

IgE, COX-2, and IL-4 are Expressed by DEHP through p38 MAPK and Suppressed by Plant Glycoprotein (75 kDa) in ICR Mice

Phil-Sun Oh¹ and Kye-Taek Lim^{2,3}

Abstract—The aim of the present study was to evaluate the inhibitory effect of glycoprotein isolated from *Cudrania tricuspidata* Bureau (CTB glycoprotein) on di(2-ethylhexyl) phthalate (DEHP)-induced allergic inflammatory response in mice. We evaluated the activity of β -hexosaminidase, expression of cyclooxygenase (COX)-2, p38 mitogen-activated protein kinase (MAPK), and activator protein (AP)-1, and production of immunoglobulin (Ig)E and interleukin (IL)-4 in DEHP-treated RBL-2H3 cells and ICR mice. Our results revealed that the CTB glycoprotein inhibited the activity of β -hexosaminidase and production of IgE and IL-4 in serum from DEHP-treated mice. We also found that the CTB glycoprotein reduced arachidonic acid release, COX-2 expression, and AP-1 transcriptional activation through p38 MAPK phosphorylation in DEHP-treated RBL-2H3 cells. The activation of AP-1 was completely blocked by treatment with p38 MAPK inhibitor (SKF86002). The results from these experiments indicate that CTB glycoprotein effectively protects against the allergic inflammation response, mainly through downregulation of MAPK/AP-1 in the mast cell degranulation stage. In conclusion, we suggest that the CTB glycoprotein may be one component of health supplements for the prevention of allergic inflammation.

KEY WORDS: *Cudrania tricuspidata* Bureau (CTB) glycoprotein; di(2-ethylhexyl) phthalate; immunoglobulin E; interleukin-4; arachidonic acid.

INTRODUCTION

Allergy is a hypersensitive response that is characterized by immunoglobulin (Ig)E-dependent mast cell activation. Recent studies have shown that the occurrence rate of allergic disorders has been increased rapidly among children and juveniles [1]. Many commonly used environmental chemicals affect the functions of the endocrine system and immune system in the body [2], thus it can

cause allergic disorders such as atopic dermatitis, allergic rhinitis, and bronchial asthma [3, 4]. Among the many environmental chemicals, di(2-ethylhexyl) phthalate (DEHP) has been widely used in PVC products, including vinyl flooring, wall coverings, food packaging, and children's toys [5]. Recent studies have revealed that absorption of some phthalates including DEHP could enhance the release of histamine from isolated human basophils [6] and stimulate differentiation of T helper precursor (Th0) cells to Th2 cells through IL-4 signaling in primary lymphocytes [7]. In addition, many researchers have reported that DEHP has an adjuvant effect on allergen-induced immunoglobulin production [8, 9].

In an allergic response, binding of antigen to the high-affinity IgE receptor (Fc ϵ RI) on the cellular surface of mast cells and basophils induces the release of pre-formed and *de novo* synthesized mediators such as histamine, arachidonic acid metabolites, protease, and cytokines [10–12]. Among allergic inflammatory mediators, arachidonic acid is rapidly metabolized to leukotriene C₄/B₄ and PGD₂

¹ Molecular Biochemistry Laboratory, Biotechnology Research Institute and Center for the Control of Animal Hazards Using Biotechnology (BK21), Chonnam National University, 300 Yongbong-Dong, Gwang-ju, 500-757, South Korea

² Molecular Biochemistry Laboratory, Biotechnology Research Institute, Chonnam National University, 300 Yongbong-Dong, Gwang-ju, 500-757, South Korea

³ To whom correspondence should be addressed at Molecular Biochemistry Laboratory, Biotechnology Research Institute, Chonnam National University, 300 Yongbong-Dong, Gwang-ju, 500-757, South Korea. E-mail: ktlim@chonnam.ac.kr; kyetaklim@hotmail.com

via lipoxygenase and cyclooxygenase (COX) pathways, respectively [13, 14]. Also, IL-4 can be generated in the mast cell and stimulate allergic inflammatory immune responses through the subsequent induction of other inflammatory mediators [12, 15]. Activation of mast cells leads to phosphorylation of protein kinases and production of reactive oxygen species (ROS). This is followed by activation of mitogen-activated protein kinases (MAPKs) and redox-sensitive activator protein (AP)-1, thereby producing various inflammatory mediators. Therefore, the modulation of degranulation-related mediators from mast cells is one rational strategy for regulating allergic responses.

It is generally accepted that traditional medicines isolated from natural sources have helpful properties on the prevention and healing of various immune disorders. Recently, many glycoproteins have been purified from various natural materials, including mushrooms, lichens, and plants, and examined for their efficacy as anti-tumor and anti-allergy agents [16, 17]. Korean herbal plant *Cudrania tricuspidata* Bureau (CTB) has been traditionally used for treating gastritis, liver damage, and inflammation in Korea [18]. Recently, we have purified a glycoprotein from CTB (CTB glycoprotein) which consists of 27.5% protein and 72.5% carbohydrates. In our previous study, we have observed that CTB glycoprotein strongly inhibits oxidative activity of free radicals and suppresses nitric oxide production induced by lipopolysaccharide in the murine macrophage cell line RAW 264.7 [19]. In addition, the anti-oxidant activity of CTB glycoprotein suppressed the Th2 response in mouse lymphocytes through modulation of cytokine and related signaling cascade [7, 19]. Because excessive Th2 cell differentiation can be responsible for the activation of mast cells, in the current study, we have examined whether CTB glycoprotein can inhibit the allergic inflammatory mediators induced by DEHP in ICR mice. To clarify the role of the CTB glycoprotein, we determined its effect on the activities of IgE, IL-4, and COX-2.

MATERIALS AND METHODS

Chemicals

DEHP, penicillin G, and streptomycin were obtained from Sigma (St. Louis, MO, USA). DMEM and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other chemicals and reagents were of the highest analytical grade.

Preparation of CTB Glycoprotein

The CTB glycoprotein was isolated and purified from *C. tricuspidata* Bureau (CTB) fruit, as described previously [19]. Briefly, fruits of CTB were obtained in October 2007 from the Naju traditional market in the Chonnam province, South Korea. They were identified by Dr. H.T. Lim (Chonnam National University). CTB (1 kg) was chopped into small portions and soaked in distilled water (20 l, w/v) for several months in a dark basement. The water extract was passed through Whatman filter paper (No. 2) to remove debris and concentrated using a rotary evaporator (B465; Buchi, Flawil, Switzerland). The concentrated solution was freeze-dried (SFDS06; Samwon, Seoul, Korea). The dried powder (8 g) was dissolved again in distilled water. The solution was subjected to concanavalin A-sepharose 4B affinity chromatography (24–45 mm, Sigma, C9017) and eluted with 0.5 M methyl α -D-glucopyranoside containing 0.5 M NaCl at pH 7.4. The eluted solution was dialyzed (8,000–12,000 Da, MWCO dialysis membrane, Spectrum Labs, CA, USA) with a dialysis membrane against 20 mM Tris-Cl (pH 7.4) at 4°C overnight and lyophilized. The eluted solution was used for further experimentation. To confirm, we performed SDS-PAGE of a sample of protein (10–25 mg/ml) containing 0.1% SDS, using a 15% polyacrylamide mini-gel and a Mini-PROTEIN II electrophoresis cell (Bio-Rad) at 110 V and 30 mA for 2.5 h. The CTB glycoprotein was confirmed by staining with Schiff's reagent [20], which is a specific staining reagent for the glycoprotein detected through a redox reaction. The purity of the CTB glycoprotein used in our studies was more than 98%.

Animals and Treatments

Female ICR mice (25 g), aged 6 weeks, were purchased from Daehan Lab (Animal Research Center Co., Ltd, DaeJeon, Co. Ltd., Daejeon, South Korea) and housed according to the "Guiding principles in the use of animals in toxicology," adopted by the Society of Toxicology in 1989 at the experimental animal room of the Veterinary College of Chonnam National University. All mice were fed a commercial diet and water *ad libitum* and kept for at least 1 week prior to the experiments. The body weight and food intake of each group were recorded once weekly. No significant differences of body weight and food intake between treatments groups were observed. Mice were divided into the following five groups:

Group 1: control ($n=6$)

Group 2: 10 mg/kg CTB glycoprotein ($n=6$)

Group 3: 10 mg/kg DEHP ($n=6$)

Group 4: 10 mg/kg DEHP+5 mg/kg CTB glycoprotein ($n=6$) and

Group 5: 10 mg/kg DEHP+10 mg/kg CTB glycoprotein ($n=6$)

Mice in group 1 were fed a commercial diet and water *ad libitum*. Mice in group 2 were orally given 10 mg/kg CTB glycoprotein alone daily for 5 weeks. Mice in group 3 received intraperitoneal injection of 0.1 ml of olive oil containing 10 mg/kg DEHP five times a week for 4 weeks. The mice in groups 4 and 5 were treated with CTB glycoprotein (5 and 10 mg/kg) daily for 5 weeks and co-treated with 10 mg/kg DEHP five times a week for 4 weeks. At week 5, the blood from mice was collected by a cardiac puncture under diethyl ether anesthesia and then centrifuged at 10,000 g for 5 min at 4°C. The supernatant was separated and stored at -70°C to measure the levels of β -hexosaminidase, immunoglobulin (Ig)E, and interleukin (IL)-4.

β -Hexosaminidase Secretion Assay

The level of β -hexosaminidase was measured according to the methods of Matsubara *et al.* [21]. Briefly, an aliquot (20 μ l) of serum was incubated with an equal volume of 1 mM p-nitrophenyl-N-acetyl β -D-glucosaminide in 0.1 M sodium citrate buffer (pH 4.5) at 37°C for 1 h. The reaction was terminated by adding 200 μ l of stopping buffer (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). The absorbance was measured with a microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm.

Enzyme-Linked Immunosorbent Assay

The amounts of IgE and IL-4 were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies specific to IgE and IL-4 (BD Biosciences, San Diego, CA, USA). ELISA plates were coated with rat anti-mouse IgE and IL-4, respectively. After coating, serially diluted sera were added to the plates and incubated overnight at 4°C. Murine recombinant IgE and IL-4 were used as a standard for quantitation of IgE and IL-4 in the serum. After washing, biotinylated anti-mouse IgE and IL-4 were added, respectively. The plates were then washed and streptavidin-horseradish peroxidase was added. After additional washes, TMB solution was added

and developed for 10 min. The OD was determined at 405 nm.

Cell Culture

RBL-2H3 cells were provided by Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C and 5% CO₂. The medium was renewed twice a week. The cells (2.3×10^6 cells/ml) were dispensed into 6-well plates. The cellular cytotoxicity was determined by assessing the release of lactate dehydrogenase (LDH) into the medium according to the method of Bergmeyer and Bernt [22]. Briefly, the cells were treated with DEHP (100 μ M) or co-treated with 100 μ M DEHP and the CTB glycoprotein (50–100 μ g/ml). After that, the culture medium (35 μ l) was mixed with reaction mixture containing 0.6 mM pyruvate in 48 mM potassium phosphate buffer (pH 7.5), and the final volume of the reaction mixture was brought up to 3.15 ml. The reaction was initiated by the addition of 0.18 mM β -NADH (reduced nicotinamide adenine dinucleotide), and LDH activity was measured as the rate of loss of β -NADH absorption at 340 nm for 2 min. For the total LDH determination, the cells were treated with Triton X-100 (1%) for 30 min and centrifuged at 1,000 $\times$ g for 10 min. The resulting supernatants were added to the reaction mixtures, and the LDH assay was carried out as described above. Results were expressed as relative percentage, compared to the Triton X-100 treatment alone.

Arachidonic Acid (AA) Release Assay

The [³H]AA release experiments were performed using a modification of the method reported by Xing *et al.* [23]. RBL-2H3 cells were incubated for 24 h in DMEM containing 0.5 μ Ci/ml [³H]AA. The cells were then washed three times with DMEM and incubated at 37°C for 24 h in culture medium. At the end of the incubation period, the culture medium was transferred to ice-cold tubes containing 100 μ l of 55 mM EGTA and EDTA (final concentration, 5 mM each). The culture medium was then centrifuged at 12,000 $\times$ g to eliminate the cell debris. The level of radioactivity in the supernatant was determined by placing samples in scintillation vials containing scintillation fluid and measuring the radioactivity using a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA).

Preparation of Cell Extracts

Protein extracts were prepared as previously described [16]. The cells were treated with DEHP (100 μ M) or co-treated with 100 μ M DEHP and the CTB glycoprotein (50 or 100 μ g/ml) for 2 h. The cells were then rinsed with PBS after removing the medium and lysed in 300 μ l of buffer A (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP40, 25% glycerol) containing a protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN, USA). For lysis, the cells were freeze-thawed, shaken for 30 min at 4°C and centrifuged at 14,000 $\times g$ for 30 min. The supernatant was designated as whole cell extract.

To prepare nuclear extracts, the cells were scraped in 400 μ l of hypotonic buffer B (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40) and then centrifuged at 3,000 $\times g$ for 5 min. The pelleted nuclear proteins were resuspended in 200 μ l of NE buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40) containing a protease inhibitor cocktail and centrifuged at 12,000 $\times g$ for 15 min. The amount of protein was measured by the Lowry method [24]. Samples of protein were stored at -70°C.

Immunoblot Analysis

Intracellular protein extracts were analyzed by 10% polyacrylamide mini-gel electrophoresis at 100 V for 2 h at room temperature using a Mini-PROTEIN II electrophoresis cell (Bio-Rad). After transfer onto nitrocellulose membranes (Millipore, Bedford, MA, USA), the membranes were incubated for 1 h at room temperature in TBS-T solution (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween-20) containing 5% BSA. The membranes were subsequently incubated for 2 h at room temperature with rabbit polyclonal antibodies [1:3,000 dilution: COX-2, AP-1 (c-jun and c-fos) and α -tubulin] in TBS-T solution. After three time washes with TBS-T, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Cell Signaling, MA, USA) in TBS-T solution for 1 h at room temperature. The resulting protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK). Immunoblot assay results were calculated

as relative intensity using Scion imaging software (Scion Image Beta 4.02, MO, USA).

Statistical Analysis

All experiments were carried out in triplicate, and data were expressed as means $\pm$ SD. One-way analysis of variance and the Duncan test were carried out to determine significant differences of multiple comparisons (SPSS program, ver 11.0).

RESULTS

Effect of CTB Glycoprotein on IL-4 Production in Serum from DEHP-Treated ICR Mice

As shown in Fig. 1, addition of DEHP caused a 158.1 pg/ml increase in IL-4 levels compared to control. However, administration of the CTB glycoprotein considerably attenuated the DEHP-induced increase of IL-4 levels in a dose-dependent manner. The values of IL-4 amounts decreased 77.1 pg/ml at addition of 10 mg/kg (BW) CTB glycoprotein in the presence of DEHP, compared with that for the DEHP treatment alone.

Effect of CTB Glycoprotein on IgE Production in Serum from DEHP-Treated ICR Mice

We observed an increase in IgE antibody levels of 126 ng/ml in DEHP-treated mice compared to the control group. However, administration of the CTB glycoprotein in the presence of DEHP decreased IgE

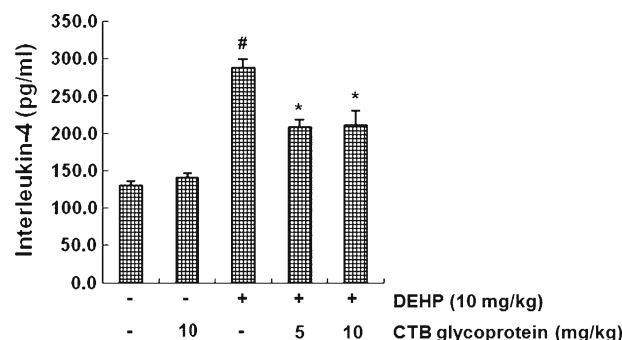


Fig. 1. Effect of the CTB glycoprotein on IL-4 production in serum from DEHP-treated ICR mice. The amount of IL-4 was determined by sandwich ELISA using monoclonal antibody specific to IL-4 (BD Biosciences, San Diego, CA, USA). Data are the means $\pm$ SD from triplicate experiments. Single number sign represents a significant difference between treatments and control, $p < 0.05$. Single asterisk indicates a significant difference between DEHP treatment and the CTB glycoprotein treatment in the presence of DEHP, $p < 0.05$.

levels considerably in a dose-dependent manner. When the mice were administered with 10 mg/kg (BW) CTB glycoprotein, IgE antibody levels decreased 133 ng/ml compared with the DEHP treatment alone (Fig. 2).

Effect of CTB Glycoprotein on β -Hexosaminidase Activity in Serum from DEHP-Treated ICR Mice

As shown in Fig. 3, addition of DEHP caused a 1.35-fold increase in β -hexosaminidase activity compared to the control. However, treatment with the CTB glycoprotein at 10 mg/kg suppressed the DEHP-induced increase of β -hexosaminidase activity. That is, the values of β -hexosaminidase activity decreased by 0.3-fold in the case of administration of 10 mg/kg (BW) CTB glycoprotein in the presence of DEHP, compared with that for the DEHP treatment alone.

Effect of CTB Glycoprotein on Arachidonic Acid Release and COX-2 Expression in DEHP-Stimulated RBL-2H3 Cells

As shown in Fig. 4a, treatment of RBL-2H3 cells with DEHP stimulated release of arachidonic acid by 1.65-fold as compared to control. However, treatment with the CTB glycoprotein attenuated the DEHP-induced increase of arachidonic acid release without any cytotoxic effects (Fig. 4a and b). That is, the value of arachidonic acid release decreased 0.26-fold in the case of addition of 100 μ g/ml CTB glycoprotein in the presence of DEHP, compared with that for the DEHP treatment alone. In expression of COX-2, treatment with

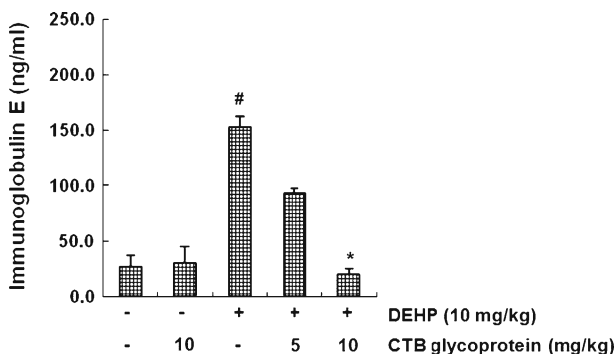


Fig. 2. Effect of the CTB glycoprotein on IgE antibody production in serum from DEHP-treated ICR mice. The amount of IgE was determined by sandwich ELISA using monoclonal antibody specific to IgE (BD Biosciences, San Diego, CA, USA). Data are the means $\pm$ SD from triplicate experiments. *Single number sign* represents a significant difference between treatments and control, $p < 0.05$. *Single asterisk* indicates a significant difference between DEHP treatment and the CTB glycoprotein treatment in the presence of DEHP, $p < 0.05$.

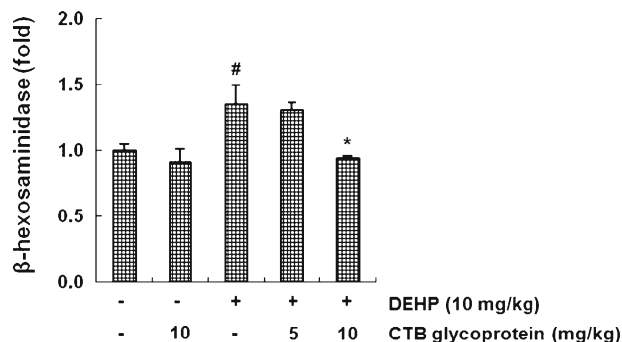


Fig. 3. Effect of the CTB glycoprotein on degranulation in serum from DEHP-treated ICR mice. Data are the means $\pm$ SD from triplicate experiments. *Single number sign* represents a significant difference between treatments and control, $p < 0.05$. *Single asterisk* indicates a significant difference between DEHP treatment and the CTB glycoprotein treatment in the presence of DEHP, $p < 0.05$.

DEHP induced expression of COX-2 at 1 h. That is, the band intensity of COX-2 significantly increased by 15.4-fold in the presence of DEHP as compared with that in the control. Treatment with the CTB glycoprotein resulted in a concentration-dependent attenuation of DEHP-induced COX-2 expression in RBL-2H3 cells; COX-2 expression decreased by 0.43-fold after the addition of 100 μ g/ml CTB glycoprotein to DEHP-treated cells compared with expression after DEHP treatment alone.

Effect of CTB Glycoprotein on p38 MAPK Phosphorylation and AP-1 Activity in DEHP-Treated RBL-2H3 Cells

As shown in Fig. 5a, treatment with DEHP for 0.5 h induced 1.41-fold increase in p38 MAPK phosphorylation compared to control, without affecting total p38 MAPK protein expression. However, treatment with 100 μ g/ml CTB glycoprotein decreased p38 MAPK phosphorylation in DEHP-treated RBL-2H3 cells by 0.46-fold compared to treatment with DEHP alone. In AP-1 activity, the nuclear levels of c-jun and c-fos increased 1.33- and 1.32-fold in the presence of DEHP, respectively, compared with that in the control. However, treatment with the CTB glycoprotein in the presence of DEHP inhibited the expression of c-jun and c-fos in a concentration-dependent manner, compared with the level of expression after DEHP treatment. As shown in Fig. 5b, the nuclear levels of c-jun and c-fos decreased by 0.57- and 0.59-fold, respectively, after the addition of 100 μ g/ml CTB glycoprotein in the

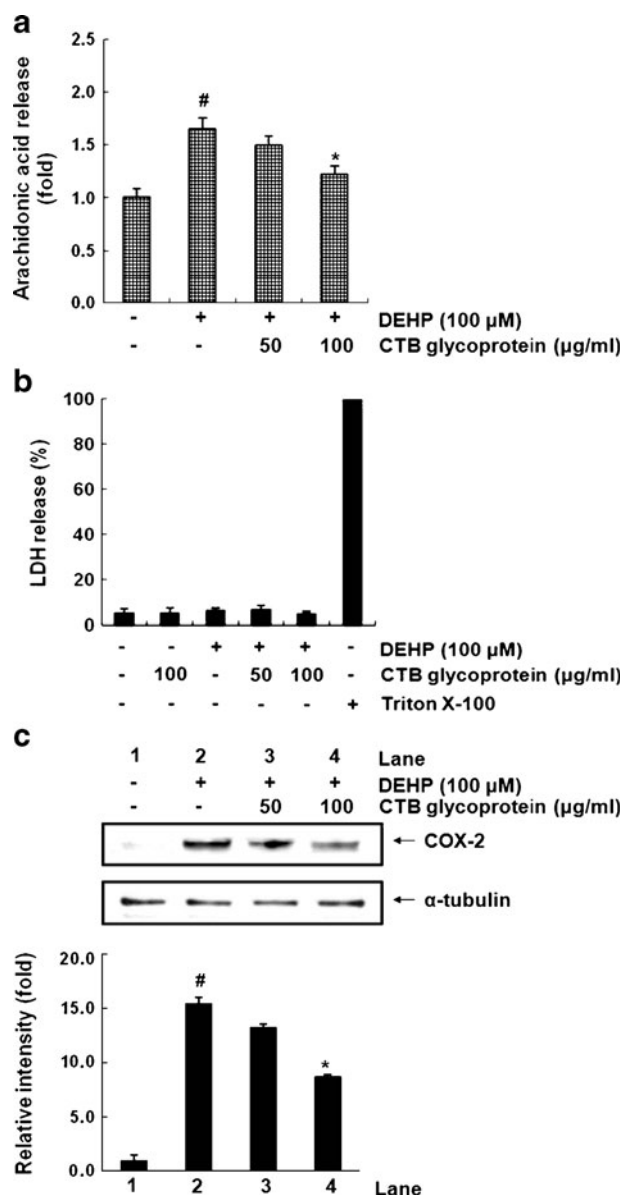


Fig. 4. Inhibitory effect of the CTB glycoprotein on arachidonic acid release and COX-2 expression in DEHP-treated RBL-2H3 cells. Cells were treated with DEHP alone (100 μ M) or with the combination of CTB glycoprotein (50 or 100 μ g/ml) and DEHP. The cellular cytotoxicity was determined by assessing the release of LDH into the medium. Relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, Maryland, USA). All data are the means $\pm$ SD from triplicate experiments. *Single number sign* represents a significant difference between treatments and control, $p < 0.05$. *Single asterisk* indicates a significant difference between DEHP treatment and CTB glycoprotein treatment in the presence of DEHP, $p < 0.05$. Lane 1, control; lane 2, DEHP alone; lane 3, 50 μ g/ml CTB glycoprotein in the presence of DEHP; and lane 4, 100 μ g/ml CTB glycoprotein in the presence of DEHP.

presence of DEHP compared with nuclear levels after DEHP treatment alone. To investigate whether p38 MAPK involved in DEHP-induced translocation of AP-1 into the nucleus, we determined whether AP-1 activation in DEHP-treated cells was affected by p38 MAPK inhibitor. Pretreatment of DEHP-treated cells with the p38 inhibitor SKF 86002 decreased nuclear

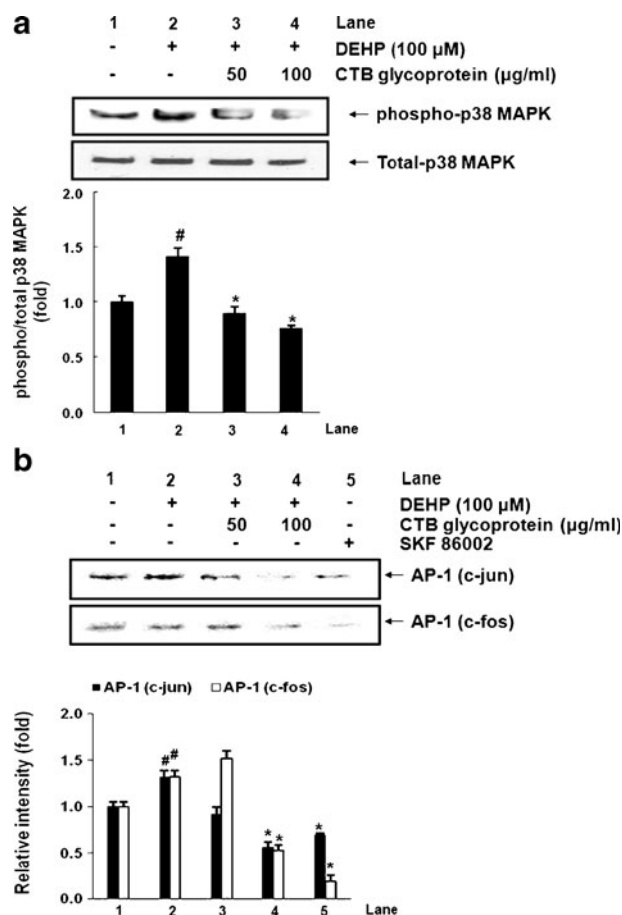


Fig. 5. Inhibitory effect of the CTB glycoprotein on MAPK and AP-1 activation in DEHP-treated RBL-2H3 cells. Cells were treated with DEHP alone (100 μ M) or with the combination of CTB glycoprotein (50 or 100 μ g/ml) and DEHP or with p38 MAPK inhibitor (SKF 86002). The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, Maryland, USA). All data are the means $\pm$ SD from triplicate experiments. *Single number sign* represents a significant difference between treatments and control, $p < 0.05$. *Single asterisk* indicates a significant difference between DEHP treatment alone and treatment with the CTB glycoprotein in the presence of DEHP, $p < 0.05$. Lane 1, control; lane 2, DEHP alone; lane 3, 50 μ g/ml CTB glycoprotein in the presence of DEHP; lane 4, 100 μ g/ml CTB glycoprotein in the presence of DEHP; and lane 5, SKF 86002 (p38 MAPK inhibitor) in the presence of DEHP.

levels of c-jun and c-fos by 0.47- and 0.85-fold, respectively, compared with nuclear levels in cells with DEHP treatment alone.

DISCUSSION

There is increasing evidence supporting the therapeutic potential of traditional medicines or health foods for a variety of pathological conditions such as allergic disorders without any cytotoxicity and side effects [25]. In our previous study, the CTB glycoprotein suppresses differentiation of Th precursor cells to Th2 cells via modulation of STAT6 phosphorylation and GATA-3 activation in primary immune cells [7]. Since excessive Th2 cell differentiation can be responsible for the activation of mast cells, in the present study we examined whether the CTB glycoprotein can inhibit the DEHP-induced production of IgE and cytokine and arachidonic acid metabolites via modulation of MAPK in ICR mice and RBL-2H3 cells.

Many studies have shown that phthalate esters including DEHP have the ability to impact on allergy, showing potential of adjuvant to influence this process. It has been reported that exposure to DEHP enhances the production of IL-4 in T cells from mice [26] and causes an inflammatory response in cultured neutrophils of both humans and rodents [27]. In addition, at high concentration it can increase serum IgG1 and the number of lung inflammatory cells in animal experiments [9]. These observations indicate that DEHP has the potential to interact with the immune system, thereby enhancing the progress of allergic inflammatory diseases. One of potent possibilities is that it may be related to their ability to increase expression of inflammatory cell proteins called cytokines or chemokines. This means that DEHP could be indirectly involved in induction of allergy-related cytokines like classical allergic response. In the present study, our results showed that the CTB glycoprotein inhibits the production of IgE and IL-4 and activity of β -hexosaminidase in DEHP-treated ICR mice. In addition, the CTB glycoprotein suppresses the release of arachidonic acid and expression of COX-2 induced by DEHP in RBL-2H3 cells. Our results suggest that the inhibitory effect of the CTB glycoprotein is related to the modulation of p38 MAPK phosphorylation and ROS production induced by DEHP. Thus, the CTB glycoprotein may attenuate allergic response via inhibition of mast cell degranulation and cytokine production induced by DEHP *in vivo* and *in vitro*.

The results in Figs. 1 and 2 indicate that the CTB glycoprotein has a dose-dependent suppressive effect on

production of IgE and IL-4 in DEHP-treated mice. It has been known that the IgE antibody is related closely to allergic responses, which are thought to be due to the development and activation of Th2 cells [28]. Also, IL-4 is an eosinophil chemoattractant that induces endothelial cells to produce eosinophil chemotactic factor and eotaxin [29] and enhances expression of endothelial cell surface adhesion molecules such as vascular cell adhesion molecule-1 [30]. Moreover, IL-4 has an important role in Th2 cells and B cells through stimulation of the switch of the antibody isotype to IgE [31, 32]. Therefore, inhibition of IgE antibody and IL-4 production is a reliable parameter for predicting possible anti-allergic activity of natural compounds. Our results showed that treatment with DEHP increased the production of IgE antibody and IL-4 in mice serum. This means that DEHP could stimulate the degranulation of mast cells or activation of Th2 cells though enhancement of IgE antibody level and IL-4 production in mice. To confirm whether the CTB glycoprotein inhibits the degranulation of mast cells, we measured the activity of β -hexosaminidase in serum. Among the various inflammatory mediators, β -hexosaminidase is stored in the secretory granules of the cells and is released by exocytosis when mast cells are immunologically activated [12]. Indeed, our results showed that treatment with DEHP in serum increased the release of β -hexosaminidase, indicating its stimulatory activity of mast cell degranulation (Fig. 3). However, administration of the CTB glycoprotein inhibits the production of IgE antibody and IL-4 and activity of β -hexosaminidase in DEHP-treated mice. Results from our experiments suggest that the CTB glycoprotein is an anti-allergic agent that can inhibit degranulation of mast cells.

In addition, the results of this study clearly indicated that the CTB glycoprotein has inhibitory effect on the level of COX-2 and arachidonic acid in DEHP-treated RBL-2H3 cells (Fig. 4). Several recent studies have proposed that arachidonic acid and COX-2 are the most important mediators of allergic inflammatory disorders. During cell activation, cell injury, or inflammatory processes, free arachidonic acid is accumulated. Once released, arachidonic acid can be metabolized by different enzymatic pathways leading to modulation of the inflammatory process [33]. In contrast to the constitutive COX isoforms, it has been reported that during inflammation development COX-2 is upregulated by allergen in cells including fibroblasts, monocytes, and vascular endothelium [34]. Consistent with these reports, our result indicates that treatment with DEHP stimulates arachidonic acid level and

COX-2 activity in RBL-2H3 cells. However, the CTB glycoprotein suppresses the release of arachidonic acid and expression of COX-2 in DEHP-treated RBL-2H3 cells, suggesting that CTB glycoprotein is a potential inhibitor of the pro-inflammatory mediators in mast cells.

We also investigated whether the CTB glycoprotein has a suppressive effect on the signal pathway responsible for mast cell degranulation. Recent studies demonstrated that activation of proinflammatory mediators is regulated by protein kinase and transcriptional factors, and these events can lead to pathogenesis of allergic inflammation in asthma and other allergic diseases [26, 35]. In particular, MAPKs are serine and/or threonine kinases that transfer mitotic signals to the nucleus in response to extracellular stimuli, leading to cell proliferation, differentiation, and cytokine expression [36, 37]. The activation of the MAPK cascade eventually results in direct or indirect activation of transcriptional factors. AP-1 transcriptional activation is responsible for allergic reaction by inducing the expression of proinflammatory mediators including cytokines, adhesion molecules, and cell proliferation growth factors [38, 39]. Our results showed that treatment with DEHP stimulates the phosphorylation of p38 MAPK and activation of AP-1 in RBL-2H3 cells. However, the activities of p38 MAPK and AP-1 in DEHP-treated RBL-2H3 cells were effectively inhibited by treatment with the CTB glycoprotein, suggesting that the anti-allergic activity of the CTB glycoprotein is related to the modulation of p38 MAPK and AP-1 activation in these cells. Indeed, the DEHP-induced AP-1 activation was inhibited by pretreatment with SKF 86002. These results suggest that the activation of AP-1 is influenced by p38 MAPK phosphorylation in DEHP-treated RBL-2H3 cells (Fig. 5). On the basis of our findings, we postulate that the CTB glycoprotein may have anti-allergic potential by suppressing MAPK/AP-1 activation and subsequent activation of proinflammatory mediators in DEHP-stimulated RBL-2H3 cells.

In conclusion, our results showed that the CTB glycoprotein inhibited degranulation of mast cells via modulation of IgE antibody, IL-4, and β -hexosaminidase release in mice. In addition, our results suggest that the CTB glycoprotein suppressed the release of arachidonic acid and expression of COX-2 in mast cells via inhibition of the activities of MAPK/AP-1. The results from these experiments indicated that the CTB glycoprotein has anti-allergenic potential *in vivo* and *in vitro*. Further study is necessary to elucidate the precise mechanism underlying the anti-allergenic effects of CTB glycoprotein at the molecular level.

ACKNOWLEDGMENT

This work was partially supported by Biotechnology Research Institute, Chonnam National University in 2009.

REFERENCES

1. Beasley, R., P. Ellwood, and I. Asher. 2003. International patterns of the prevalence of pediatric asthma, the ISAAC program. *Pediatric Clinics of North America* 50: 539–553.
2. Chalubinski, M., and M.L. Kowalski. 2006. Endocrine disrupters—potential modulators of the immune system and allergic response. *Allergy* 61: 1326–1335.
3. Bornehag, C.G., J. Sundell, C.J. Weschler, T. Sigsgaard, B. Lundgren, M. Hasselgren, and L. Hagerhed-Engman. 2004. The association between asthma and allergic symptoms in children and phthalates in house dust: a nested case-control study. *Environmental Health Perspectives* 112: 1393–1397.
4. Jaakkola, J.J., H. Parise, V. Kislitsin, N.I. Lebedeva, and J.D. Spengler. 2004. Asthma, wheezing, and allergies in Russian schoolchildren in relation to new surface materials in the home. *American Journal of Public Health* 94: 560–562.
5. Schettler, T. 2006. Human exposure to phthalates via consumer products. *International Journal of Andrology* 2(9): 134–139.
6. Glue, C., M.H. Platzer, S.T. Larsen, G.D. Nielsen, P.S. Skov, and L.K. Poulsen. 2005. Phthalates potentiate the response of allergic effector cells. *Basic & Clinical Pharmacology & Toxicology* 96: 140–142.
7. Oh, P.S., and K.T. Lim. 2009. Suppressive effect of CTB glycoprotein (75 kDa) on IL-4 expression in primary-cultured lymphocytes treated with di(2-ethylhexyl) phthalate. *Naunyn-Schmiedeberg's Archives of Pharmacology* 380: 115–124.
8. Larsen, S.T., R.M. Lund, G.D. Nielsen, P. Thygesen, and O.M. Poulsen. 2002. Adjuvant effect of di-n-butyl-, di-n-octyl-, di-isononyl- and di-iso-decyl phthalate in a subcutaneous injection model using BALB/c mice. *Pharmacology & Toxicology* 91: 264–272.
9. Larsen, S.T., J.S. Hansen, E.W. Hansen, P.A. Clausen, and G.D. Nielsen. 2007. Airway inflammation and adjuvant effect after repeated airborne exposures to di-(2-ethylhexyl)phthalate and ovalbumin in BALB/c mice. *Toxicology* 235: 119–129.
10. Stassen, M., L. Hultner, C. Muller, and E. Schmitt. 2002. Mast cells and inflammation. *Archivum Immunologiae et Therapiae Experimentalis* 50: 179–185.
11. Galli, S.J., M. Tsai, and A.M. Piliponsky. 2008. The development of allergic inflammation. *Nature* 454: 445–454.
12. Gilfillan, A.M., and C. Tkaczuk. 2006. Integrated signalling pathways for mast-cell activation. *Nature Reviews. Immunology* 6: 218–230.
13. Ashraf, M., M. Murakami, and I. Kudo. 1996. Cross-linking of the high-affinity IgE receptor induces the expression of cyclooxygenase 2 and attendant prostaglandin generation requiring interleukin 10 and interleukin 1b in mouse cultured mast cells. *The Biochemical Journal* 320: 965–973.
14. Igarashi, Y., J.D. Lundgren, J.H. Shelhamer, M.A. Kaliner, and M. V. White. 1993. Effects of inhibitors of arachidonic acid metabolism on serotonin release from rat basophilic leukemia cells. *Immunopharmacology* 25: 131–144.
15. Theoharides, T.C., and D. Kalogeromitos. 2006. The critical role of mast cells in allergy and inflammation. *Annals of the New York Academy of Sciences* 1088: 78–99.
16. Oh, P.S., and K.T. Lim. 2008. Protective activity of 30 kDa phytoglycoprotein from glucose/glucose oxidase-induced cell death in primary cultured mouse thymocytes. *Environmental Toxicology and Pharmacology* 25: 114–120.

17. Ooi, V.E., and F. Liu. 2000. Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. *Current Medicinal Chemistry* 7: 715–729.
18. Park, K.H., Y.D. Park, J.M. Han, K.R. Im, B.W. Lee, I.Y. Jeong, T. S. Jeong, and W.S. Lee. 2006. Anti-atherosclerotic and anti-inflammatory activities of catecholic xanthenes and flavonoids isolated from *Cudrania tricuspidata*. *Bioorganic & Medicinal Chemistry Letters* 16: 5580–5583.
19. Joo, H.Y., and K.T. Lim. 2008. Glycoprotein isolated from *Cudrania tricuspidata* Bureau inhibits iNO and COX-2 expression through modulation of NF- κ B in LPS-stimulated RAW 264.7 cells. *Environmental Toxicology and Pharmacology* 27: 247–252.
20. Neville Jr., D.M., and H. Glossmann. 1974. Molecular weight determination of membrane protein and glycoprotein subunits by discontinuous gel electrophoresis in dodecyl sulfate. *Methods in Enzymology* 32: 92–102.
21. Matsubara, M., S. Masaki, K. Ohmori, A. Karasawa, and K. Hasegawa. 2004. Differential regulation of IL-4 expression and degranulation by anti-allergic olopatadine in rat basophilic leukemia (RBL-2H3) cells. *Biochemical Pharmacology* 67: 1315–1326.
22. Bergmeyer, H.U., and E. Bernt. 1974. Lactate dehydrogenase. In *Methods of enzymatic analysis*, vol. 2, 2nd ed, ed. H.U. Bergmeyer, 574–579. San Francisco and London: Verlag Chemie Weinheim. Academic press Inc New York.
23. Xing, M., L. Tao, and P.A. Insel. 1997. Role of extracellular signal-regulated kinase and PKC in cytosolic PLA2 activation by bradykinin in MDCK-D1 cells. *American Journal of Physiology. Cell Physiology* 272: C1380–C1387.
24. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry* 193: 265–275.
25. Li, X.M., C.K. Huang, T.F. Zhang, A.A. Teper, K. Srivastava, B.H. Schofield, and H.A. Sampson. 2000. The Chinese herbal medicine formula MSSN-002 suppresses allergic airway hyperreactivity and modulates Th1/Th2 responses in a murine model of allergic asthma. *The Journal of Allergy and Clinical Immunology* 106: 660–668.
26. Lee, M.H., J. Park, S.W. Chung, B.Y. Kang, S.H. Kim, and T.S. Kim. 2004. Enhancement of interleukin 4 production in activated CD4⁺ T cells by dipthalate plasticizers via increased NF-AT binding activity. *International Archives of Allergy and Immunology* 134: 213–222.
27. Gourlay, T., I. Samartzis, D. Stefanou, and K. Taylor. 2003. Inflammatory response of rat and human neutrophils exposed to di-(2-ethyl-hexyl)-phthalate-plasticized polyvinyl chloride. *Artificial Organs* 27: 256–260.
28. Romagnani, S. 1995. Atopic allergy and other hypersensitivities editorial overview: Technological advances and new insights into pathogenesis prelude novel therapeutic strategies. *Current Opinion in Allergy and Clinical Immunology* 7: 745–750.
29. Rothenberg, M.E., A.D. Luster, and P. Leder. 1995. Murine eotaxin: An eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. *Proceedings of the National Academy of Sciences of the United States of America* 92: 8960–8964.
30. Schleimer, R.P., S.A. Sterbinsky, J. Kaiser, C.A. Bickel, D.A. Klunk, K. Tomioka, W. Newman, F.W. Luscinskas Jr., M.A. Gimbrone, and B.W. McIntyre. 1992. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. *Journal of Immunology* 148: 1086–1092.
31. Murphy, K.M., and S.L. Reiner. 2002. The lineage decisions of helper T cells. *Nature Reviews. Immunology* 2: 933–944.
32. Herrick, C.A., H. MacLeod, E. Glusac, R.E. Tigelaar, and K. Bottomly. 2000. Th2 responses induced by epicutaneous or inhalational protein exposure are differentially dependent on IL-4. *The Journal of Clinical Investigation* 105: 765–775.
33. Smith, W.L., D.L. DeWitt, and R.M. Garavito. 2000. Cyclooxygenases: Structural, cellular, and molecular biology. *Annual Review of Biochemistry* 69: 145–182.
34. Parente, L., and M. Perretti. 2003. Advances in the pathophysiology of constitutive and inducible cyclooxygenases: Two enzymes in the spotlight. *Biochemical Pharmacology* 65: c153–c159.
35. Abdel-Raheem, I.T., I. Hide, Y. Yanase, Y. Shigemoto-Mogami, N. Sakai, Y. Shirai, N. Saito, F.M. Hamada, N.A. El-Mahdy, A.-D. Elsisy, S.S. Sokar, and Y. Nakata. 2005. Protein kinase C- α mediates TNF release process in RBL-2H3 mast cells. *British Journal of Pharmacology* 145: 415–423.
36. Yamashita, M., M. Kimura, M. Kubo, C. Shimizu, T. Tada, R.M. Perlmutter, and T. Nakayama. 1999. T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. *Proceedings of the National Academy of Sciences of the United States of America* 96: 1024–1029.
37. Duan, W., and W.S. Wong. 2006. Targeting mitogen-activated protein kinases for asthma. *Current Drug Targets* 7: 691–698.
38. Lee, W., P. Mitchell, and R. Tjian. 1987. Purified transcription factor AP-1 interact with TPA-inducible enhancer elements. *Cell* 49: 741–752.
39. Lee, Y.N., J. Tuckerman, H. Nechushtan, G. Schutz, E. Razin, and P. Angel. 2004. c-Fos as a regulator of degranulation and cytokine production in Fc ϵ RI-activated mast cells. *Journal of Immunology* 173: 2571–2577.