng/mL), again indicating that the OVA antigen induced an allergic reaction in the mice. After i.p. injections with various Class A rDioscorins, the histamine levels significantly decreased to 238–560 ng/mL in all rDioscorin-treated mice.

Effects of Class A rDioscorins on the Th1/Th2 cell responses

To elucidate whether the Class A rDioscorins regulated the Th1/Th2 cell response in OVA-sensitized mice, we determined the IFN- γ (a Th1 cytokine) and IL-5 (a Th2 cytokine) levels in the sera after i.p. injections of various Class A rDioscorins. The results showed that the positive (155 ± 124 pg/mL) and negative (156 ± 40 pg/mL) control mice exhibited comparable levels of IFN- γ (Figure 5A); however, the IFN- γ levels significantly increased to 902 ± 522 , 566 ± 158 and 576 ± 263 pg/mL after the mice were treated with rDa-dioscorinA3, rDj-dioscorinA3 and rDp-dioscorinA2, respectively. These levels were 3.7–5.8 times the IFN- γ levels of the positive control mice. Compared with the negative control mice (22 ± 7 pg/mL), the IL-5 levels of the positive control mice increased 1.5-fold to 33 ± 10 pg/mL (Figure 5B). The IL-5 dramatically decreased, though statistically non-significant, to the basal level (24 – 27 pg/mL) after the mice were given i.p. injections of rDa-dioscorinA3, rDj-dioscorinA3 or rDp-dioscorinA2.

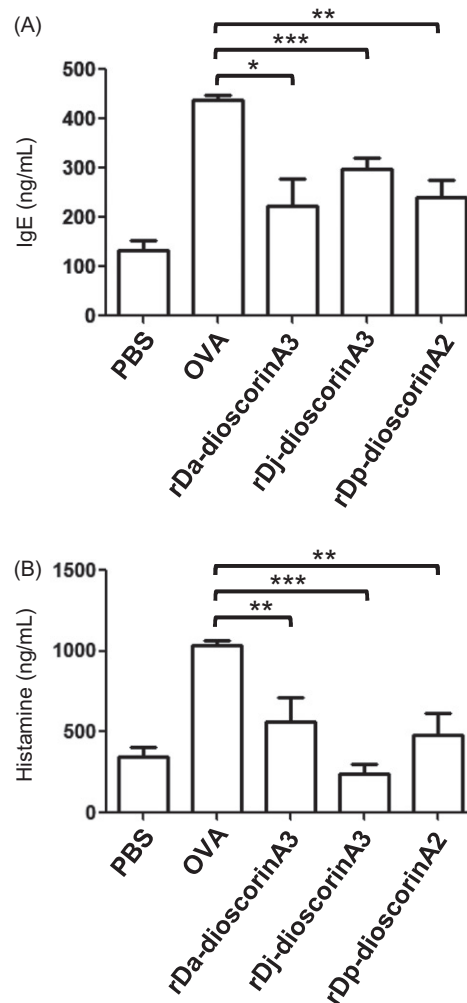


Figure 4. Effects of various rDioscorins on the production of IgE and histamine in OVA-sensitized mice. OVA-sensitized BALB/c mice were intraperitoneally injected with rDa-dioscorinA3, rDj-dioscorinA3 or rDp-dioscorinA2. The sera at 27 days after OVA sensitization were collected for measuring the levels of IgE (A) and histamine (B). Three replicates for each measurement were performed. Results represent the mean $\pm$ SD of five mice ($n = 5$). Asterisk (*) represented a significant difference of the IgE or histamine levels between rDioscorin-treated and OVA-sensitized mice if $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

To further study the Th1/Th2 cell responses exhibited by OVA-sensitized mice after i.p. injections of various Class A rDioscorins, we measured the IgG2a (secreted by Th1 cells) and IgG1 (secreted by Th2 cells) levels in the sera. Figure 6(A) shows that the serum levels of IgG2a in rDioscorin-treated mice were approximately 3- to 5-fold (351 – 603 μ g/mL) higher than those in the positive control mice were (124 ± 72 μ g/mL). By contrast, the serum levels of IgG1 in the rDioscorin-treated mice (592 – 630 μ g/mL) did not decrease, and even were higher than those in the positive control mice (519 ± 99 μ g/mL) (Figure 6B).

Effects of Class A rDioscorins on the production of IFN- γ and IL-5 by splenocytes

To elucidate the effect of Class A rDioscorins on the production of IFN- γ and IL-5 by the splenocytes of rDioscorins-treated mice, we isolated splenocytes from the negative control, positive control and rDioscorin-treated mice, re-stimulated these cells with mitogen ConA and

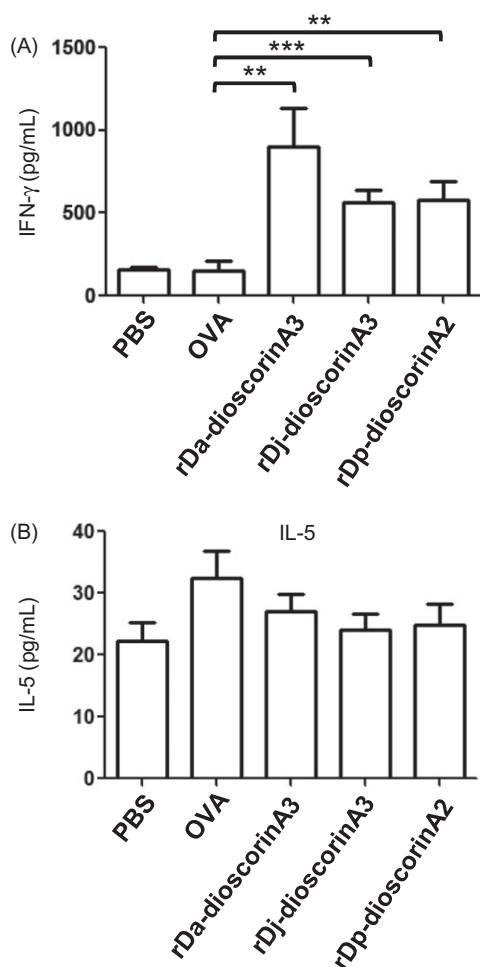


Figure 5. Effects of various rDioscorins on the production of IFN- γ and IL-5 in OVA-sensitized mice. OVA-sensitized BALB/c mice were intraperitoneally injected with rDa-dioscorinA3, rDj-dioscorinA3 or rDp-dioscorinA2. The sera at 27 days after OVA sensitization were collected for detecting IFN- γ (A) and IL-5 (B). Three replicates for each measurement were performed. Results represent the mean $\pm$ SD of five mice ($n=5$). Asterisk (*) represented a significant difference of the IFN- γ or IL-5 levels between rDioscorin-treated and OVA-sensitized mice if $p < 0.01$ (**) or $p < 0.001$ (***).

subsequently measured the IFN- γ and IL-5 levels they produced. Compared with the IFN- γ levels produced by the splenocytes of the positive control mice in response to ConA re-stimulation, those of the rDioscorin-treated mice were 17%–20% higher (Figure 7A). By contrast, although not statistically significant, the levels of IL-5 produced by the splenocytes of the rDioscorin-treated mice in response to ConA re-stimulation were 18%–24% lower than those of the positive control mice (Figure 7B).

Discussion

Type I allergic responses involve IgE-dependent mast-cell and basophil-cell degranulation and eosinophil accumulation in sites of inflammation^{1–3}. During the allergic reactions, activation of Th2 cells leads to the production of IL-4 and IL-13, which induce immunoglobulin class switching to IgE production. Binding of antigens to the mast cell- and basophil cell-bound IgE leads to degranulation of histamine. Therefore, regulating IgE production and histamine release

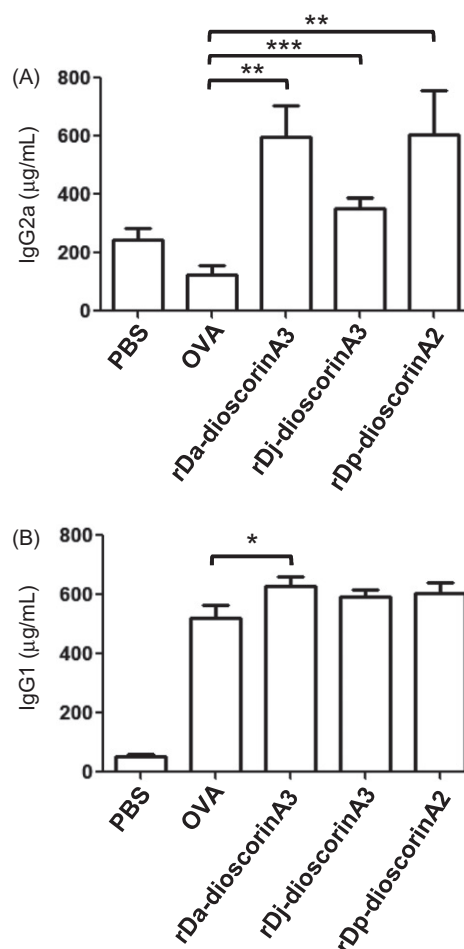


Figure 6. Effects of rDioscorins on the production of IgG1 and IgG2a in OVA-sensitized mice. OVA-sensitized BALB/c mice were intraperitoneally injected with rDa-dioscorinA3, rDj-dioscorinA3 or rDp-dioscorinA2. The sera at 27 days after OVA sensitization were collected for detecting IgG2a (A) and IgG1 (B). Three replicates for each measurement were performed. Results represent the mean $\pm$ SD of five mice ($n=5$). Asterisk (*) represented a significant difference of the IgG2a or IgG1 levels between rDioscorin-treated and OVA-sensitized mice if $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

is of clinical importance. In this study, we demonstrated that the Class A dioscorins of various yam species exhibited immunomodulatory activities for suppressing allergic reactions. The i.p. injection of various Class A rDioscorins into OVA-sensitized mice significantly reduced the serum IgE and histamine levels (Figure 4). The decrease in the IgE and histamine levels was concomitant with the significant increase in the IFN- γ and IgG2a levels in sera (Figures 5A and 6A), suggesting that the Class A dioscorins suppressed the allergic reaction by promoting the Th1 cell response. We observed that i.p. injecting Class A rDioscorins into OVA-sensitized mice reduced the IL-5 levels to the basal level in all the rDioscorin-treated mice (Figure 5B), suggesting that the Class A dioscorins suppressed the allergic reaction by down-regulating the Th2 cell response. Moreover, the production of IFN- γ and IL-5 by the spleen cells in response to ConA re-stimulation coincides with the production of IFN- γ and IL-5 in the sera of OVA-sensitized BALB/c mice in response to the Class A rDioscorin treatment (Figure 7). The combined results suggest that the Class A dioscorins suppressed the

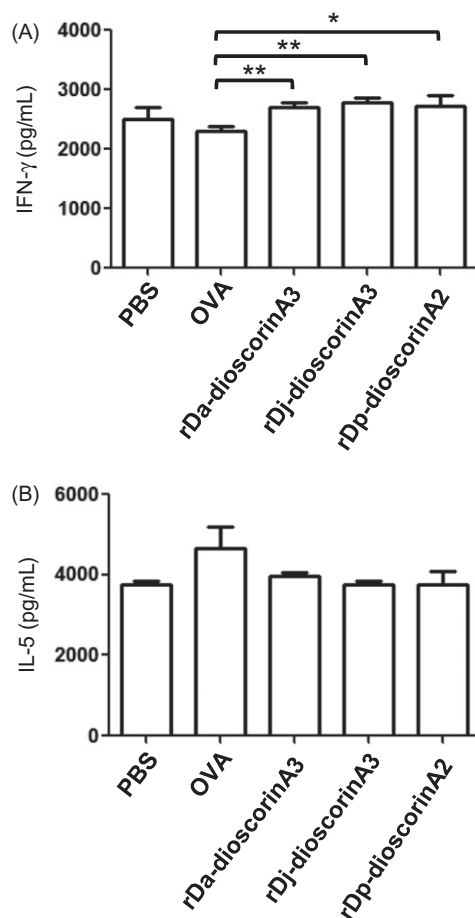


Figure 7. Effects of ConA on the production of IFN- γ and IL-5 by the splenocytes of rDioscorin-treated mice. The splenocytes isolated from the negative control, OVA-sensitized and rDioscorin-treated mice were treated with ConA for 48 h. The supernatants of the treated splenocytes were collected for determination of IFN- γ (A) and IL-5 (B) levels. Three replicates for each treatment were performed. Results represent mean $\pm$ SD of five mice ($n=5$). Asterisk (*) represented a significant difference of the cytokine levels between OVA-sensitized and rDioscorin-treated mice if $p < 0.05$ (*) or $p < 0.01$ (**).

OVA-induced allergic reaction by modulating the Th1/Th2 imbalance and promoting the Th1 cell response. Because the native Da-dioscorins and Dj-dioscorins isolated from the yam tubers of *D. alata* and *D. japonica*, respectively, mainly consisted of Class A dioscorins, the antiallergic activity of native Da-dioscorins and Dj-dioscorins was attributed to the Class A dioscorins^{18,20}.

However, the Class A dioscorins of various yam species exhibited similar immunomodulatory activities in OVA-sensitized mice, which were different from the activities demonstrated by native dioscorins²⁰. We observed that i.p. injecting Class A rDioscorins into OVA-sensitized mice reduced the IL-5 levels to the basal level in all of the rDioscorin-treated mice (Figure 5B). This result was different from that observed in the native dioscorin-treated mice in which the IL-5 levels dramatically decreased to the basal level after the mice were i.p. injected with native Da-dioscorins or Dj-dioscorins, whereas they significantly increased to nearly 4-fold higher in the Dp-dioscorin-treated mice compared with that in the positive control mice²⁰. A similar result was also observed for the IgG1 in that the serum levels of IgG1 in all

the rDioscorin-treated mice were slightly higher than that in the positive control mice; however, for the native dioscorins, the levels of IgG1 in the Da-dioscorin-treated and Dj-dioscorin-treated mice were comparable to those in the positive control mice, whereas the level of IgG1 in the Dp-dioscorin-treated mice significantly increased to 2.5-fold higher than that in the positive control mice²⁰. Because the native Dp-dioscorins in yam tubers of *D. pseudojaponica* contain a higher amount of Class B dioscorins than those of the other two yam species, all these results suggest that the different immunomodulatory effects on OVA-sensitized mice produced by the Dp-dioscorins but not by the Class A rDp-dioscorins might be attributed to the Class B dioscorins in the yam tubers. Although we could not disregard the possibility that the different immunomodulatory effects on OVA-sensitized mice between the Dp-dioscorins and the Class A rDp-dioscorins might be caused by a lack of glycosylation of the Class A rDp-dioscorins, we considered this scenario to be less likely because the Class A rDa-dioscorins and the Class A rDj-dioscorins that are not glycosylated produced immunomodulatory effects similar to those produced by the native dioscorins¹⁹. Further research using purified recombinant Class B dioscorins with conformational structures similar to the corresponding native dioscorins is required for elucidating this hypothesis.

IgG2a is secreted by B cells that are stimulated by activated Th1 cells²¹. Th1 cells modulate cellular immunity by producing IL-2 and IFN- γ . IFN- γ activates B cells, undergoing isotype switching and producing IgG2a, but suppressing switching to IgG1^{7,22}. Although the increase in the IgG2a levels was concomitant with the increase in the IFN- γ levels in all rDioscorin-treated mice, the IgG1 levels did not decrease, and even increased slightly in the sera of the rDioscorin-treated mice (Figures 5A and 6). Studies have reported the suppression of an allergic reaction by down-regulation of the Th2 cell response, but without a concomitant decrease in the IgG1 levels for many herbal medicines. Hsu et al. (2003) demonstrated that dehulled adlay exhibited a modulating ability, shifting the balance from Th2 to Th1 dominance in OVA-sensitized mice²³. Orally administering dehulled adlay decreased the serum IgE and IL-5 levels, but increased both the IgG1 and IgG2a levels. Sy et al. (2006) reported that propolis extract might enhance the Th1 cell response and inhibit the Th2 cell response²⁴. Orally administering a high dose of propolis extract to mice for 16 weeks suppressed the serum IgE level, but significantly increased both the IgG1 and IgG2a levels. Lin et al. (2006) observed that i.p. injections of polysaccharides purified from *Ganoderma lucidum* promoted Th1 cell response in OVA-sensitized BALB/c mice²⁵. IgG2a production significantly increased in response to the polysaccharide treatment, whereas the IgG1 levels remained similar. Further research is required to elucidate why the IgG1 levels were not suppressed by IFN- γ in the mice treated with the native or recombinant dioscorins.

Conclusion

Various Class A dioscorins, like the native dioscorins, suppress allergic reactions in OVA-sensitized BALB/c mice,

possibly by modulating imbalanced Th1/Th2 immune responses to OVA by promoting Th1 cell responses; thus, the Class A dioscorins can serve as potential therapeutic agents for treating IgE-mediated hypersensitivity. The Class A dioscorins of various yam species exhibited similar immunomodulatory activities on OVA-sensitized mice that were different from those of the native Dp-dioscorins, which contain a higher amount of Class B dioscorins, suggesting that the distinct immunomodulatory effects of native dioscorins on mice might be attributed to the various dioscorin isoforms in different yam species.

Acknowledgements

We thank Drs Yi-Hsiung Tseng (Tzu Chi University) and Jung-Hsin Hsu (National Dong Hwa University) for kindly providing ELISA kits and cell culture facility, respectively.

Declaration of interest

The authors declare that they have no conflict of interest.

References

- Larché M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 2006;6: 761–771.
- Ozdemir C, Akdis M, Akdis C. T-cell response to allergens. *Chem Immunol Allergy* 2010;95:22–44.
- Umetsu DT, Akbari O, DeKruyff RH. Regulatory T cells control the development of allergic disease and asthma. *J Allergy Clin Immunol* 2003;112:480–487.
- Sehmi R, Wardlaw AJ, Cromwell O, et al. Interleukin-5 enhances the chemotactic response of eosinophils obtained from normal but not eosinophilic subjects. *Blood* 1992;79:2952–2959.
- Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol* 2010;125:S73–S80.
- Viola JPB, Rao A. Molecular regulation of cytokine gene expression during the immune response. *J Clin Immunol* 1999;19: 98–108.
- Kawano Y, Noma T, Yata J. Regulation of human IgG subclass production by cytokines. IFN- γ and IL-6 act antagonistically in the induction of human IgG1 but additively in the induction of IgG2. *J Immunol* 1994;153:4948–4958.
- Mosmann TR, Coffmann RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989;7:145–173.
- Li SC. Pen-T'sao Kang Mu (Systematic Pharmacopoeia). Nanjing, China; 1596.
- Harvey PJ, Boulter D. Isolation and characterization of the storage protein of yam tubers (*Dioscorea rotundata*). *Phytochem* 1983;11: 1687–1693.
- Fu SL, Hsu YH, Lee PY, et al. Dioscorin isolated from *Dioscorea alata* activates TLR4-signaling pathways and induces cytokine expression in macrophages. *Biochem Biophys Res Commun* 2006; 339:137–144.
- Liu YW, Shang HF, Wang CK, et al. Immunomodulatory activity of dioscorin, the storage protein of yam (*Dioscorea alata* cv. Tainong No. 1) tuber. *Food Chem Toxicol* 2007;45:2312–2318.
- Lin PL, Lin KW, Weng CF, Lin KC. Yam storage protein dioscorins from *Dioscorea alata* and *Dioscorea japonica* exhibit distinct immunomodulatory activities in mice. *J Agric Food Chem* 2009;57: 4606–4613.
- Liu YW, Liu JC, Huang CY, et al. Effects of oral administration of yam tuber storage protein, dioscorin, to BALB/c mice for 21-days on immune responses. *J Agric Food Chem* 2009;57:9274–9279.
- Fu LS, Ko YH, Lin KW, et al. Dioscorin protects tight junction protein expression in A549 human airway epithelium cells from dust mite damage. *J Microbiol Immunol Infect* 2009;42:457–463.
- Conlan RS, Griffiths LA, Napier JA, et al. Isolation and characterisation of cDNA clones representing the genes encoding the major tuber storage protein (dioscorin) of yam (*Dioscorea cayenensis* Lam.). *Plant Mol Biol* 1995;28:369–380.
- Gaidamashvili M, Ohizumi Y, Iijima S, et al. Characterization of the yam tuber storage proteins from *Dioscorea batatas* exhibiting unique lectin activities. *J Biol Chem* 2004;279:26028–26035.
- Tsai WY, Jheng YJ, Chen KS, et al. Molecular cloning, structural analysis, and mass spectrometric identification of native dioscorins of different species of yam tubers. *J Sci Food Agric* 2013;93: 761–770.
- Jheng YJ, Tsai WY, Chen KH, et al. Recombinant dioscorins of the yam storage protein expressed in *Escherichia coli* exhibit antioxidant and immunomodulatory activities. *Protein Expr Purif* 2012;85: 77–85.
- Hsu YJ, Weng CF, Lin KW, Lin KC. Suppression of allergic reactions in ovalbumin-sensitized mice by yam storage proteins dioscorins. *J Agric Food Chem* 2013;61:11460–11467.
- Snapper CM, Peschel C, Paul WE. IFN- γ stimulates IgG2a secretion by murine B cells stimulated with bacterial lipopolysaccharide. *J Immunol* 1988;140:2121–2127.
- Finkelman FD, Katona IM, Mosmann TR, Coffman RL. IFN- γ regulates the isotypes of Ig secreted during in vivo humoral immune responses. *J Immunol* 1988;140:1022–1027.
- Hsu HY, Lin BF, Lin JY, et al. Suppression of allergic reactions by Dehulled adlay in association with the balance of Th1/Th2 cell responses. *J Agric Food Chem* 2003;57:3763–3769.
- Sy LB, Wu YL, Chiang BL, et al. Propolis extracts exhibit an immunoregulatory activity in an OVA-sensitized airway inflammatory animal model. *Inter Immunopharm* 2006;6:1053–1060.
- Lin YL, Lee SS, Hou SM, Chiang BL. Polysaccharide purified from *Ganoderma lucidum* induced gene expression changes in human dendritic cells and promotes T helper 1 immune response in BALB/c mice. *Mol Pharm* 2006;70:637–644.