A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Allergen-Denaturing Activities of Constituents Identified in *Eriobotrya japonica* leaves against Egg Allergen Ovalbumin

비파엽(***Eriobotrya japonica***) 유래 화합물의 계란 알러젠 오발부민(**Ovalbumin**)에 대한 알러젠 중화활성

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ABSTRACT

Egg allergy is the second most common cause of food allergies in children. It causes an overreaction of the immune system which may lead to severe physical symptoms for millions of infant around the world. Most cases are derived from egg white which induces allergic conjunctivitis, itchiness, and a runny nose. Early studies reported that one of the major allergen ovalbumin (OVA, *Gal d 2*, 45 kDa) constitutes about 54 % of egg white proteins. It is very difficult to control of ovalbumin physically or chemically because their stability to heat and digestive enzymes, reflecting their capacity to stimulate a specific immune response. Medication such as anti-histamines, oral corticosteroids and epinephrine has been used to reduce symptoms of allergic disorders. They have not only low efficacy but also side effects. As an allergen denaturant, promethazine is affordable but their residual property is criticized. There is therefore a critical need for the

development of new improved allergen denaturants with novel target sites. *Eriobotrya japonica* has been used in Korean and Chinese traditional medicine for inflammatory disease and chronic bronchitis. A traditional therapy using it in an ancient book has shown that it is effective to treat asthma, coughs, and high fever as to drink its tea.

This research is focused on isolating allergen denaturing activity compound from the leaves of *E. japonica* and an assessment was made of allergen-denaturing activities of constituents identified in *E. japonica* leaves against egg allergen ovalbumin. The allergen denaturing activities of active principle from *E. japonica* are compared with those of the commercial allergen denaturant, promethazine. The activities were analyzed by measuring of intensity and IC_{50} about Immunoblot and Competitive Indirect enzyme-linked immunosorbent assay (ciELISA), respectively. Moreover, fragmentation of protein induced by treatment of natural products was examined, allergenicity and antigenicity of residual materials was compared between human sera from allergic patients and commercial rabbit antibodies.

The active principles of *E. japonica* leaves were separated using column chromatography, glass thin layer chromatography and preparative HPLC. In immunoblot using rabbit anti-OVA antibodies, active principle exhibited antigenic inhibition. The results from the immunoblot bioassay demonstrated that the allergen denaturing activity of compound 1 (relative antigenicity, 99.18 % at 100 μg/mL) did not differ significantly from that of promethazine (99.34 % at 100 μg/mL), whereas compound 2 (95.83 % at 10 μg/mL) and compound 3(83.33 % at 25 μg/mL) treatments were much higher than that of promethazine. In *E. japonica* derived compounds, ellagic acid suppressed the binding of

OVA and specific IgG effectively followed by lactic acid, hydrobenzoic acid, rutin trihydrate, (D)-(-)-tartaric acid, riboflavin and palmitic acid (85.2–96.7 %). The activities of these compounds were comparable to that of promethazine. In the ciELISA inhibition, the concentration of treated compounds, which were reduced to 10 μg/mL. Tartaric acid was the most active compound (IC_{50}, 114.18 μg/mL) as compared with that of native OVA (11.49 μg/mL) and ellagic acid treatments (13.11 μg/mL) did not inhibit the antigenicity of the OVA effectively as that of promethazine (12.81 μg/mL). However, in isolated compounds, compound 3 has been shown to be most effective, which was more active than compound 1 and compound 2. The antigenicity was reduced to 1/10 along with oleanolic acid, palmitic acid, lactic acid and *p*-coumaric acid (106.63-100.52 μg/mL). Other compounds failed to show good effects on the reduction of the antigenicity of OVA.

In the SDS-PAGE bioassay, treatment with urea clearly indicates that *E. japonica* leaf extract and promethazine caused appearance of ovalbumin protein and residual material bands in pellet solutions. In addition, high concentration of each sample extracts strongly increased the content of protein by exposing particular four amino acids. In the immunoblot assay, allergenicity of residual materials treated with *E. japonica* leaf extract remained, whereas antigenicity of residual materials treated with *E. japonica* leaf extract was removed. However, the three isolated compounds either physically or chemically modified egg white whole protein and ovalbumin, their binding activities to allergic patient's specific IgE and rabbit-specific IgG were removed. Moreover, the denaturing activity of *E. japonica* leaf extract was higher than that of promethazine.

E. japonica leaves principle as well as its constituents possess allergen denaturing

activity against OVA protein, respectively. They merit further study as potential allergen denaturants or lead molecules which control OVA populations through suppression of IgG-mediated allergic response for the prevention or eradication of egg allergic disorders. In addition, in the light of global efforts to reduce the level of toxicity and side effects of drugs, it is suggested that the use of plant materials could considerably modify symptoms of allergic disorders as allergen denaturants are warranted.

Key words: Allergen denaturing activity, Egg allergy, Ovalbumin, *Eriobotrya japonica*, Ovalbumin, Antigenicity, Allergenicity

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INTRODUCTION

Allergy to egg is one of the most common food hypersensitivities in infants and young children. The estimated prevalence of egg allergy varies between 1.6 % and 3.2 % and was documented in two thirds of the children found to be food sensitive by ascertainment method or definition in Oslo, Norway (Heine et al., 2006). The range of clinical signs is broad which produce a variety of allergens causing allergic symptoms, such as asthma, atopic dermatitis, conjunctivitis, and perennial rhinitis in sensitive humans. It encompasses life-threatening anaphylactic shocks in 11.6 % of cases, who had food induced anaphylaxis (Morisset et al., 1995). These costs will impact directly, indirectly and intangibly on both individuals and society in general. In the UK, the market for allergies and intolerances of foods has increased by 165% since 2000 and was forecasted to increase of 138 million ECU by 2007 (Miles et al., 2005). Strict avoidance of the offending food remains the most common recommendation for egg allergic individuals; however, strict elimination diets might be lead to malnutrition. Furthermore, the omnipresence of egg-derived components in cooked or manufactured food products renders total avoidance difficult and thus makes inadvertent exposure and ensuing adverse reactions. (Allen et el., 2007). Egg is composed of 56-61 % of egg white and 27-32 % of egg yolk and egg white contains more allergenic proteins than the yolk such as ovomucoid (*Gal d 1*), ovalbumin (*Gal d 2*), ovotranferrin (*Gal d 3*), and lysozyme (*Gal d 4*) (Mine and Yang, 2008). One of the dominant allergens ovalbumin constitutes about 54 % of egg white consisting of 385 amino acids with molecular

weight of 45 kDa (Nisbet et al., 1981). Promethazine is a currently available protein-denaturing agent as the antihistamine substances and illustrated in the protection which gives against the edema and haemoconcentration caused by injection of egg-white into normal or adrenalectomized rats (Halpern et al., 1950). Antihistamines have been used in an attempt to modify symptoms of food-induced allergic disorders; however, they have not only minimal efficacy but side effects which are not unacceptable to patients (Sampson, 1999). These are, therefore, a critical need for the development of selective egg allergen-denaturing alternatives with novel target sites.

Plant secondary substances have been suggested as alternative sources for anti-allergic products, largely because they constitute a potential source of bioactive chemicals that have been perceived by the general public as relatively safe, with minimal impacts to human health, and often act at multiple and novel target sites (Raskin et al., 2002; Wink, 2006). Much effort has been focused on plant preparations as potential sources of commercial allergen-denaturing products. These potential new products can be applied to egg allergen populations in the same manner as the egg allergen denaturants currently used. Historically, *Eriobotrya japonica* Lindl. (Rosaceae) has long been used as a hypoglycaemic and anti-inflammatory agent for the treatment of chronic bronchitis, coughs, phlegm, high fever and ulcers (Perry, 1980). No information has been obtained concerning the potential of *E. japonica* leaf-derived materials to modify egg allergic disease), despite its excellent pharmacological action such as anticancer, antiallergic, blood glucose-lowering, antihepatopathic, and antinephropathic activities (Onogawa et al., 2009).

In this study, an assessment is made of the egg allergen-denaturing activity of the constituents that comprise *E. japonica* leaves and another 26 known compounds of the plant leaves against ovalbumin using a immunoblot assay and a competitive indirect enzyme-linked immunosorbent assay (ciELISA) based on the inhibition of IgG-mediated type III allergy. In addition, IgE-binding reactivity related to type I allergy was investigated using human serum. The allergen-denaturing activity of the constituents was compared with that of a conventional antihistamine agent promethazine.

LITERATURE REVIEW

1. Clinical presentation of allergy

Certain foreign substances of various origins can invade the body and cause a hypersensitive reaction of the immune system, called allergy. Allergic reactions occur to normally harmless environmental substances also known as allergen (Mine and Yang, 2008). Harmless substances include pollen, food, genetic basis and the venom of stinging insects (Figure 1).

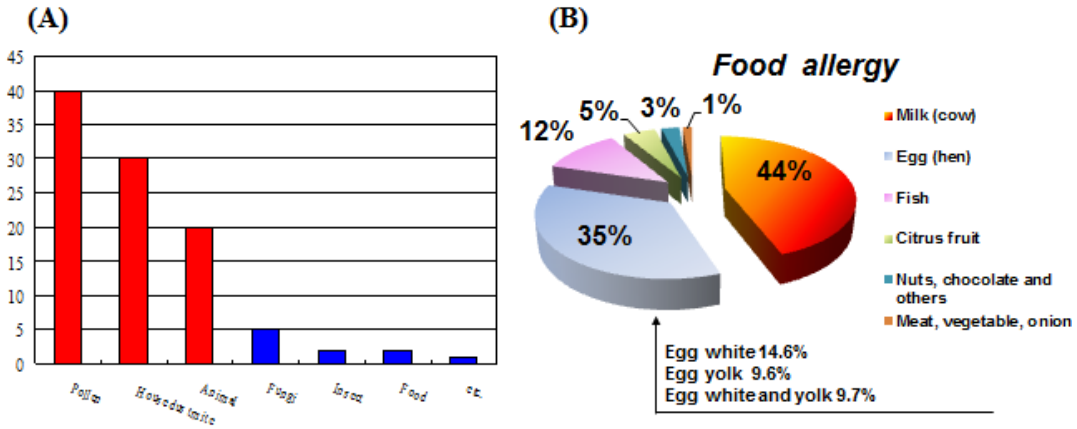

Figure 1. Diagram of main allergies (a) and food allergy prevalence (b) (Ahn et al., 2006)

These reactions are acknowledged, predictable, and rapid. Various factors and complex environmental interactions generate allergic reaction including not only air pollution and genetic basis but also westernized our lifestyle and a habit. Allergic disorder now poses a major public health problem and occurs as life-threatening

anaphylactic shock as well as rhinitis, asthma, and atopic dermatitis (Mine and Yang, 2008). The socio-economic costs of the major allergic diseases are estimated at 10 billion ECU (European Currency Unit) for direct costs and 19 billion ECU for indirect costs (Van Moerbeke, 1997). A food allergy results in the development of further socio-economic problems. Pilot studies have suggested that food allergy prevalence is increasing in some parts of the world, approximately 9.7 % of mans and 12.9 % of women are affected by food allergy in Korea (Han et al., 1997). The first indication for this increase is cross sensitization, which means that food allergy often occurs in combination with other allergy such as atopic dermatitis, allergic rhinitis, allergic asthma and urticaria (Jansen and Brussaard, 2001). Another indication is provided by the observation that allergy is becoming more common in younger generations and continue to suffer from symptoms as they get order whereas food allergy usually resolves by school age in the past (Hourihane et al., 1996). The last indication is the fact that, people is increasingly exposed to a wider diversity of food antigens as a consequence of the increase of global trade (Jansen and Brussaard, 2001). Indeed, factors related to western lifestyle in developing countries appear to be the strongest determinant in the increasing of food allergy, so these varieties of allergies hasn’t yet become an issue in Korea; however, there is a high chance that it may become a serious issue in the future.

1.1 Allergic reaction

Allergic inflammation is classified into four types; type 1, type 2, type 3 and type 4 (Anderson, 1996). These are caused by the antibodies, whereas type 4 is mediated by lymphocytes or macrophages (Hayakawa et al., 1999). Anderson reported that the early stage of the allergic reaction usually occurs within minutes followed by allergen exposure and it is also commonly referred to as the immediate allergic reaction or a type 1 allergic reaction which involves IgE antibodies. Type 3 involves IgG antibodies, and neutrophils, which clump together to form large immune complexes in the blood (Sampson, 1990). Sampson reported that it is highly related to food allergy because IgG antibody tests usually detect chronic allergies, especially to milk, eggs, and gluten grains. These deposit in the organs and tissues, where they cause inflammation and tissue damage, leading to chronic degenerative symptoms or diseases, such as autoimmune conditions, arthritis or kidney disease (Hayakawa et al., 1999). A common approach to evaluating the stability of egg allergens is to examine alterations of their IgE/IgG-binding capacities, in practice, the binding activities of the four major egg allergens to egg allergic patients' specific IgE/IgG and rabbit-specific IgG were studied (Mine and Zhang, 2002).

1.2 Egg allergy

Allergens in egg are one of the most common causes of food allergic reactions, particularly in children. The prevalence of egg allergy is develops within the first two years of life and it usually disappears by age five to seven years, but may sometimes be

lifelong (Mine and Rupa, 2004). Hen's egg is among the most commonly implicated food, representing the second cause of food allergy nest to peanut, in combination with peanut and cow's milk allergies, they represent up to 80 % of food allergy cases in infants (Rolinck-Werninghaus et al., 2005). A few studies have suggested that early childhood sensitization to hen's egg may favor the subsequent development of respiratory allergies and occupational asthma has been associated with the inhalation of aerosolized dried egg powder in adults, leading to the development of IgE-mediated food allergey upon egg ingestion (Tariq et al., 2000; Escudero et al., 2003). Dietary avoidance of egg-containing food is the main principle of treating egg-allergic individuals. However, elimination diets in young children may be associated with adverse nutritional outcomes, and growth parameters should be closely monitored (Isolauri et al., 1998). On the other hand, oral immunotherapy with raw or cooked preparations of egg may represent a safe alternative, immediately available to allergic subjects and it is usually efficacious however this treatment has to be administered under the careful supervision of a trained physician and with the standby support of emergency treatment to only nonanaphylactic patients (Van Moerbeke, 1997).

1.3 Current clinical issues associated with egg allergy

In recent years, egg hypersensitivity has raised other issues related to the potential development of anaphylaxis subsequent to the administration of viral vaccines including influenza, yellow fever and measles-mumps-rubella (MMR) (Wong and Yuen, 2005). Importantly, they are prepared directly from egg embryos and this conventional method

of viral culture has dependence on high quality eggs. They may therefore have an immunologically more significant content of allergens, for example, ovalbumin and ovomucoid which can be induce adverse reactions when used in individuals with egg allergy (Teuber et al., 2006). Influenza is one of the most common infectious diseases and possesses high epidemic and pandemic potential. A total of 2,292 deaths have been reported since April 2009 in Europe and 14,378 deaths are documented all over the world (European Centre for Disease Prevention and Control). Clinical reports pertaining to the safety of influenza vaccines revealed controversy; even if current recommendations strongly advocate its safe use, it is necessary to perform skin patch test to egg and skin testing of influenza vaccine. Egg allergy is, therefore, still considered a relative contraindication to immunization against influenza, particularly in individuals with a history of egg anaphylaxis (Heine et al., 2006).

2. Medical importance of egg allergen

The major egg allergens were found to be mainly egg white proteins, but there are uncommonly those who are allergic to the yolk proteins apovitellenins I, apovitellenins VI and phosvitin (Mine and Yang, 2008). Early studies involving a cohort of 342 patients reported that the major egg allergens were, in increasing order, ovomucoid, ovalbumin, ovomucin, lysozyme, based on skin tests (Miler et al., 1950). Whereas major egg allergen ovomucoid has been the focus of a number of investigations, only a few studies have explored the immunological properties minor egg allergens including

ovalbumin. The latter study strongly suggests that their clinical significance warrants further investigations (Mine and Yang, 2008).

2.1 Allergenic components of the egg

Among these allergens, ovalbumin is a phosphoglycoprotein and the major protein in avian egg white, comprising 54 % of its total protein content (Mine and Yang, 2008). Previously, ovalbumin was considered the main egg allergen but later studies suggested that the glycoprotein ovomucoid was the immunodominant allergen in egg white (Bernhisel-Broadbent, 1994). Ovalbumin has been widely used as a standard model for studies of protein various properties because it has been unveiled on its structural (Shirai et al., 1997), functional (Doi and Kitabatake, 1997) and immunological properties (Renz et al., 1993). The previous study also has revealed relationship between functional properties and structure of ovalbumin which gives that conformational changes in ovalbumin resulted from the addition of anionic, cationic and amphoteric detergents, denaturants, organic and inorganic acids induced changes of various functional properties (Zemser et al., 1994). Epitope mapping of the entire ovalbumin molecule has been reported very recently. Studies involving both egg-allergic patients and murine model sera have examined both IgE- and IgG-binding sites present on ovalbumin (Mine and Rupa, 2003). With regard to T-cell epitopes were specifically recognized by T-cell obtained from atopic dermatitis egg-allergic patients (Katsuki et al., 1996).

2.2 Biochemical properties of ovalbumin

Ovalbumin consists of a five-stranded ß-sheet running parallel to the long axis of the molecule and an α-helix protruding as a loop that forms the reactive center (Mine and Yang, 2008). Ovalbumin are treated various additives or heating during preservation food processes and are partially denatured, modifying their physical, chemical, and biological properties. However, regarding the effects of treatment on ovalbumin structure, it is possible that some of ovalbumin fragments still retain their antigenicity. In the previous study, their results demonstrate that dramatic conformational changes in ovalbumin induced by irradiation, treatment with heat and urea and guanidine denaturation can be effected; however, the newly antigenic sites are then exposed on the molecular surfaces (Masuda et al., 2000). On the other hand, in case of ovomucoid, the peptic digestion and cyanogen bromide cleavage induced conformational changes and decreased the trypsin inhibitory activity but did not completely destroy antigenic sites analyzed by immunodiffusion and Passive Cutaneous Anaphylaxis (PCA) test (Beeley, 1976; Matsuda et al., 1983).

3. Clinical manifestations of egg allergy

Allergic rhinitis is usually called hay fever and describes an inflammation of internal nose. The chances of allergic rhinitis are higher in developed countries whereas chances of asthma are higher in underdeveloped and developing countries (Van Moerbeke, 2004). Asthma is a common chronic inflammatory disease of the airways characterized by variable and recurring symptoms (Mine and Yang, 2008). According to recent studies in

the United States, nearly 25 million Americans suffer from allergic asthma (Asthma and Allergy Foundation of America) and 250,000 people died from asthma throughout the world in 2009 (Global Strategy for Asthma Management and Prevention, 2011). Atopic reactions cause by localized hypersensitivity reaction to an allergen and show a strong hereditary component (Mine and Yang, 2008). Also, atopic reactions developed 10 to 20 percent under age 7 in recent ten years (Van Moerbeke, 2004). Gastrointestinal manifestations account for a minority of egg allergic manifestations, however, involved in a variety of gastrointestinal disorders, including allergic proctocolitis and eosinophilic esophagitis (Spergel et al., 2002). In a study of 95 infants with food protein-induced proctocolitis, 19 % responded to dietary elimination of egg (Lake, 2000).

3.1 Diagnosis of egg allergy

The diagnosis of egg allergy is determined by skin prick tests (SPT), radio-allergosobent (RAST) assay and the double-blind placebo-controlled food challenge (DBPCFC). SPT is easy to do and results are available in minutes. This test is performed to identify which allergen is responsive for certain patients, they used tiny amount of the suspected allergen with a needle and inject it on the patient's skin (Sampson, 1999). This is a process to detect if a person is allergic to any particular antigen or not. RAST assay detects the presence of IgE antibodies to a particular allergen as a blood test another useful diagnostic tool for evaluating IgE-mediated food allergies. The method is that IgE antibodies which drive immediate allergic reaction are reacted with certain antigen and IgE-binding antibodies are labeled with radioactive isotopes for quantifying

the levels of IgE antibody in the blood (Sampson and Ho, 1997). DBPCFC is a good standard for diagnosis of food allergies. Blind food challenges involve packaging the suspected allergen into a capsule or food, giving it to the patient, and observing the patient for signs or symptoms of an allergic reaction (Bock et al., 1988). The purpose of this test is to identify that aroused allergic reaction is related to certain allergen or other factors such as food intolerance and cross sensitization. However, clinical diagnosis is possible to give different results with other methods because it is highly dependent on method used (Margreet et al., 2006).

3.2 Management of egg allergy

There are much management to control food allergen population such as avoidance, treatment and immunotherapy. An important step of food allergy management is prevention. Primary prevention means blocking of immunologic sensitization and appears to be beneficial for infants with an atopic family history (Zeiger, 2003). It is counted as an essential method however this procedure cannot identify the risk of cross-reactions generated by response of interactions among various allergens as the case with birch allergens (Smith, 1997). Secondary prevention is interpreted as suppression of disease symptoms after immunologic sensitization has occurred. The method is associated with avoidance of the allergen, especially in food case, strict elimination diets may lead to malnutrition (Sampson, 1999). Also, an elimination of a single food can be difficult when the food is used in many other food products, such as egg. Tertiary prevention represents the stage in which symptoms are treated and worsen of the patient's situation. Apart from H_1 receptor antagonists, the most frequently prescribed

drugs are selective ß2 agonists and inhaled corticosteroids (Van Moerbeke, 1997). It should be noted, however, that where the steroid, topical antihistamines and cromoglycates are concerned, there are major differences in prescribing and frequencies. Consequently, it is nothing more than treatment of the symptoms and side effects unacceptable to patients are reported (Sampson, 1999). Immunotherapy provides some protection against the effects of natural allergenic exposure by providing regular administration of the allergens. It is usually efficacious however this treatment has to be administered under the careful supervision of a trained physician and with the standby support of emergency treatment (Van Moerbeke, 1997). Given that these still represent an important part of food allergy management, it is necessary to develop the complete strategy to decrease the food allergy prevalence effectively.

4. Current preventive and therapeutic options for egg allergy

Food-processing methods are expected alter the allergenicity of food proteins in various ways. With the intention to develop approaches for reducing egg allergenicity in food products, a number of studies have investigated the use of food-processing methods (Mine and Yang, 2008). Earlier in vitro studies showed that thermal treatment of egg white led to a >50% decrease of RAST binding intensity using 16 egg allergic patient's sera (Anet et al., 1985). On the other hand, residual IgG-binding activity against ovalbumin and ovomucoid could still be detected in boiled eggs, as tested by radio-immunoelectrophoresis (Hoffman, 1983). Enzymatic processing represents a specific approach and reduction in egg allergenicity would be expected when egg

allergen epitopes are eliminated by the enzymatic fragmentation (Wal, 2003). The allergenicity of egg proteins, however, depends a great deal on their strong resistance digestive enzymes (Astwood, 1996) and another major hurdle is to maintain the unique functional properties of egg protein, for example, foaming and gelling (Mine and Yang, 2008). In addition, γ-irradiation superior to 10 kGy altered the structure of ovalbumin and led to a significantly decreased immunogenicity, thus make a hypoallergenic form of the protein (Seo et al., 2007).

4.1 Clinical chemotherapeutic agents and their modes of action

The effects of chemicals on controlling food allergy and their allergen for the production of hypoallergenic egg-containing products have been studied. Certain chemicals exhibit reduction in IgG binding activity, especially carboxymethylation decreased their antigenicity (Mine and Zhang, 2002). A number of attempts were also made to chemically modify allergens for use in specific immunotherapy that glutaraldehyde and formaldehyde were among the first reagents to be used (Hayglass and Stefura, 1990), followed by carbamylation strategies (Bagnasco et al., 2001). In addition, many chemicals are involved in medication of symptoms, for instance, indomethachin, aspirin and salicylate are used as anti-inflammatory drugs which are strongly inhibited the synthesis of prostaglandins (Ferreira and Vane, 1974) (Figure 2).

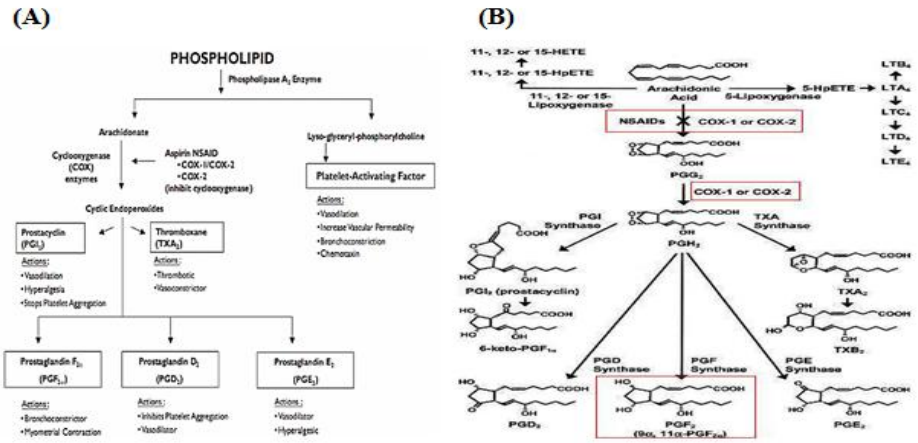

Figure 2. Prostaglandin biosynthesis pathway (a) and inhibiting mechanism of aspirin (b) (Palmer, 1990)

There are also aspirin-like drugs which seem to be a unique character, for compounds representing many other type of pharmacological activity were active. These included chloroquine, morphine, mepyramine, probenecid, azathioprine, bydroxybenzodic acid, promethazine, atropine, methysergide and phenoxybenzamine (Ferreira and Vane, 1974). However, as mentioned above, studies found that ovalbumin can retain its human IgE binding capacity after chemical treatment such as reduction and carboxymethylation, heat, or urea, indicating that IgE epitopes are linear and thermostable (Mine and Zhang, 2002).

4.2 Alternative medicine

Promethazine has an action on capillary permeability and this is clearly illustrated in the protection which these substances give against the edema and haemoconcentration caused by injection of egg-white into normal or adrenalectomized rats (Halpern and

Briot, 1950). This action of antihistamine substances on edema produced by egg-white has also been found by other studies (Legar and Masson, 1948; Clark and Mackay, 1949). The mode of action of promethazine was reported that the same explanation with adrenaline could account for the reduction by promethazine of the haemoconcentration produced by histamine. The adrenaline acts directly by reducing the sensitivity of the capillaries to the action of histamine, which increases their permeability (Halpern and Wood, 1950). On the other hand, other finding have also been reported which support this action of the synthetic anti-histamine substances on capillary permeability. When histamine or other irritant substance is injected intraperitoneally in rabbits, exudation of fluid occurs into the peritoneal cavity but the rate of diffusion is much reduced when the animal has previously received promethazine (Halpern, 1948). It is probable that the protective action of promethazine against the lethal effect of histamine. The only registered antihistamine for parenteral use in Australia, promethazine, can worsen vasodilation and hypotension, and its use is not advised (Brown, 2006). The use of traditional antihistamines such as promethazine is often associated with a number of unwanted and undesirable central side effects, the most troublesome of which is sedation (Hindmarch and Shamsi, 1999). Also, there are many other side effects which include vertigo, drowsiness, dry mouth, irritability, nervousness, insomnia and disorientation. It has been shown that promethazine in wheal suppression had high mean side effect score compared with chlorpheniramine and tripelennamine which, however, ranked second in effectiveness and duration of wheal size suppression using skin test (Cook et al., 1973).

5. Natural products and egg allergen

New antiallergic medication should be developed not only in terms of their immediate beneficial effect on symptoms and comparative costs, but also with respect to their impact on the quality of life and the long term reduction of allergic reactions. Therapy targets fall into four categories: down-regulation of inflammation, relief of symptoms, avoidance of exacerbation and maintenance of the quality of life (Van Moerbeke, 2004). These targets will have a considerable impact on the treatment of allergy. Natural products of plant origin have an advantage in these targets based on their long term use by humans. The value of plants is as a methodology of medicinal agents, which, according to the World Health Organization (WHO), almost 65 % of the world's population has incorporated into their primary modality of health care (Farnsworth et al., 1985). One might expect any bioactive compounds obtained from such plants to have patentable entities of low human toxicity and/or higher activity (Fabricant et al., 2001). The Central Drug Research Institute (CDRI) evaluated approximately 2,000 plant species for several biologic activities, including anti-inflammatory, antitumor, cytotoxicity, antibacterial, antidiabetic, antifungal, cardiovascular and others (Dhar et al., 1968). Botanical active molecules have been widely investigated for their anti-inflammatory values in humans and trials as a form for alternative treatment for immunological conditions such as asthma or allergy (Bielory, 2007). It demonstrates that variety of phytochemicals have an immunomodulatory function, suggesting its potential use in the alleviation of egg allergic symptoms.

5.1 Approaches to drug discovery using plants

Plant secondary substances may provide potential sources of antiallergen agents. This approach is appealing, in part, because they constitute a potential source of bioactive chemicals that have been perceived by the general public as relatively safe and often act at multiple and novel target sites (Raskin et al., 2002; Wink, 2006). Much effort has been focused on plant preparations and their constituents as potential sources of commercial antiallergen products for prevention or eradication of egg allergy. It is difficult to use because of limited either by the natural occurrence or the availability of a given plant species as well as knowledge about the potential pharmaceutical application (Hänsel and Sticher, 2009). Each phytochemical has different effect because of characteristic of plant species, difference in a plant species, part, geographical location, and extraction and application methods (Fabricant and Farnsworth, 2001). Therefore, collecting plants on the basis of their ethnomedical claims requires considerable preliminary planning to determine where each plant grows, what the abundance of each plant is, whether any of the plants are threatened or endangered, what local arrangements must be made to collect the plants and whether local botanists familiar with the flora of the region are available to assist (Fabricant and Farnsworth, 2001).

5.2 Anti-inflammatory properties of plants

Many plant secondary substances present significant anti-inflammatory effects. For this reason, they represent potential molecules for the development of new drugs, especially designed for the treatment and control of chronic inflammatory states such as

rheumatism, asthma, inflammatory bowel diseases, and atherosclerosis (João et al., 2003). A substantial body of evidence obtained from both *in vivo* and *in vitro* studies supports the concept that various plant-derived compounds with anti-inflammatory properties exert their effects through the modulation of the cytokine system (Habtemariam, 2000). For instance, one study has reported that luteolin quertcetin and the isoflavonoid genistein, a class of phenolic compounds widely distributed throughout the plant kingdom, inhibited LPS-stimulated TNFα and interleukin-6 release in RAW 264.7 macrophages (Xagorari et al., 2001). In addition, celastrol, a class of terpenes, decreased the production of the pro-inflammatory cytokines TNFα and IL-1ß in human monocytes and macrophages (Sylvester et al., 2001), the alkaloid piperlactam S isolated from *Piper kadsura* inhibited C5a-induced release of TNFα and IL-1ß in RAW 264.7 macrophages (Chiou et al., 2003).

5.3 Natural history of *Eriobotrya japonica*

Historically, the loquat, *Eriobotrya japonica*, has been used for inflammatory disease and chronic bronchitis that is widely cultivated for its succulent fruit. The loquat is effective to treat asthma, coughs, and high fever as to drink its tea. In particular, leaves of the loquat have been employed to control dermal disorders or relieve inflammation, pain, cough, and sputum (Liang et al., 1990). The leaves contain polyphenols and have the blood glucose-lowering (De Tommasi et al., 1991), anti-inflammatory (Huang et al., 2006), and anticancer actions (Ito et al., 2000). Various triterpenes, flavonoids, tannins and megastigmane glycosides have been found in the leaves of *E. japonica*, and some of

these compounds such as oleanolic acid, 2-α-hydroxyursolic acid, maslinic acid and 7-O-ß-D-glucopyranoside are reported to have wide range of activities such as antiallergic, anti-inflammatory, anti-HIV and antibacterial action (Shimizu et al., 1986; Taniguchi et al., 2002). *E. japonica* seeds contain amygdalin (Gray and Fowden, 1972), unsaturated fatty acid, such as linoleic acid and linolenic acid, and plant sterols, such as ß-sitosterol, caffeic acid, chlorogenic acid and various essential amino acids (Yokota et al., 2006). They are effective for prevention and treatment of disorders, such as hepatopathy (Nishioka et al., 2002), nephropathy (Hamada et al., 2004), type 2 diabetis (Tanaka et al., 2008) and allergic contact dermatitis (Sun et al., 2007).

6. Perspectives of development of new drugs for egg allergy

It is essential to achieve a better understanding of the development of allergen denaturants leading to long term efficacy and quality control of life. Measuring egg allergen exposure will become progressively important, as too will the impact of identified allergens due to the increased patients by the westernized lifestyle and diet. In the investigative sphere, cells, mediators, cytokines and new lead molecules should be further studied to treat allergic reaction effectively (Van Moerbeke, 2004). Antagonists to mediators other than histamine are already emerging from basic research, while the new research field of natural compounds offers interesting prospects. Nontoxic medications for human are needed; efforts are being made to improve the efficacy of therapy and such newly identified compounds may give greater prominence to the role of therapy in the future.

CHAPTER 1. Allergen denaturing activity of *Eriobotrya japonica* leaf constituents

INTRODUCTION

Studies have yield evidence that 15 to 30% of allergic infants remain allergic to milk or egg for longer periods, while allergy to peanut and fish appears to persist for several years in the majority of cases (Van Moerbeke, 2004). However, use of traditional antihistamines has until recently been associated a number of undesirable side effects, the most troublesome of which is sedation (Hindmarch and Shamsi, 1999). Therefore, development of egg products safe for human consumption complemented with efficient forms of therapy is highly warranted (Mine and Yang, 2008). These problems highlighted the need for the development of alternative allergy treatments. Most of plant derived compounds affect minimal toxicity to humans and are accepted relatively safe to general, over 160 natural ingredients have identified that are used or promoted for treating or preventing allergic disorders including asthma (Woldemariam et al., 2012). In the screening of plants for allergen denaturing activity, a methanol extract from the leaves of *Eriobotrya japonica* was shown to have allergen denaturing activity toward ovalbumin. No information, however, has been done to assess the potential of E. japonica leaf-derived materials to treat or prevent allergic disorders.

In this chapter, an assessment is made of the allergen denaturing activity of three constituents identified in *E. japonica* leaves and another twenty-seven previously known constituents (Sun et al., 2007) toward commercial ovalbumin. The allergen denaturing activities of the active principles were compared with those of the most commonly used antihistamine promethazine.

MATERIALS AND METHODS

1. Instrumental analyses

^{1}H and ^{13}C NMR spectra were recorded in CD_3OD on a Bruker AM-500 spectrometer (Rheinspettem, Germany) at 600 and 150 MHz (tetramethylsilane as an internal standard), respectively, and chemical shifts are given in δ (ppm). UV spectra were obtained in methanol with a Kontron UVICON 933/934 spectrophotometer (Milan, Italy) and mass spectra on Jeol GSX 400 spectrometer (Tokyo, Japan). Merck silica gel (0.063–0.2 mm) (Darmstadt, Germany) was used for column chromatography. Merck precoated silica gel plates (Kieselgel 60 F_{254}) were used for analytical thin-layer chromatography (TLC). A Biotage Isolera One medium-pressure liquid chromatograph (MPLC) (Uppsala, Sweden) and an Agilent 1200 series high-performance liquid chromatograph (HPLC) (Santa Clara, CA) were used for isolation of active principles.

2. Materials

The twenty-seven chemicals used in this study are listed in Table 1. Ovalbumin, Coomassie brilliant blue, sodium dodecyl sulfate (SDS), Promethazine, and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS) and skim milk were purchased from Sigma-Aldrich and BD Difco (Becton Drive, NJ), respectively. Precision Plus Protein Standards was supplied by Bio-Rad Laboratories (Hercules, CA). Rabbit anti-ovalbumin IgG and goat polyclonal secondary antibody-H&L(HRP) were purchased from Abcam (Cambridge, UK). ECL Western blotting detection reagent and nitrocellulose membrane strips were purchased from GE

Healthcare Life Sciences (Buckinghamshire, UK). 96 well plates, 3,3',5,5' tetramethylbenzidine liquid substrate system for ELISA was supplied by SPL life science (Pochen, Korea) and Sigma-Aldrich, respectively. All of the other chemicals and reagents were of reagent-grade quality and available commercially.

Table 1. The twenty-seven compounds examined in this study

Compound	Source	Compound	Source
p-anisaldehyde	S-A[a]	n-Hexadecan	S-A
(L)-(+)-ascorbic acid	MC[b]	3-hydroxybenzoic acid	S-A
Caffeic acid	S-A	Kaempferol	S-A
Campesterol	TM[c]	Lactic acid	HW[d]
Chlorogenic acid	S-A	Methyl Salicylate	KT[e]
Citric acid	S-A	Nerolidol	S-A
p-coumaric acid	S-A	Oleanolic acid	WK[f]
Ellagic acid	S-A	Palmitic acid	S-A
(-)-Epicatechin	S-A	Quercetin	TCI[g]
(-)-Epigallocatechin gallate	S-A	Rivoflavin	S-A
Farnesol	S-A	Rutin trihydrate	TCI
Ferulic acid	S-A	β-sitosterol	S-A
Gallic acid	S-A	(D)-(-)-tartaric acid	S-A
Heptadecan	S-A		

[a] Purchased from Sigma-Aldrich (St. Louis, MO).

[b] Purchased from Merck (Washington, USA).

[c] Purchased from Tama (Kawasaki, Japan).

[d] Purchased from Hanawa (Fukushima, Japan).

[e] Purchased from Kanto (Tokyo, Japan).

[f] Purchased from Wako (Osaka, Japan).

[g] Purchased from Tokyo Chemical Industry (Tokyo, Japan).

3. Plant

Air-dried leaves of *E. japonica* was purchased from Boeun medicinal herb shop (Kyoungdong market, Seoul) and used for extraction. A voucher specimen (HDB–01) was deposited in the Research Institute for Agriculture and Life Science, Seoul National University.

4. Ovalbumin extracts

Commercial ovalbumin (5 mg/ml) was prepared in 0.01 M phosphate buffer (pH 7.2) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE). For immunoblot assay, the mixture was heated for 10 min at 60℃ and centrifuged for 10 min at 10,000 × *g*. The supernatant was diluted with phosphate buffer to 3 mg/mL. Ovalbumin solutions were stored at –20℃ until use.

5. Extraction and isolation

The dried leaves (600 g) from *E. japonica* were pulverized and extracted with methanol (3 L) three times at room temperature and filtered using Whatman no. 2 filter paper (Maidstone, UK). The combined filtrate was concentrated under vacuum at 42℃ to yield approximately 11.70% on the dark green tar (based on the weight of the dried leaves). The extract was subsequently partitioned into hexane-, chloroform-, ethyl acetate-, butanol-, and water-soluble portions for subsequent bioassay (Figure 3). The organic solvent-soluble fractions were concentrated to dryness by rotary evaporation at 42℃, and the water-soluble fraction was concentrated at 48℃. For isolation of active

principles, on the basis of preliminary results, 25 mg/ mL of each *E. japonica* leaf-derived material was tested in an immunoblot bioassay.

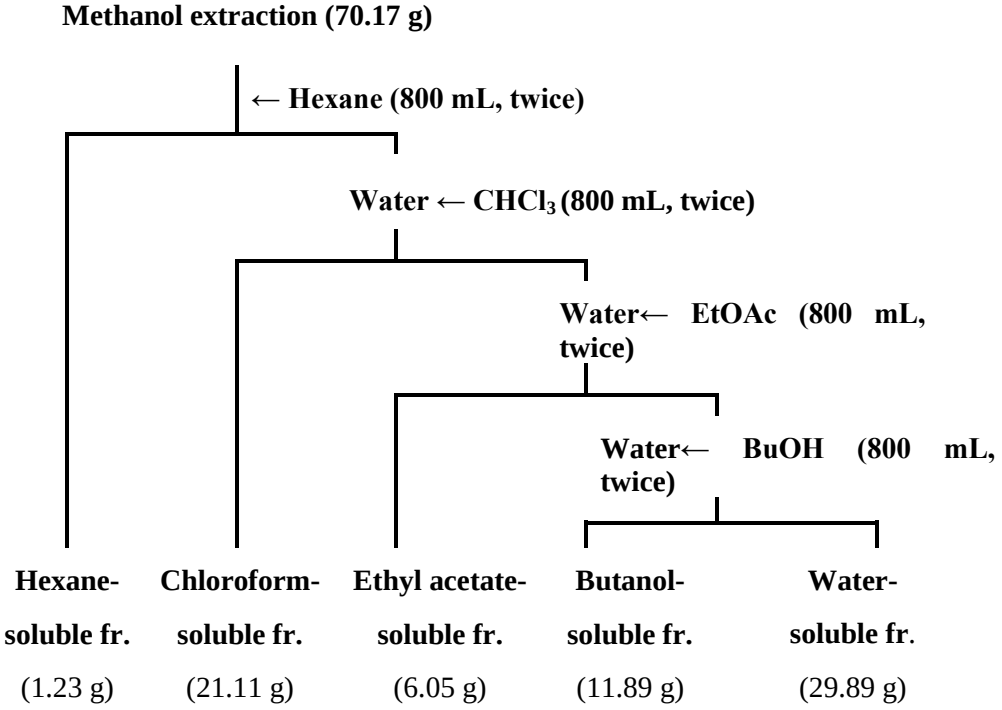

Figure 3. Solvent fraction procedures of methanol extract from *E. japonica*

Due to its potent allergen denaturing activity toward ovalbumin, a MPLC was used for separation of the constituents from the chloroform-soluble fraction (21.11 g) (Figure 4). The column was a 71 mm i.d. × 168 mm Biotage SNAP silica (340 g) column and eluted with a gradient of chloroform and methanol [100:0 (0.5 L), 99:01 (0.5 L), 98:02 (3 L), 97:03 (2 L), 95:5 (4 L), 90:10 (4 L), 80:20 (5 L) and 0:100 (1 L) by volume] to provide 66 fractions (each about 240 mL) at a flow rate of 1 mL/min. Column fractions were monitored by TLC on silica gel plates with chloroform and methanol (95:5 by volume). Fractions with similar R_f values on the TLC plates were pooled. Spots were

detected by spraying with 2% H_2SO_4 and then heating on a hot plate. Of the eight fractions, the active fractions 30 to 36 (C5, 7.32 g) was separated by MPLC with SNAP (120 g) column, eluted with a gradient of chloroform and methanol [100:0 (1 L), 99:01 (1 L), 98:02 (1 L), 97:03 (2 L), 95:5 (3 L), 90:10 (4 L) and 0:100 (1 L) by volume] to provide 130 fractions (each about 22 mL) at a flow rate of 1 mL/min. Column fractions were monitored by TLC on silica gel plates with chloroform and methanol (95:5 by volume). Of the eight fractions, the active fractions 103 to 129 (3.32 g) was separated by MPLC with SNAP (120 g) column, eluted with a gradient of chloroform and methanol [100:0 (4 L), 99:01 (4 L), 98:02 (6 L), 97:03 (5 L), 96:04 (4 L), 95:5 (3 L), 90:10 (3 L) and 0:100 (1 L) by volume] to provide 190 fractions (each about 22 mL) at a flow rate of 1 mL/min. The active fractions 120 to 154 (C574, 1.1 g) was chromatographed on a 5.5 cm i. d. × 70 cm silica gel (580 g) column by elution with methanol and water (95:5 by volume) to provide 44 fractions (each about 250 mL). Column fractions were monitored by TLC on silica gel plates (Merck RP-18 $F_{254}S$) developed with methanol and water (95:5 by volume). Two active fractions 12 to 15 (C5746, 244 mg) and 40 to 44 (C5748, 302.1 mg) were obtained. A preparative HPLC was used for separation of the constituents from the C5746. The column was a 7.8 mm i.d. × 300 mm Waters μBondapak C18 (Milford, MA) with a mobile phase of methanol and water (8:2 by volume) at a flow rate of 1 mL/min. Chromatographic separations were monitored using a UV detector at 210 nm. Finally, two active principles **1** (C57463, 9.44 mg) and **2** (57468, 8.52 mg) were isolated and identified at a retention time of 50.09 min using GC and 20.59 min using HPLC, respectively. Another active fraction C5748 were purified

by preparative TLC [chloroform: methanol (95:5) by volume] to provide compound **3** (35.11 mg, R_f = 0.21).

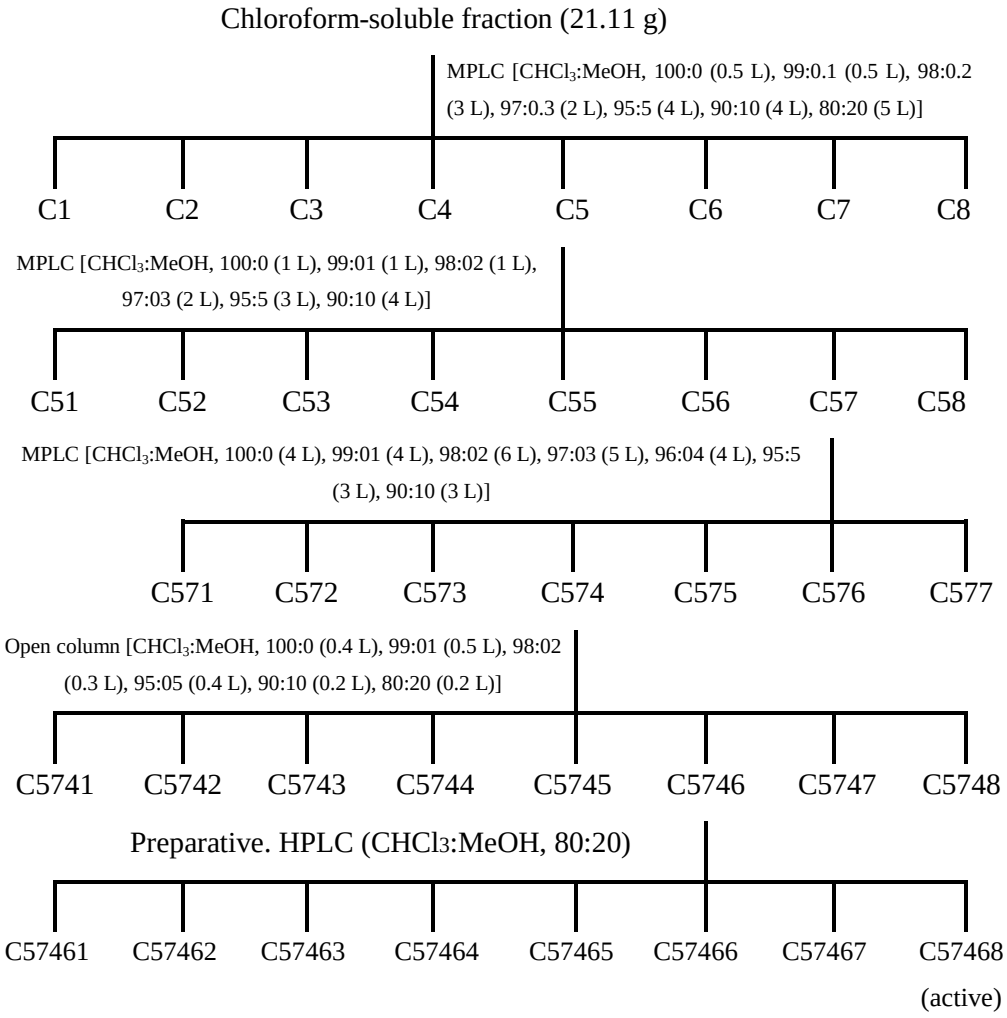

Figure 4. Isolation procedures of *E. japonica* leaves derived compound

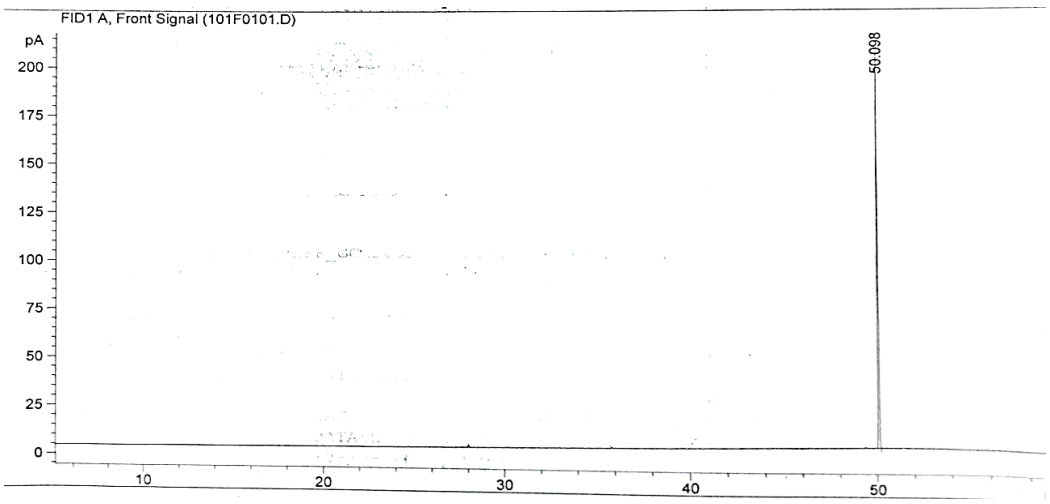

Figure 5. GC chromatogram of compound 1

Table 2. Operating conditions for preparative HPLC

HPLC system	Agilent 1200 series with binary solvent pump
Column	Reverse phase ZORBAX Eclipse XDB-C18 Particle: 5 µm Pore size: 80 Å Column size: 4.6 mm ID x 250 mm
Solvent system	Methanol 80%, Water 20%
Flow rate	1 ml/min
Detector	Variable Wavelength Detector, Agilent Technologies

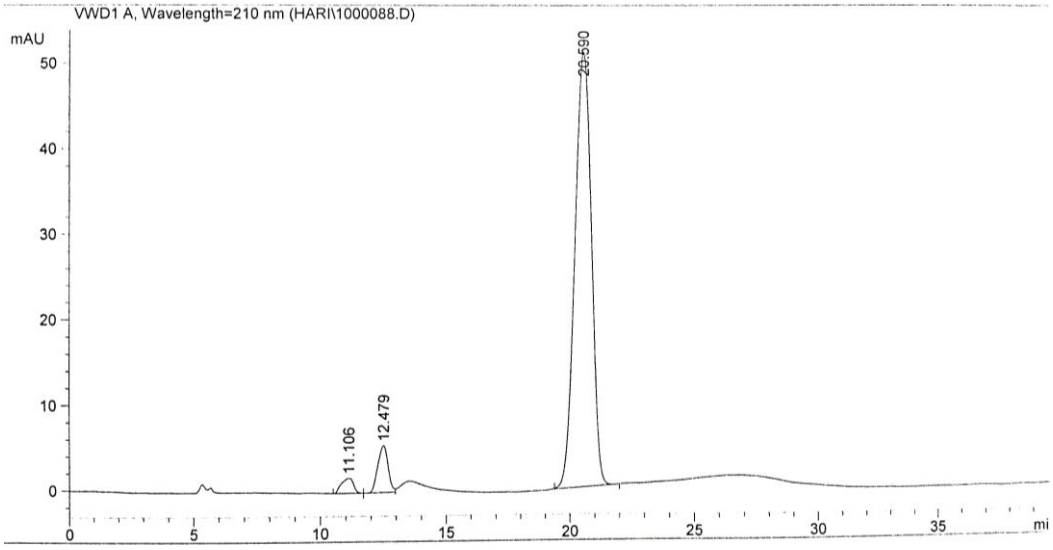

Figure 6. HPLC chromatogram of compound 2

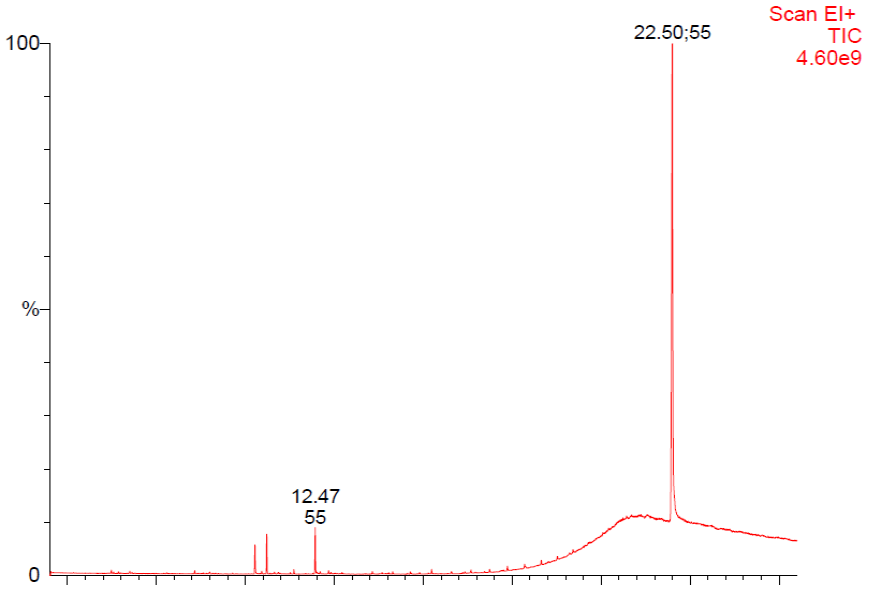

Figure 7. GC chromatogram of compound 3

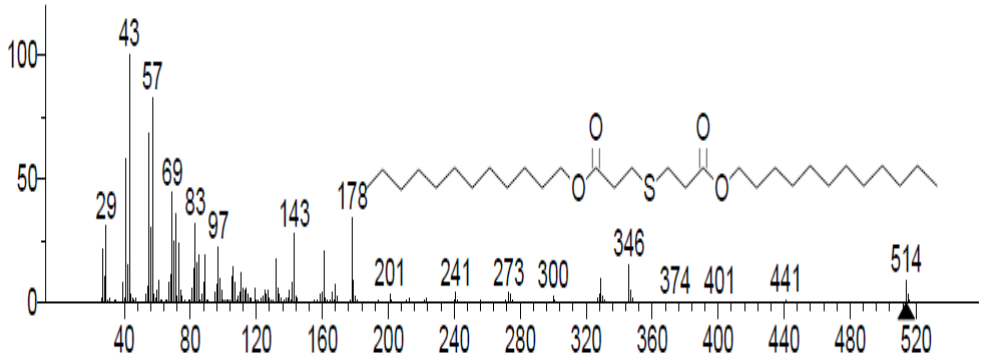

Figure 8. GC-MS spectrum of retention time 22.50 peak

(Dilauryl Thiodipropionate)

6. Sodium dodecyl sulfate-poly acrylamide gel electrophoresis analysis

SDS-PAGE was performed as described by Laemmli (1970) in 12% (w/v) polyacrylamide gels by using Bio-Rad mini-protean 3 electrophoresis cell (Hercules, CA). Ovalbumin extract (5 μg) and each test compound (1, 250, and 500 each) dissolved in 5 μL of dimethyl sulfoxide (DMSO) were incubated for 1 h at 25 ± 1℃. Samples were mixed with 5 × sample buffer (4% SDS, 4% mercaptoethanol, and 100 mM Tris-HCl) (pH 8.0). After boiling for 10 min, each sample was centrifuged for 5 min at 12,000 rpm at 4℃. The supernatant was then loaded onto gel. Gels were run at 80 V for 30 min and then 110 V for 50 min. The proteins were visualized by staining Coomassie brilliant blue. The intensity of the gel was determined using a Bio-Rad Gel Dox XR image analyzer (Herculex, CA) with Bio-Rad Quantity One software according to the

manufacturer's instructions. Negative controls consisted of 5 μL of DMSO. Promethazine served as a positive control and was similarly prepared on the test compounds.

7. Immunoblot assay

A Western-blot immunoassay for ovalbumin-specific IgG-binding reactivity were performed as described previously (Rupa and Mine, 2003). Each concentration of the test sample was dissolved in 5 μL of DMSO and protein solution which contains 3 μg of ovalbumin protein was mixed together. The mixture was incubated for 1 h in room temperature. After incubation, the mixture was electro-blotted onto the nitrocellulose membrane strips using a Bio-Rad Western blot apparatus (San Francisco, CA). It was blocked with PBS-T solution including 5% skim milk with shaking in the rotary shaker for 1 h. Then it was washed 4 times with PBS-T solution, each time for 15 min and incubated for overnight at 4℃ with rabbit anti-ovalbumin IgG which diluted in 1:20,000 with PBS-T solution including 5% skim milk. The membrane was moved into the PBS-T solution and washed 4 times, the membrane was bounded for 2 h with goat polyclonal secondary antibody-H&L (HRP) conjugate antibody which diluted in 1:20,000 with PBS-T solution including 5% skim milk on the rotary shaker. The membrane was washed 4 times with PBS-T for 15 min. The antibody was reacted with an ECL Western blotting detection reagent for 1 min and then covered by OHP film and X-ray film. It was exposed for 30 s or 1 min in darkness. The denaturing activities (DA) of each test sample were calculated by Bio-Rad Quantity one software (Hercules, CA) and Gel Doc XR image analyzer (Bio-Rad, Hercules, CA). The value of DA was calculated by

following formula: DA = [($C - T$)/C] × 100, where C is intensity in control, and T is intensity of the treated.

8. Competitive indirect enzyme-linked immunosorbent assay

A sandwich ELISA method was used for the detection of egg allergen as reported previously (Yasuko et al., 2001). Ovalbumin was diluted with 0.01 M phosphate buffer into 2 μg/mL and coated the well of a microtiter plate by pipetting 100 μL each. The plate was covered and incubated for overnight at 4℃. The coating solution was removed and washed the plate 4 times by filling the wells with 200 μL 0.05% PBS-T. Then, it was blocked with 200 μL PBS-T solution including 5% skim milk with shaking in the rotary shaker for 2 h. Each concentration of the ovalbumin solution was dissolved in 0.01 M phosphate buffer and test sample which contains 10 μg of compounds was mixed together. This mixture was incubated for 1 h in room temperature. After washing the membrane with PBS-T 4 times, 75 μL of the ovalbumin extract, diluted serially with phosphate buffer, was mixed with the same volume of the rabbit anti-ovalbumin IgG which diluted in 1:8,000 ratio with PBS-T solution including 5% skim milk. The mixtures were added to the wells of the plates 100 μL each and incubated for 2 h room temperature. The plate was washed 5 times with PBS-T solution, the plate was bounded for 2 h with HRP conjugate antibody which diluted in 1:120,000 with PBS-T solution including 5% skim milk on the rotary shaker. The plate was washed with PBS-T 5 times, the antibody was reacted with 100 μL TMB substrate reagent for 30 min and add 100 μL of stop solution, 1 N sulfuric acid to the wells. It was read the absorbance at 450 nm using a Molecular Devices Versa Max microplate reader (Sunnyvale, CA).

RESULTS

1. Identification of allergen denaturing constituents

A Coomassie brilliant blue-stained SDS-PAGE showed that, at 500, 250 and 100 μg of *E. japonica* leaves, all ovalbumin protein bands completely disappeared (Figure 9). The band intensity of the gels treated with *E. japonica* leaves was comparable to that of the gels treated with promethazine.

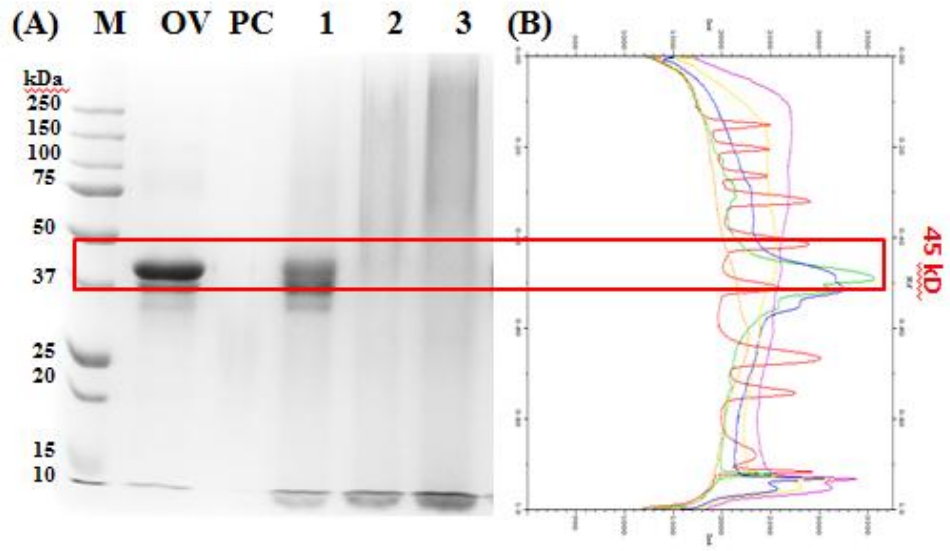

Figure 9. SDS-PAGE profile (A) and band intensity (B) of commercial ovalbumin extracts treated with or without test materials. Proteins were separated under reducing condition on 12% SDS-PAGE. Separated proteins of whole ovalbumin extract (5 μg) were visualized by staining with Coomassie brilliant blue. Lanes : M, protein marker; OV, ovalbumin extract (5 μg); PC, Promethazine; 100 μg; 1, *Eriobotrya japonica* leaves ext., 100 μg; 2, *Eriobotrya japonica* leaves ext., 250 μg; 3, *Eriobotrya japonica* leaves ext., 500 μg.

The IgG-binding reactivity to commercial ovalbumin extracts of rabbit anti-ovalbumin by fractions obtained from the solvent hydrolysable of the methanol extract of *E. japonica* leaves was examined using the immunoblot (Figure 10, Table 3). The chloroform-, butanol- and ethyl acetate-soluble fractions showed potent allergen denaturing activity. The chloroform-soluble fraction was used to identify peak activate fractions for the next step in the purification.

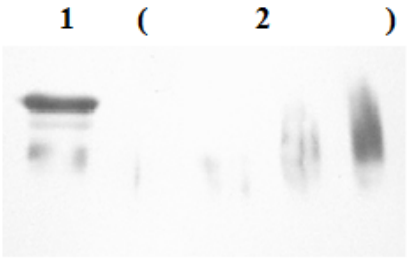

Figure 10. Immunoblot analysis of IgG-binding reactivity to 3 μg of commercial ovalbumin extracts by positive control promethazine

Table 3. The antigenicity inhibition of positive control promethazine toward 3 μg of commercial ovalbumin extract

No.	Fraction	Inhibition of antigenicity activity (%)			
		250 μg of sample	100 μg of sample	50 μg of sample	25 μg of sample
1	Control	-	-	-	-
2	Promethazine	99.88	99.38	79.86	26.46

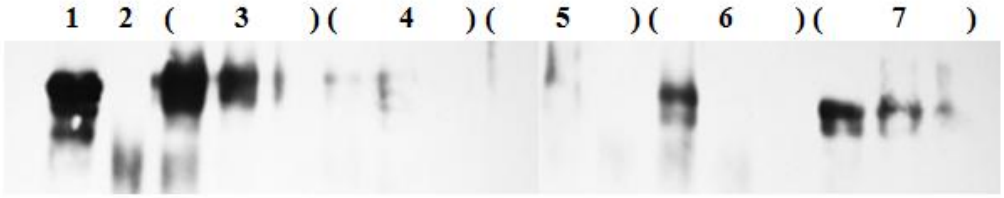

Figure 11. Immunoblot analysis of IgG-binding reactivity to 3 μg of commercial ovalbumin extracts by fractions obtained from the solvent hydrolysable of the methanol extract of *E. japonica* leaves

Table 4. The antigenicity inhibition of fractions obtained from the solvent hydrolysable of the methanol extract of *E. japonica* leaves toward 3 μg of commercial ovalbumin extract

No.	Fraction	Inhibition of antigenicity activity (%)		
		100 μg of sample	250 μg of sample	100 μg of sample
1	Control	-	-	-
2	Promethazine	56.22	-	-
3	Hexane-soluble fr.	2.61	25.63	49.63
4	$CHCl_3$-soluble fr.	49.69	45.52	44.65
5	EtOAc-soluble fr.	41.34	81.49	82.10
6	BuOH -soluble fr.	45.68	79.62	76.00
7	Water-soluble fr.	0	17.90	54.25

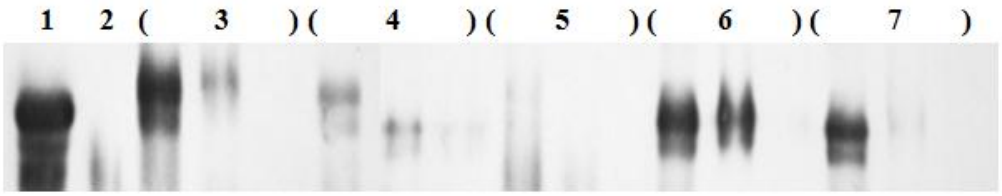

Figure 12. Immunoblot analysis of IgG-binding reactivity to 3 μg of commercial ovalbumin extracts by chloroform-soluble subfractions derived from *E. japonica* leaves

Table 5. The antigenicity inhibition of chloroform-soluble subfractions derived from *E. japonica* leaves toward 3 μg of commercial ovalbumin extract

No.	Fraction	Inhibition of antigenicity activity (%)		
		100 μg of sample	250 μg of sample	500 μg of sample
1	Control	-	-	-
2	Promethazine	79.22	-	-
3	C1	11.69	61.47	79.17
4	C4	47.51	55.58	76.53
5	C5	72.46	84.26	84.70
6	C6	6.28	20.51	79.69
7	C7	7.83	70.74	72.82

Bioassay-guided fractionation of *E. japonica* leaves extract (Figure 12 and Table 5, Figure 13 and Table 6, Figure 14 and Table 7, Figure 15 and Table 8) afforded a potent allergen denaturing principle C57468 (Figure 16 and Table 9). The activity of 10 μg the principle was slightly higher than that of promethazine (data not shown) whereas

promethazine does not shown good activity under concentration 100 μg. In addition, another active principle C57463 has potent activity similar with promethazine (Figure 17 and Table 10). In SDS-PAGE analysis, all of fractions were examined their allergen-denaturation activity (data not shown) and only active fractions were identified their IgG binding activity.

Figure 13. Immunoblot analysis of IgG-binding reactivity to 3 μg of commercial ovalbumin extracts by C5 subfractions derived from *E. japonica* leaves

Table 6. The antigenicity inhibition of C5 subfractions derived from *E. japonica* leaves toward 3 μg of commercial ovalbumin extract

No.	Fraction	Inhibition of antigenicity activity (%)
		50 μg of sample
1	Control	-
2	Promethazine (100 μg)	98.76
3	C54	77.99
4	C55	92.90
5	C56	94.35
6	C57	98.61
7	C58	97.47

Figure 14. Immunoblot analysis of IgG-binding reactivity to 3 μg of commercial ovalbumin extracts by C57 subfractions derived from *E. japonica* leaves

Table 7. The antigenicity inhibition of C57 subfractions derived from *E. japonica* leaves toward 3 μg of commercial ovalbumin extract

No.	Fraction	Inhibition of antigenicity activity (%)
		50 μg of sample
1	Control	-
2	Promethazine (100 μg)	99.92
3	C571	28.73
4	C572	97.43
5	C573	91.80
6	C574	99.93

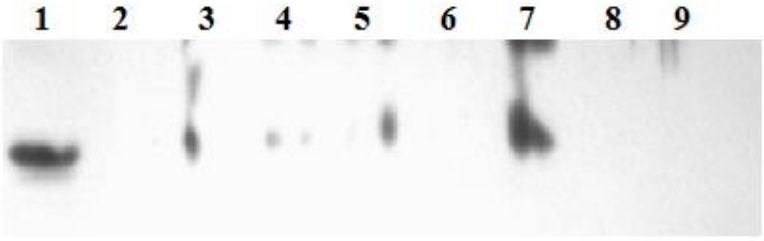

Figure 15. Immunoblot analysis of IgG-binding reactivity to 3 μg of commercial ovalbumin extracts by C574 subfractions derived from *E. japonica* leaves

Table 8. The antigenicity inhibition of C574 subfractions derived from *E. japonica* leaves toward 3 μg of commercial ovalbumin extract

No.	Fraction	Inhibition of antigenicity activity (%)
		25 μg of sample
1	Control	-
2	Promethazine (100 μg)	99.18
3	C5742	80.33
4	C5743	94.03
5	C5745	82.03
6	C5746	99.32
7	C5746 ($CHCl_3$-soluble fr.)	17.85
8	C5747	90.75
9	C5748	83.33

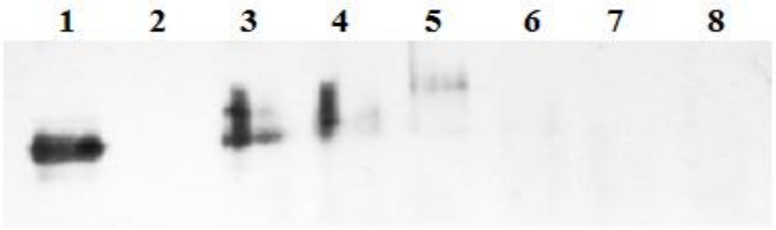

Figure 16. Immunoblot analysis of IgG-binding reactivity to 3 μg of commercial ovalbumin extracts by C5746 subfractions derived from *E. japonica* leaves

Table 9. The antigenicity inhibition of C5746 subfractions derived from *E. japonica* leaves toward 3 μg of commercial ovalbumin extract

No.	Fraction	Inhibition of antigenicity activity (%)
		10 μg of sample
1	Control	-
2	Promethazine (100 μg)	90.85
3	C57463	14.75
4	C57465	44.73
5	C57466	94.33
6	C57467	94.30
7	C57468	95.83
8	C57469	94.67

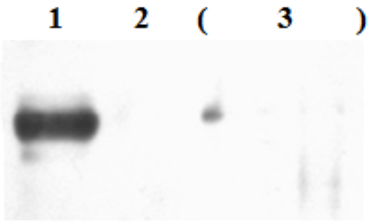

Figure 17. Immunoblot analysis of IgG-binding reactivity to 3 μg of commercial ovalbumin extracts by C57463 derived from *E. japonica* leaves

Table 10. The antigenicity inhibition of C57463 derived from *E. japonica* leaves toward 3 µg of commercial ovalbumin extract

NO.	Fraction	Inhibition of antigenicity activity (%)	
		50 µg of sample	100 µg of sample
1	Control	-	-
2	Promethazine	-	99.34
3	C57463	92.85	99.18

The immunoblot bioassay-guided fractionation afforded an active principle (compound 1). The active principle (compound 1) was obtained as a clear liquid and identified as di-n-octyl-phthalate by spectroscopic analysis, including EI-MS (Figure 18), ^{1}H NMR (Figure 19) and ^{13}C NMR (Figure 20). EI-MS revealed a molecular ion at m/z 261 $[M]^{+}$ and its ^{13}C NMR spectra showed 24 carbons in the molecule comprising two chain and two ester groups as indicated in, suggesting the molecular formula $C_{24}H_{38}O_4$. This compound was thus identified as di-n-octyl-phthalate (Fig 21). The interpretations of proton and carbon signals were largely consistent with those of Kim and Jonas (1998) and Edward and Napier (1973).

Di-n-octyl-phthalate (compound 1): clear liquid. EI-MS: *m/z* (relative intensity) = 261 $[M]^{+}$ (73), 167 (45), 149 (59), 132 (3), 112 (94), 104 (12), 83 (32), 70 (100, base peak), 57 (32).^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 400 MHz): See (Tables 11)

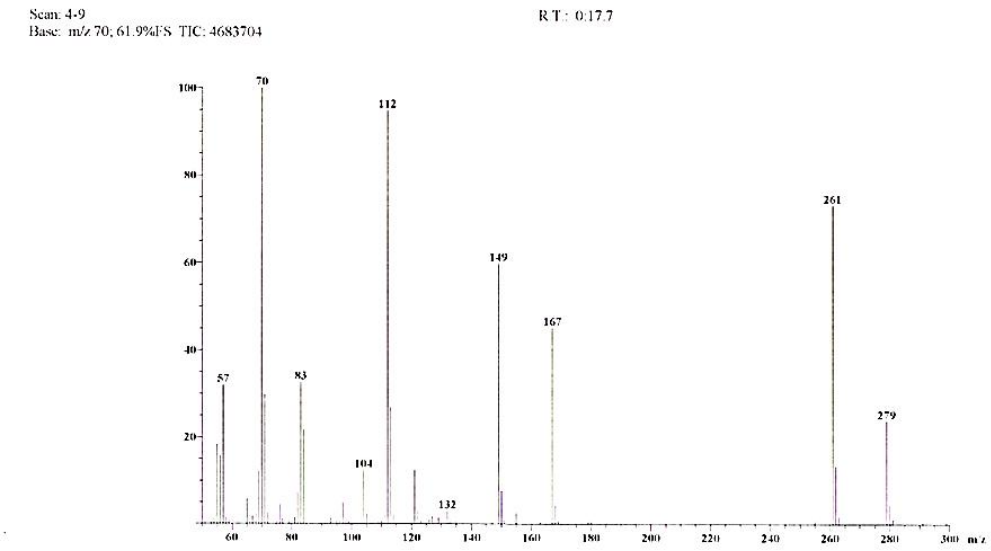

Figure 18. EI-MS spectrum of compound 1

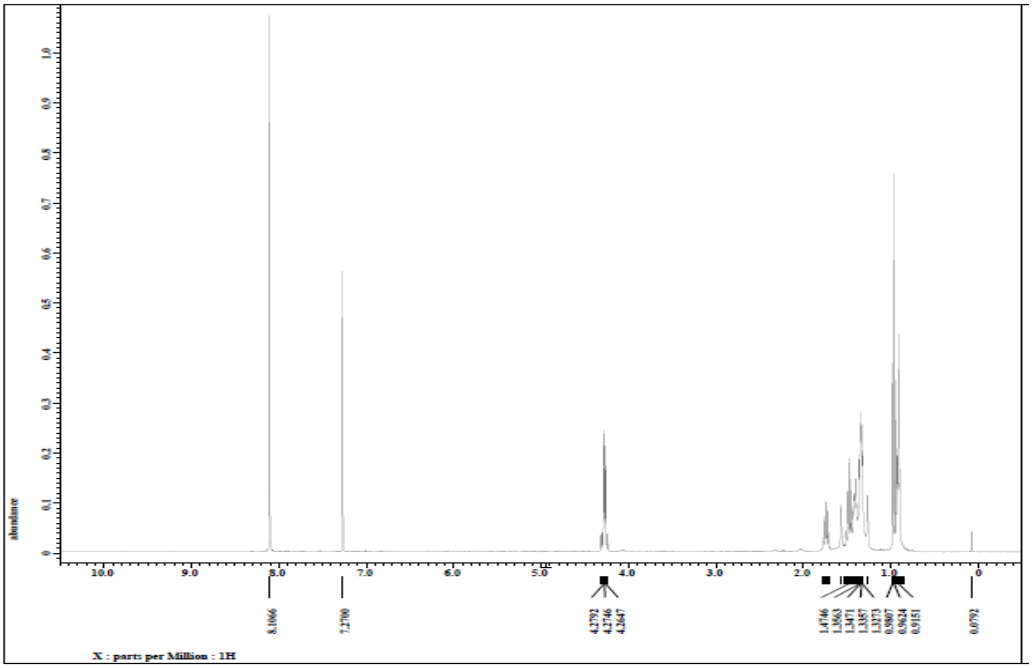

Figure 19. ^{1}H NMR spectrum of compound 1

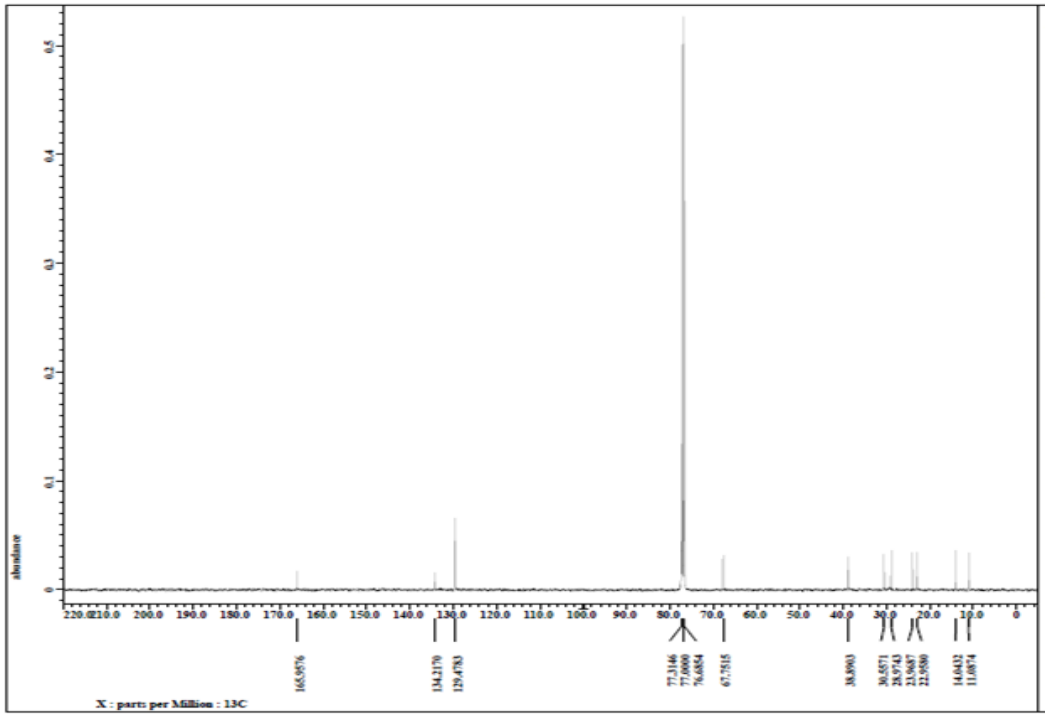

Figure 20. ^{13}C NMR spectrum of compound 1

Table 11. ^{1}H NMR (400 MHz, $CDCl_3$) spectral data for compound 1

Carbon	Partial structure	δ_H (ppm)	Di-n-octyl-phthalate, δ_H (300 MHz) (Edward and Napier, 1973)
A	CO		
B	C		
C	CH_2	8.10, s	7.68, m
D	CH_2	7.27, s	
1	CH_2	4.27, dd (J = 5.8, 3.96 Hz)	4.28, t (J = 6 Hz)
2	CH_2	1.74, m	
3	CH_2	1.47, dd (J = 7.64, 6.4 Hz)	
4	CH_2	1.40, m	
5	CH_2	1.40, m	0.8-1.9, complex
6	CH_2	1.40, m	
7	CH_2	1.34 , tt (J = 4.56, 7.92 Hz)	
8	CH_3	0.91, t	

Table 12. ^{13}C NMR (400 MHz, $CDCl_3$) spectral data for compound 1

Carbon	Partial structure	δ_C (ppm)	Di-n-octyl-phthalate, δ_C (300 MHz) (Kim and Jonas, 1998)
A	CO	165.9	166.9
B	C	134.2	132.8
C	CH_2	129.4	128.9
D	CH_2	129.4	130.8
1	CH_2	67.7	65.4
2	CH_2	28.9	28.8
3	CH_2	23.9	26.2
4	CH_2	30.5	29.52
5	CH_2	30.5	29.49
6	CH_2	38.8	32.1
7	CH_2	22.9	22.8
8	CH_3	14.0	14.1

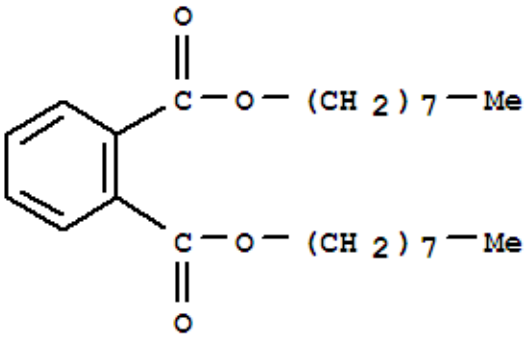

Figure 21. Structure of Di-n-octyl-phthalate

Compound 2 was obtained as a white powder and identified as the gypsogenin dimer by spectroscopic analysis, including Fab-MS (Figure 22), ^{1}H NMR (Figure 23) and ^{13}C NMR (Figure 24). The Fab-MS revealed a molecular ion at m/z 967 $[M]^{+}$ and its ^{13}C NMR spectra showed 30 carbons in the molecule of gypsogenin comprising aglycones of oleanene type. This compound was thus identified as gypsogenin dimer (Figure 25). The interpretations of proton and carbon signals were largely consistent with those of Murakami et al (2001).

Gypsogenin dimer (compound 2): white powder. Fab-MS: *m/z* (relative intensity) = 967 $[M]^{+}$ (0.4), 789 (0.1), 701 (2.1), 657 (3), 569 (5.7), 495 (18.9), 437 (6.7), 409 (4.9), 393 (4.6), 248 (6.9), 185 (33.4), 115 (100, base peak), 93 (68.9), 45 (32.4), 23 (28.6). ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 600 MHz): See Tables 13.

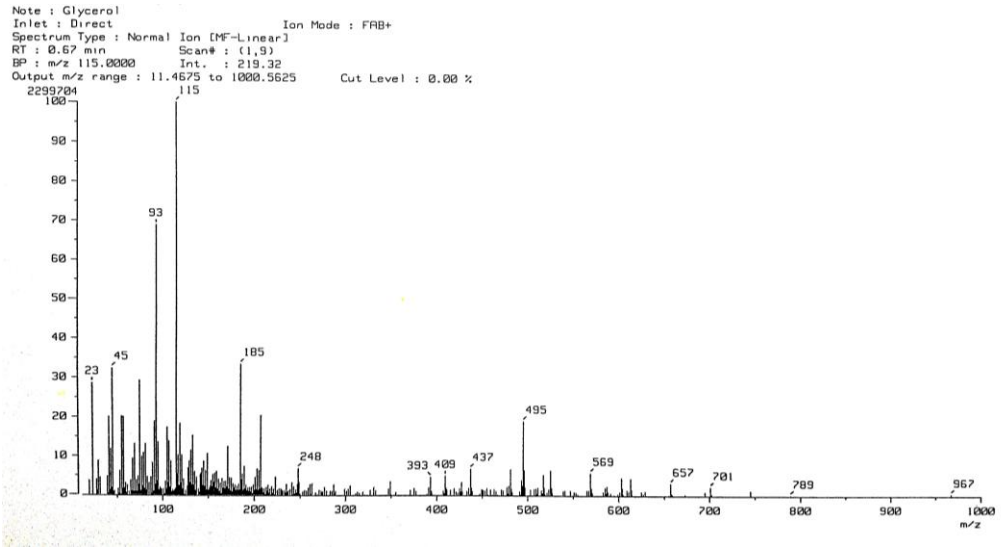

Figure 22. Fab-MS spectrum of compound 2

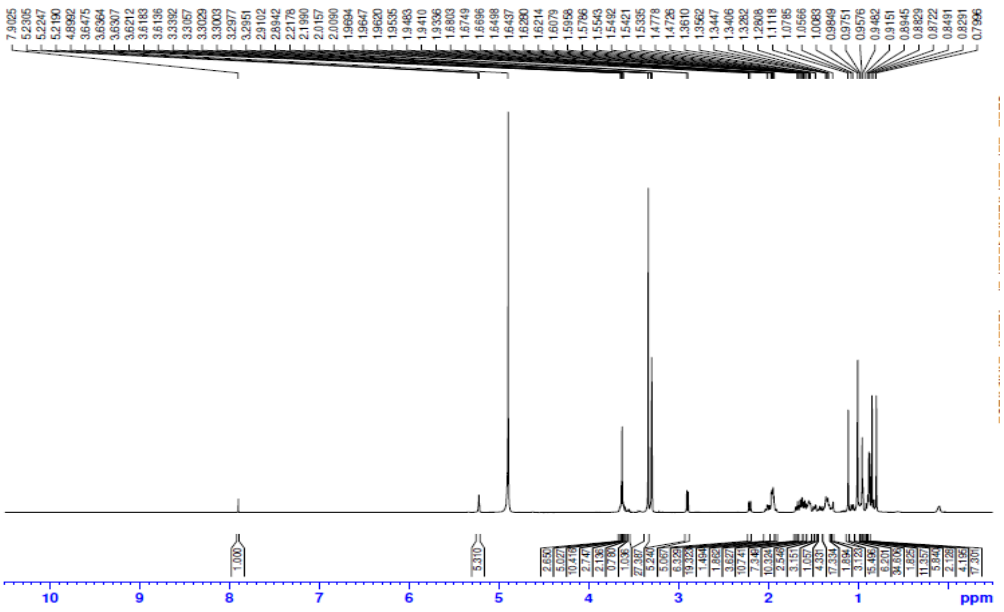

Figure 23. ^{1}H NMR spectrum of compound 2

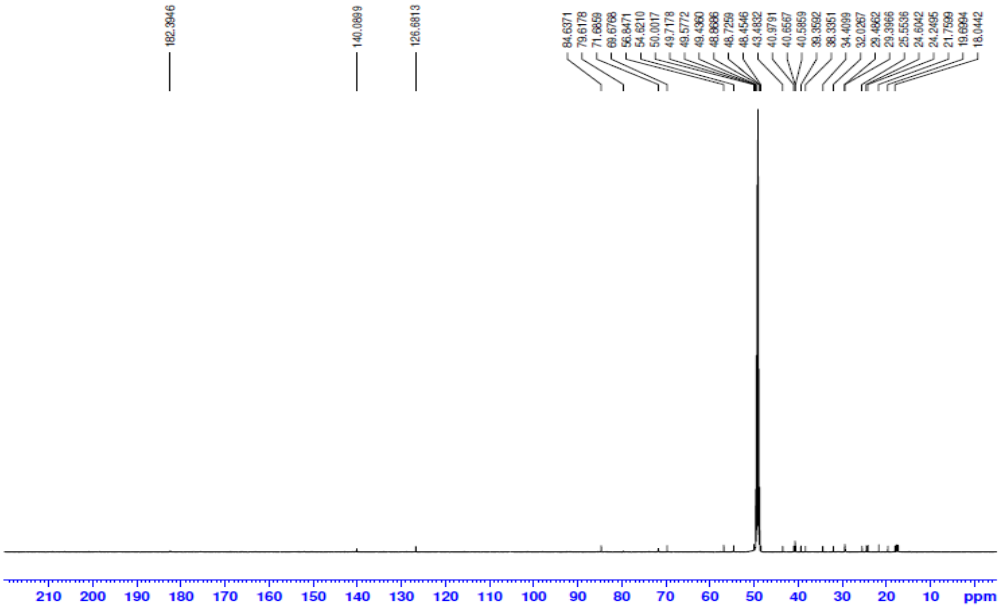

Figure 24. ^{13}C NMR spectrum of compound 2

Table 13. ^{1}H NMR (600 MHz, MeOD) spectral data for compound 2

Carbon	Partial structure	δ_H (ppm)	Gypsogenin dimer, δ_H (125 MHz) (Murakami, 2001)
1	CH_2	1.56 and 1.34, dd (J = 9.36, 2.46 Hz)	
2	CH_2	1.70, t and 1.47, d (J = 3.12 Hz)	
3	CH	3.18, dd (J = 3.18, 17.64 Hz)	3.18, dd (J = 4.3, 11.9 Hz)
4	C		
5	CH	1.62, d (J = 3.96 Hz)	
6	CH_2	1.53, dd (J = 9.42, 5.16 Hz) and 1.28	
7	CH_2	1.56 and 1.34, dd (J = 9.36, 2.46 Hz)	
8	C		
9	CH	1.44, d (J = 3.96 Hz)	
10	C		
11	CH_2	2.03, d (J = 4.02 Hz) and 1.77	
12	CH	5.23, t	5.44, br s
13	C		
14	C		
15	CH_2	1.38, m and 1.15	
16	CH_2	1.60, d (J = 7.26 Hz) and 1.36, m	
17	C		

18	CH	3.27, dd (J = 3.2, 20.1 Hz)	3.27, dd (J = 4.6, 14.0 Hz)
19	C		
20	CH_2	1.49, dd (J = 3, 13.2 Hz) and 1.21	
21	CH_2	2.01, d (J = 4.02 Hz) and 1.77	
22	CH_2	1.56 and 1.34, dd (J = 9.36, 2.46 Hz)	
23	CH_3	1.15, s	1.14, s
24	CH_3	1.05, s	1.03, s
25	CH_3	0.79, s	0.80, s
26	CH_3	0.97, s	0.97, s
27	CH_3	1.30, d (J = 4.02 Hz)	1.30, s
28	CO		
29	CH_3	0.95, d (J = 5.64 Hz)	0.95, s
30	CH_3	1.00, s	1.00, s

Table 14. ^{13}C NMR (600 MHz, MeOD) spectral data for compound 2

Carbon	Partial structure	δ_C (ppm)	Gypsogenin dimer, δ_C (125 MHz) (Murakami, 2001)
1	CH_2	40.6	38.1
2	CH_2	25.5	26.5
3	CH	84.6	90.0
4	C	40.9	39.6
5	CH	56.8	55.8
6	CH_2	21.7	18.5
7	CH_2	34.4	33.2
8	C	43.4	39.8
9	CH	50.0	48.0
10	C	40.5	37.1
11	CH_2	24.2	23.8
12	CH	126.6	122.6
13	C	140.1	144.9
14	C	48.4	42.2
15	CH_2	29.4	28.4
16	CH_2	24.2	23.8
17	C	49.7	46.7
18	CH	48.4	42.0
19	C	49.5	46.5
20	CH_2	32.0	31.0
21	CH_2	39.3	34.3
22	CH_2	34.4	33.2

23	CH_3	29.3	27.8
24	CH_3	18.0	16.4
25	CH_3	18.0	15.4
26	CH_3	19.6	17.4
27	CH_3	24.6	26.2
28	CO	182.3	180.1
29	CH_3	38.3	33.3
30	CH_3	24.2	23.8

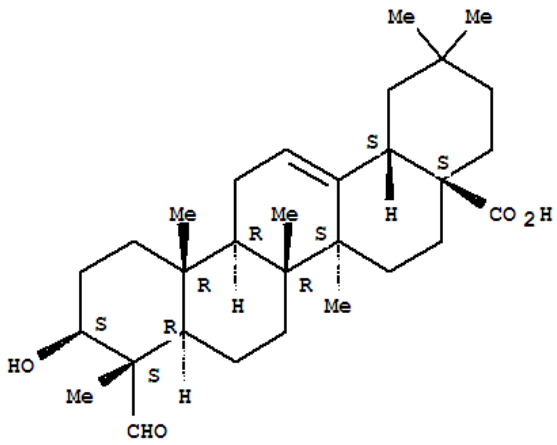

Figure 25. Structure of Gypsogenin

2. Allergen denaturing activity of test compounds

The IgG binding reactivity to commercial ovalbumin extracts of rabbit anti-ovalbumin by 27 test compounds, and promethazine were investigated using the immunoblot (Figure 26, Table 15). Responses varied according to test compound. Ellagic acid showed the most potent allergen denaturing compound, followed by lactic acid, 3-hydroxybenzoic acid, rutin trihydrate, (D)-(-)-tartaric acid, riboflavin and palmitic acid. Isolated compounds has more than 90 % inhibition rate of IgG binding reactivity as discussed Chapter 1. and the activities of these compounds were comparable to that of promethazine. Moderate or low allergen denaturing activity was obtained from 9 (77.2–50.4%) and 5 compounds (19–0%), respectively.

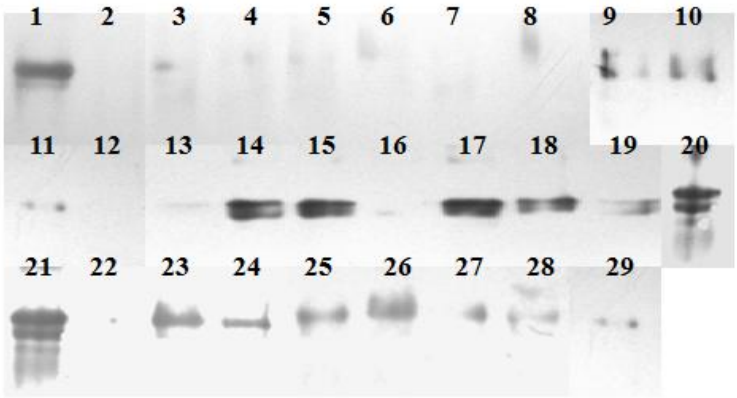

Figure 26. Immunoblot analysis of IgG-binding reactivity to commercial ovalbumin extracts by test compounds and promethazine. The number is same as in Table 13.

Table 15. The antigenicity inhibition activities of 27 compounds toward 3 μg of commercial ovalbumin protein

No.	Compounds	Inhibition of antigenicity activity (%)
		25 μg of sample
1	Control	0
2	Promethazine (100 μg)	99.9
3	(D)-(-)-tartaric acid	89.0
4	Lactic acid	95.3
5	Ellagic acid	96.7
6	Heptadecan	85.8
7	n-Hexadecan	82.1
8	Methyl Salicylate	69.5
9	(L)-(+)-ascorbic acid	77.2
10	Citric acid	72.3
11	Palmitic acid	85.2
12	Rivoflavin	86.1
13	(-)-Epigallocatechin gallate	59.0
14	Chlorogenic acid	10.1
15	Gallic acid	16.9
16	Farnesol	51.5
17	Campesterol	19.0
18	Quercetin	47.6
19	Nerolidol	64.4
20	Caffeic acid	14.3
21	Ferulic acid	0
22	Keampferol	50.4
23	(-)-Epicatechin	66.2

24	β-sitosterol	82.9
25	Oleanolic acid	82.8
26	*p*-anisaldehyde	73.2
27	*p*-coumaric acid	84.6
28	3-hydroxybenzoic acid	90.2
29	Rutin trihydrate	89.4

The anti-allergic effect of isolated compounds, 27 selected compounds and promethazine to commercial ovalbumin was examined using the competitive indirect ELISA bioassay in two formulas (Table 16). As judged by the change rate values, (D)-(-)-tartaric acid, oleanolic acid, palmitic acid, lactic acid and *p*-coumaric acid did not differ significantly in anti-allergic effect from each other which are much more higher. The gypsogenin dimer was 8 times higher effect than promethazine.

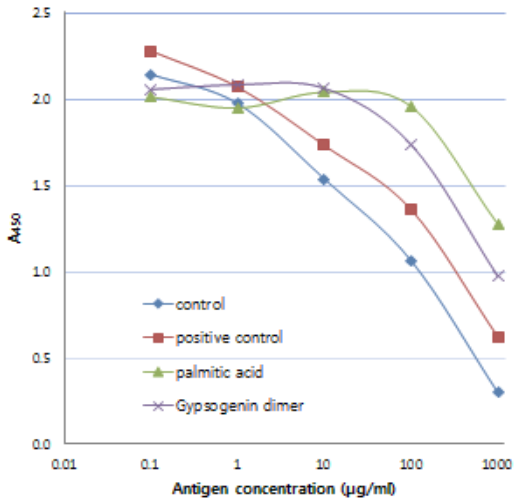

Figure 27. Inhibition analysis of binding between compounds treated commercial ovalbumin and rabbit anti-ovalbumin. The bindings of rabbit anti-ovalbumin to commercial ovalbumin were inhibited competitively by test compounds treated ovalbumin. Ovalbumin was treated 10 μg of test compounds for 1 hr at 25 ± 1℃ and diluted with phosphate buffer.

Table 16. Anti-allergic effect of 3 isolated compounds, 27 selected compounds and promethazine to commercial ovalbumin using competitive indirect ELISA bioassay

Material	IC_{50} (μg/ml)	Change rate
Control	-	-
Promethazine	12.8	1.15
Palmitic acid	109.4	9.52
Oleanolic acid	106.6	9.47
Lactic acid	102.2	9.08
(D)-(-)-tartaric acid	114.1	10.14
p-coumaric acid	100.5	8.77
Rivoflavin	11.9	1.89
Ellagic acid	13.1	1.16
Hexadecane	12.6	1.11
Methyl Salicylate	12.4	1.11
Farnesol	12.7	1.10
Ascorbic acid	12.4	1.10
Hydroxybenzoic acid	12.6	1.10
Kaempferol	11.0	0.96
Chlorogenic acid	12.2	1.09
Anisaldehyde	12.2	1.08
(-)-epigallocatechin gallate	12.2	1.08
Gallic acid	12.3	1.07

Nerolidol	12.2	1.07
b-sitosterol	12.1	1.05
Quercetin	10.9	0.97
Rutin	11.8	1.03
Caffeic acid	11.6	1.02
Heptadecane	11.6	1.01
Ferulic acid	11.5	1.00
Epicatechin	11.4	1.00
Citric acid	11.4	0.99
Campesterol	11.3	0.98
Dilauryl Thiodipropionate	11.8	1.03
Di-n-Octyl Phthalate	12.5	1.09
Gypsogenin dimer	101.6	8.87

DISCUSSION

Egg allergy accounts for one of the most prevalent food hypersensitivities, especially in children (Mine and Yang, 2008). A very recent study, involving a cohort of more than 850 egg-allergic patients 2-18 years of age, reported that outgrowth of egg allergy may in fact occur at a later stage than previously reported (Sevage et al., 2007). In egg-allergic patients, the clinical signs and symptoms involve various organs such as the skin (e.g., urticaria, angioedema), the respiratory system (e.g., asthma, rhinoconjuctivitis), and/or the gastrointestinal system (e.g., vomiting, diarrhea) (Martorell Aragonés et al, 2001). The most medically advised approach is a preventive intervention consisting in complete exclusion of the egg from the patient's diet. However, an avoidance diet may not represent the safest alternative for egg allergic patients and may, in fact, have a negative impact by lowering the patient egg reactivity threshold (Morisset et al., 2007).

The loquat, *Eriobotrya japonica*, is a small tree native to Japan and China, that is widely cultivated for its succulent fruit and its leaves have been used as a folk medicine for the treatment of chronic bronchitis, coughs, phlegm, high fever and ulcers in Japan and other Asian countries (Sun et al., 2007). Terpenoids and flavonoids have been found in the leaves and some of these compounds have been reported to be biologically active, exhibiting anti-inflammatory, andti-HIV, or hypoglycaemic properties (Shimizu et al., 1996; Jung et al., 1999).

In this study, SDS-PAGE clearly indicates that *E. japonica* leaves extract caused dissapperance of ovalbumin protein bands In the immunoblot assay, ellagic acid, lactic

acid, 3-hydroxybenzoic acid, rutin trihydrate, (D)-(-)-tartaric acid, riboflavin and palmitic acid strongly inhibited the IgG-binding reactivity to commercial ovalbumin extract of rabbit anti-ovalbumin. The anti-egg allergic principles of *E. japonica* were identified as the di-n-octyl phthalate, gypsogenin dimer and dilauryl thiodipropionate. Of the compounds examined in competitive indirect ELISA, high activity was obtained from (D)-(-)-tartaric acid, oleanolic acid, palmitic acid, lactic acid and *p*-coumaric acid against commercial ovalbumin. The activity of all compounds is comparable to that of promethazine. The isolated compounds were more active than promethazine and were less active than active five compounds. In addition, the 25 μg treatment containing the *E. japonica* resulted in good control efficacy compared with promethazine. This original finding indicates that the *E. japonica* and the compounds described may hold promise for the development of novel and effective products for the control of egg allergen ovalbumin.

In conclusion, treatments derived from *E. japonica* containing di-n-octyl phthalate, gypsogenin dimer, dilauryl thiodipropionate and other compounds described could be useful as allergen denaturant for protection from humans from various allergic diseases caused by ovalbumin. For the practical use of these materials as novel denaturant to proceed, further research is needed to establish their human and animal safety. In addition, combining treatments with these compounds may have a synergic effect on the reduction of antigenicity of ovalbumin because previous reported that reactivity may have been removed effectively while antigenicity is reduced to 1/1000 measured by competitive indirect ELISA (Hisatomi et al, 1991).

CHAPTER 2. Identification of residual materials and comparison antigenicity with allergenicity

INTRODUCTION

In general, food proteins present in a processed food will be a denatured state, aggregated in protein networks, or interacting with carbohydrates and lipids, leading either to the reduction of allergen content. However, regarding the effects of various treatments on protein structure, allergenicity or antigenicity still more remain or/and form new epitopes (Eigenmann, 2000). The conformational changes and the exposure of newly antigenic sites occur not only due to irradiation, but also in response to heat, urea, and guanidine denaturation (Tetsuya et al., 2000). Despite a large number of studies into egg white allergy, no clear consensus has been reached as to relative antigenicity and allergenicity of egg white proteins. The contradictory results on egg white allergenicity may, therefore, be attributable to several aspects including purity of individual protein fractions, antibody sources, human sera, or antibodies from experimental animals and routes of administration (Mine and Zhang, 2002).

In this chapter, fragmentation of ovalbumin and egg white whole protein induced by treatment of natural products was examined, allergenicity and antigenicity in different states was compared between human sera from allergic patients and commercial rabbit antibodies. Results were compared with those of promethazine.

MATERIALS AND METHODS

1. Materials

Urea was purchased from Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid (BCA) reagents and bovine serum albumin (BSA) were purchased from Pierce (Rockford, IL). Goat anti-human IgG (H+L)-HRP and IgE-HRP conjugate were purchased from Zymed Laboratories (San Francisco, CA). The other chemicals and reagents were the same as those used in Chapter I.

2. Egg white extracts

Egg white protein was extracted as described previously (Bernhisel-Broadbent et al., 1994). The extract was prepared by adding 10 g of fresh egg white to 100 mL of PBS in 300 mL Erlenmeyer flask. The mixture was shaken overnight at 4℃ and centrifuged for 10 min at 2800 rpm. Aliquots of supernatant were micro-centrifuged at 17,000 × *g* for 15 min for clarification, then sequentially filter-sterilized through Millipore 0. 22 μm filters (Darmstadt, Germany). The filtered egg white protein extract was stored at –80℃ until use. The protein content was determined using a BCA assay kit. BSA was used as a protein standard. Control and filtered protein solutions were measured by an Optizen Pop spectrophotometer (Daejeon, ROK).

3. Human serum

The serum was supplied by child patient who was attending the allergy clinics at Seoul National University Hospital. The allergic child is sensitive to egg allergy and house dust mites. The allergic reaction was certified by skin and RAST tests. Total IgE value is 3220 which means that allergic reaction value. If the total IgE value and specific IgE is over than 200, it can be considered as allergic disease to specific target. Sera from healthy children did not show IgE-binding reactivity to house dust mite extracts.

4. Sodium dodecyl sulfate-poly acrylamide gel electrophoresis analysis

SDS-PAGE was performed as described by Laemmli (1970) in 12% (w/v) polyacrylamide gels by using Bio-Rad mini-protean 3 electrophoresis cell (Hercules, CA). Ovalbumin extract (500 μg) and each test compound (1, 250, 500, and 1000 μg each) in 5 μL of DMSO were incubated for 1 h at 25 ± 1℃. Samples were centrifuged for 15 min at 10,000 rpm at 4℃. Pellets were treated with 8 M urea and incubated with shaking in the rotary shaker for 5 min. Samples were mixed with 5 × sample buffer as stated in Chapter 1. (4% SDS, 4% mercaptoethanol, 100 mM Tris-HCl, pH 8.0). After boiling for 10 min, each sample was centrifuged for 15 min at 12,000 rpm at 4℃. The supernatant was then loaded onto gel. Gels were run at 80 V for 30 min and then 110 V for 50 min. The proteins were visualized by staining Coomassie brilliant blue. The intensity of the gel was determined as stated in Chapter I. Negative controls consisted of 5 μL of DMSO. Promethazine served as a positive control and was similarly prepared.

5. Immunoblot assay

A Western-blot immunoassay for ovalbumin-specific IgG-binding reactivity was performed as stated in Chapter 1. Human-specific IgE-binding reactivity was evaluated as described previously (Kim et al., 2002). Each concentration of the test sample in 5 μL of DMSO and 3 μg of ovalbumin protein solution were mixed together. This mixture was incubated for 1 h in room temperature. After incubation, the mixture was electro-blotted onto the nitrocellulose membrane strips. After washing the membrane with 0.1% PBS-T for 10 s, it was blocked with PBS-T solution including 5% skim milk with shaking in the rotary shaker for 1 h. Then, it was washed 4 times with PBS-T solution, each time for 15 min and incubated for overnight at 4℃ with human serum which diluted in 1:2000 with PBS-T solution including 5% skim milk. The membrane was washed with the PBS-T solution 4 times. Then, the membrane was bounded for 2 h with goat anti-human IgG (H+L)-HRP and IgE-HRP conjugate antibody which diluted in 1:20,000 with PBS-T solution including 5% skim milk on the rotary shaker. The membrane was washed 4 times with PBS-T for 15 min. The antibody was reacted with ECL Western blotting detection reagent for 1 min. The denaturing activities of each test sample were calculated as stated in Chapter I.

6. Bicinchoninic acid assay

A bicinchoninic acid assay was used to quantify ovalbumin protein as described previously (Garry et al., 2005). Ovalbumin extract (100 µg) and each test compound (1, 250, and 500 µg each) dissolved in 5 µL of DMSO were incubated for 1 h at 25 ± 1℃. Samples were centrifuged for 15 min at 10,000 rpm at 4℃. Pellet was treated with 8 M UREA and incubated with shaking in the rotary shaker for 5 min. BSA was used as a protein standard. Each sample (25 µL) was moved into a microplate well and added 200 µL of the working solution (Reagent A:B, 50:1) to each well (Reagent A: containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide, Reagent B: containing 4% cupric sulfate). After incubation at 37℃ for 30 min, the absorbance was measured at 562 nm on a Molecular Devices Versa Max microplate plate reader.

RESULTS

1. Composition of egg white

A Coomassie brilliant blue-stained SDS-PAGE analysis revealed multiple protein bands of the egg white extract (Figure 28). Intact egg white bands were observed at approximately 43 kDa to 80 kDa. The bands were presumed to be 43 kDa of the major band and 77.7 kDa of ovotransferrin. Ovalbumin and ovomucoid bands appeared around 36 to 40 kDa, respectively, and lysozyme appeared at 14.3 kDa. Similar results were reported by Bernhisel-Broadbent et al, 1994.

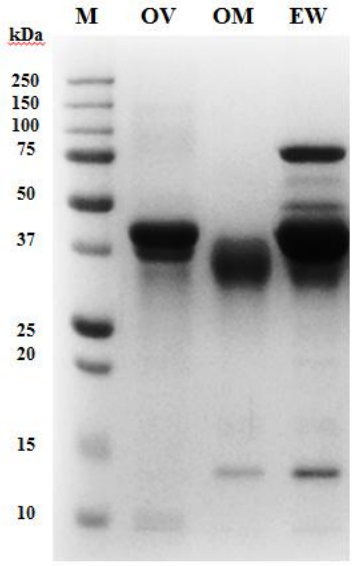

Figure 28. SDS-PAGE profile of egg white whole protein extract and commercially pure egg white proteins. Proteins were separated under reducing condition on 12% SDS-PAGE. Separated proteins of whole extracts (4 µg) were visualized by staining with Coomassie brilliant blue. Lanes: M, protein marker; OV, commercially ovalbumin; OM, commercially ovomucoid; EW, egg white whole protein extract.

2. Identification of residual materials

SDS-PAGE analysis revealed reduction of multiple protein bands of 8 M urea treated ovalbumin in presence of *E. japonica* whole plant leaf extract and promethazine, although responses varied according to treatment concentration (Figure 29). The 10 kDa ovalbumin band appeared at 250 and 500 μg of *E. japonica* whole plant leaf extract (Figure 29A). The band was fainted visible at 100 μg of the extract and completely disappeared at 50 μg of the extract. The 10 kDa ovalbumin band was fainted visible at 250 and 500 μg of promethazine, whereas the band disappeared at 50 and 100 μg of the compound (Figure 29B). These results suggest that residual material has aggregations of structurally-stable ovalbumin and fragmented proteins. In addition, more active fractions make a number of residual materials and induce fragmentation of ovalbumin structure.

The protein content of the supernatant (A) and pellet (B) of ovalbumin treated with *E. japonica* whole plant leaf extracts and promethazine was determined using a BCA assay (Figure 30). Treatment with high concentration of the whole plant leaf extracts increased the protein content of the supernatant from 1.1 to 1.7 (mg/mL) (Figure 30A). Similarly, treatment with high concentration of the whole plant leaf extracts led to a high content of proteins of the pellet (Figure 30B). In addition, treatment with high concentration of positive control promethazine increased the protein content either the supernatant and the pellet, specially, the protein content of the supernatant was increased from 2.3 to 3.8 (mg/mL). These findings suggest that more active compounds make a number of fragmented proteins.

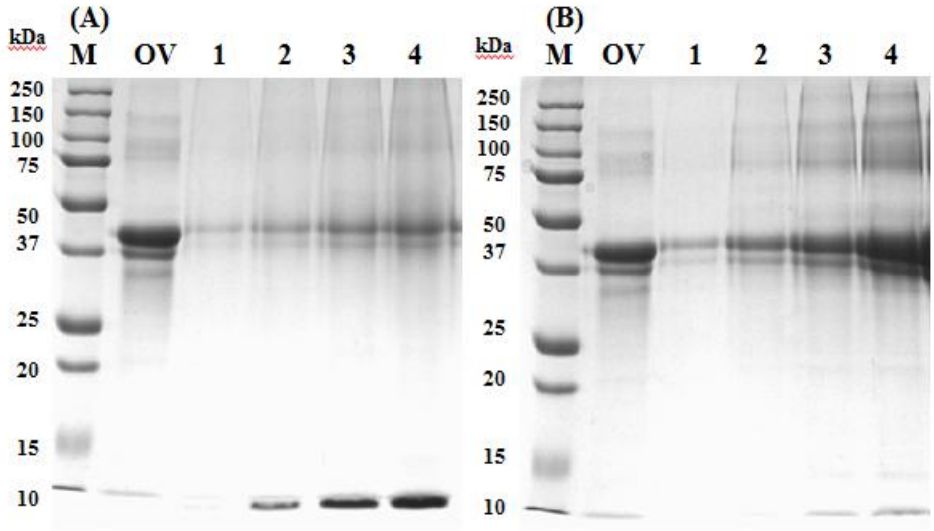

Figure 29. SDS-PAGE profile of ovalbumin treated with *E. japonica* whole plant leaf extracts and promethazine. Proteins were separated under reducing condition on 12% SDS-PAGE. Separated proteins of whole ovalbumin extract (5 µg) were visualized by staining with Coomassie brilliant blue. (A) Lanes: M, protein marker; C, ovalbumin extract; 1, *E. japonica* whole plant leaf ext., 50 µg; 2, *E. japonica* whole plant leaf ext., 100 µg; 3, *E. japonica* whole plant leaf ext., 250 µg; 4, *E. japonica* whole plant leaf ext., 500 µg. (B) Lanes: M, protein marker; C, ovalbumin extract; 1, Promethazine ext., 50 µg; 2, Promethazine ext., 100 µg; 3, Promethazine ext., 250 µg; 4, Promethazine ext., 500 µg.

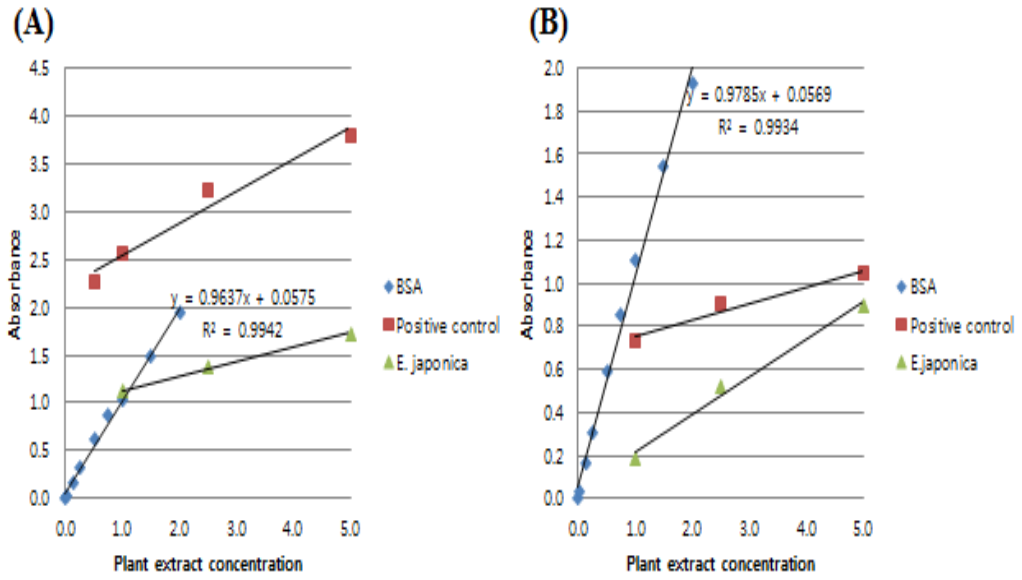

Figure 30. The protein content of supernatant (A) and pellet (B) treated with *E. japonica* whole plant leaf extracts and promethazine. Proteins were incubated for 1 h at 25 ± 1°C with test materials. Bovine serum albumin was used as a protein standard. The protein contents of each sample were measured the absorbance at 562 nm on a plate reader.

3. Comparison antigenicity with allergenicity of residual materials

The IgE-binding reactivity to crude egg white extracts and ovalbumin of an asthmatic patient's serum by fractions obtained from the solvent hydrolysable of the methanol extract of *E. japonica* leaves was examined using an immunoblot assay (Figure 31). Responses varied according to treatment concentration. Unlike promethazine, treatment with the leaf extracts showed potent intensity of residual materials in both crude egg white extracts (Figure 31A) and ovalbumin (Figure 31B).

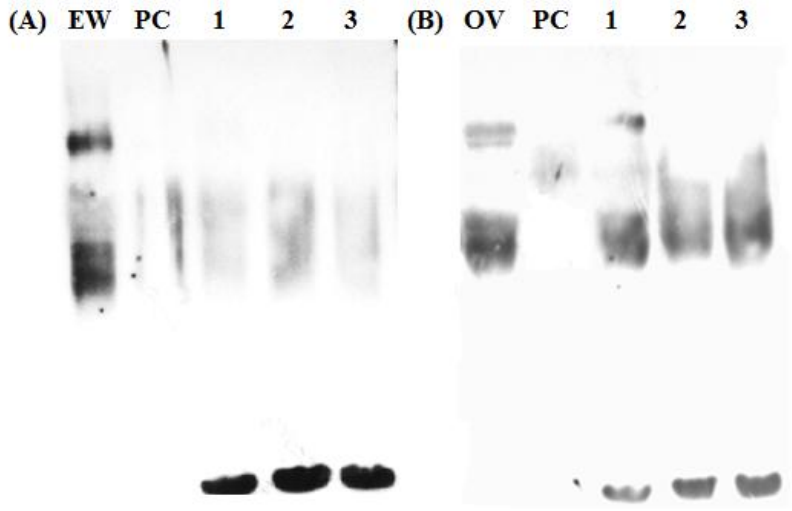

Figure 31. Immunoblot analysis of IgE-binding reactivity to 4 μg of crude egg white extracts (A) and 3 μg of commercial ovalbumin extracts (B) by fractions obtained from the solvent hydrolysable of the methanol extract of *E. japonica* leaves. Lanes: EW, egg white whole protein; OV, coomercial ovalbumin; PC, positive control (promethazine), 100 μg; 1, *E. japonica* leaves ext., 100 μg; 2, *E. japonica* leaves ext., 250 μg; 3, *E. japonica* leaves ext., 500 μg.

The suppression of IgE-binding reactivity of three isolated constituents and promethazine was examined using allergic patient serum (Figure 32). In SDS-PAGE analysis, three isolated constituents denatured egg white whole protein effectively, however, di-n-octyl phthalate 50 μg and gypsogenin dimer 25 μg showed residual material which can be arouse allergic reaction (Figure 32A). Unlike methanol extract of *E. japonica* leaves, three isolated constituents strongly inhibited IgE-binding reactivity to egg white whole protein and residual material even if they are treated with just 25 μg while positive control promethazine administered 100 μg (Figure 32B).

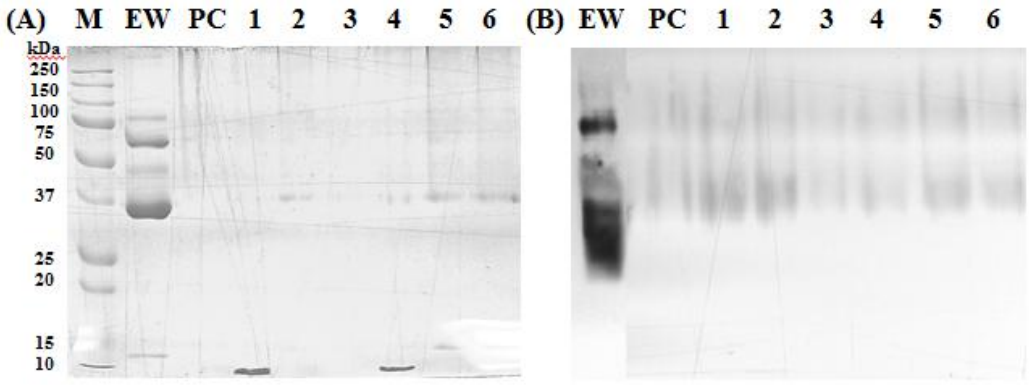

Figure 32. SDS-PAGE profile (A) and Immunoblot analysis (B) of IgE-binding reactivity to 4 μg of crude egg white extracts by three single compounds obtained from the solvent hydrolysable of the methanol extract of *E. japonica* leaves. Lanes: M, protein marker; EW, egg white whole protein; PC, positive control, 100 μg; 1, Di-n-Octyl Phthalate, 50 μg; 2, Di-n-Octyl Phthalate, 25 μg; 3, Gypsogenin Dimer, 50 μg; 4, Gypsogenin Dimer, 25 μg; 5, Dilauryl Thiodipropionate, 50 μg; 6, Dilauryl Thiodipropionate, 25 μg.

DISCUSSION

Dramatic conformational changes in protein can be induced or that some tertiary or secondary structures can be broken down by denaturation process, producing new antigenic sites (Masuda et al., 2000). These results suggested that IgE antibody recognizes more sequential epitopes and that IgG antibody recognizes both conformational and sequential epitopes which may play a crucial role in the stabilization of allergen (Mine and Zhang, 2002). The most studies have so far been based on the binding properties of specific IgG isotypes, thus detecting antigenic components rather than providing a reliable indication of allergenicity (Demeulester and Giovannacci, 2007). The IgE antibodies to egg white protein in the serum of patients differ from the IgG or IgA antibodies with respect to their binding activities with different preparations of denatured or fragment protein (Honma et al., 1996). Recently, a common approach to evaluating the stability of egg allergens is to examine alterations of their IgE/IgG binding capacities on physical, chemical, or genetic manipulations, reflecting their capacity to stimulate a specific immune response (Mine and Yang, 2008). It is important to reveal information about molecular features of allergenic and antigenic egg white proteins after treatment as therapy was made with IgE and IgG antibodies.

In this study, SDS-PAGE with urea clearly indicates that *E. japonica* leaf extract and promethazine caused appearance of ovalbumin protein and residual material bands in pellet solutions. In addition, high concentration of each sample extracts strongly increased the content of protein by exposing particular four amino acids. BCA reagent

has the potential to detect to four particular amino acids (cysteine, cysteine, tryptophan, and tyrosine), which could be exposed by fragmentation induced by heating, urea and natural compounds. The complete increase of content of proteins is probably due to fragmentation as active plants contains virtually a lot of natural compounds to induce conformational change with surface four particular amino acids. Moreover, the denaturing activity of *E. japonica* leaf extract was higher than that of promethazine. In the immunoblot assay, allergenicity of residual materials treated with *E. japonica* leaf extract remained, whereas antigenicity of residual materials treated with *E. japonica* leaf extract was removed. The reactivity to IgE suggested that residual material remains their allergenicity which means egg white allergenic sites might not be completely destroyed by *E. japonica* even antigenicity was disappeared as stated in Chapter 1. However, the three isolated compounds either physically or chemically modified egg white whole protein and ovalbumin, their binding activities to allergic patient's specific IgE and rabbit-specific IgG were removed. These findings suggest that residual material induced by treatment with isolated compounds might not be provoke allergic reaction, whereas crude extracts of *E. japonica* leaves make allergenic residual materials. The dual allergenic plus antigenic reactivity-inhibiting action of the constituents is of practical importance because a single application of natural egg allergen denaturant could not prove possibility of arousing of allergic reaction. Detailed tests are needed to fully understand the modes of action of the constituents.

In conclusion, *E. japonica*-derived products could be useful as naturally occurring egg allergen denaturants for protection from humans from various symptoms caused by

egg allergen. In addition, applications of diluted solutions of *E. japonica* constituents are potentially an effective and acceptable method for controlling egg white whole protein and their allergens, on the basis that botanical active molecules are widely available with some being relatively effective compared with chemical compounds (Bielory, 2007).

CONCLUSION

The emergence of egg allergy has had both industrial and clinical implications (Mine and Yang, 2008). Clinically relevant egg allergens have been identified in both the egg white and the egg yolk fraction. However, reports have documented that the major egg allergens were mainly contained in the egg white (Anet et al., 1985). Egg allergy tends to persist in children with severe reactions (e.g., respiratory symptoms, angioedema, and multisystemic reactions) or with positive skin test (Ford and Taylor, 1982). Avoidance diets are primarily preventive and present several hurdles as they are difficult to comply with, they may lower threshold reactivity, and they often leave the natural history of egg allergy unchanged (Morisset et al., 2007). Therefore, an active and therapeutic approach seems to be more appropriate and plant-derived constituents will be effective because their anti-inflammatory activity have been largely investigated.

To control and remove egg allergens, chemical and physical methods have been suggested. Food-processing methods may alter the allergenicity of food proteins in various ways such as heat application, enzymatic fragmentation, and γ-irradiation. However, a main obstacle to the use of food processing in reducing egg allergenicity is the risk to alter the unique functional attributes of egg proteins (Mine and Yang, 2008). Therefore, it has been suggested that novel food-processing methods, such as high pressure and pulsed electric field, possibly combined with physical and biochemical treatments, hold great promise for the development of hypoallergenic food products (Wichers et al., 2003). On the other hand, antihistamines such as promethazine are

powerful allergic symptoms control agent, it originally has minimal efficacy and side effects so not suitable to human for a long term (Sampson, 1999). These problems facilitate development of practical and effective allergen denaturing materials.

In conclusion, *E. japonica* leaf-derived products containing di-n-octyl phthalate, dilauryl thiodipropionate, gypsogenin dimer and other constituents could be useful as allergen denaturants for protection from humans from various allergic diseases caused by egg allergen. For the practical use of these materials as novel allergen denaturant to proceed, further research is needed to establish their human safety, although incorporating *E. japonica*-based products into the diet because of their health-promoting properties are in the marketplace in China. Humans have a broad range of individual characteristics in terms of allergic reaction type and/or in responses to treatments, the present results with *E. japonica* on *in vitro* should be further validated in clinical experiments. In addition, formulations for both improving allergen denaturing potency and stability need to be developed.

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비파엽 (*Eriobotrya japonica*) 유래 화합물의 계란 알러젠 오발부민 (Ovalbumin)에 대한 알러젠 중화 활성

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초 록

계란은 영 유아 및 소아에게 많이 발생하는 대표적인 알레르기이다. 최근 환경오염 및 식생활 양식의 변화에서 비롯한 계란 알레르기의 발증 빈도가 점차 증가하고 있으며 그 증상은 설사, 구토, 복통, 아토피성 피부염, 기관지 천식에서부터 전신성의 anaphylactic shock에 이르기까지 다양하여 때로는 치명적 질환으로 심하면 사망에 이르게 된다. 하지만 계란은 우수한 단백질 공급원이라는 영양적 가치와 저렴한 가격으로 인해 일반 식단이나 상업적 가공 식품에서 중요한 식품소재로 자리잡고 있으므로 그 섭취를 배제하기 어렵다. 이에 따라

알레르기를 저감화 시킨 계란 소재의 개발을 위해 물리적, 화학적 방법에 의한 단백질 변성을 이용함으로써 계란의 항원성과 알레르기성을 감소시키고자 하는 연구는 오래 전부터 시도되어 왔다. 그러나 계란이 가진 protease inhibitor activity와 난백 내에 있는 당쇄들, 열에 강한 성질, papain이나 trypsin 등의 효소작용에 저항성이 큰 특성으로 인해 계란이 가지는 알레르기성을 저하시키기가 쉽지 않았다. 그 외에도 많이 사용되고 있는 프로메타진과 같은 항 히스타민제는 식품으로 인해 유발되는 알레르기 질병의 증상을 완화시킬 수 있으나 전반적으로 약한 효과를 나타내며 환자들에게 수용적이지 못한 부작용을 발생시키기도 한다. 면역학적 요법은 일반적으로 효과적이기는 하나 응급상황에서 잘 수련된 전문가의 감독하여 이루어져야만 하는 단점이 있다.

본 연구에서는 안전성이 우수한 천연물에서 독성과 부작용이 적고, 적은 양으로도 큰 효과를 내는 활성물질을 분리하여 의약품의 지표물질로 사용될 수 있도록 하는 개발을 타진하고자 하였다. 이에 오발부민에 비파엽의 활성 물질을 처리하여 그 항원성 변화를 조사하는 과정에서 단백질젤전기영동 간이 검정법, 웨스턴브럿검정 방법, 간접경합 ELISA 등의 기술이 사용되었고 양성대조군으로 프로메타진을 사용해 그 결과를 비교하였다. 비파엽 유래 화합물에 대한 웨스턴블럿검정 결과 ellagic acid, lactic acid, 3-hydroxybenzoic acid, rutin trihydrate, (D)-(-)-tartaric acid, riboflavin 그리고 palmitic acid (85.2-96.7 %)에서 프로메타진보다 높은 알러젠 중화활성을 보였다. 비파엽 추출물은 헥산, 클로로포름, 에틸아세테이트, 부탄올, 물로 분획 후 웨스턴블럿검정으로 확인한 결

과 클로로포름 분획층에서 높은 중화활성을 보였다. 이후 중압액체크로마토그래피, 박층크로마토그래피 및 고속액체크로마토그래피로 분리하였고 핵자기 공명 분광법 분석을 통해 구조 동정을 완료하였다. 최종적으로, 프로메타진을 양성 대조군(Positive Control)로 하여 실험을 수행한 결과 분리 된 단일물질인 Di-n-Octyl-Phthalate(99.18 % at 100 μg/ml)는 프로메타진 (99.34 % at 100 μg/ml)과 비슷한 수준의 활성을 보였고 GC-MS의 성분 라이브러리 검색으로 확인된 Dilauryl Thiodipropionate (83.33 % at 25 μg/ml)과 Gypsogenin dimer (95.83 % at 10 μg/ml)는 프로메타진의 경우보다 더 낮은 농도에서 높은 활성을 보였다. 또한, 간접경합 ELISA에서 화합물의 농도를 10 μg/mL으로 낮추었을 때, (D)-(-)-tartaric acid가 가장 높은 활성을 나타냈고 (IC50, 114.18 μg/mL) ellagic acid (13.11 μg/mL)의 경우 양성대조군인 프로메타진(12.81 μg/mL)과 비슷한 활성을 보였다. 반면에, Gypsogenin dimer는 오발부민의 항원성을 1/10까지 떨어뜨려 oleanolic acid, palmitic acid, lactic acid에 이어 높은 활성을 나타냈다 (106.63-101.62 μg/mL).

오발부민뿐 아니라 계란 난백 전체의 알레르기성 분석을 위하여 알레르기 환자의 IgE 항체를 이용하여 반응성을 검토하였다. Urea 처리를 통해 획득된 residual material을 가지고 단백질젤전기영동 간이 검정법과 BCA 정량법을 수행한 결과 구조적으로 안정한 오발부민과 분해된 fragment를 확인할 수 있었고, 높은 활성을 보이는 화합물일수록 상층액과 침전물 모두에서 더 높은 단백질 농도를 나타냈다. 또한 웨스턴블럿검정으로 항원성 감소와 관련한 알레르기성의 변화를 분석한 결과, 비파엽 추출물의 경우 오발부민과 난백 전체의 항원

성을 감소시키기는 하였으나 알레르기성을 감소시키지 못하였다. 이는 화합물이 난백의 알레르겐을 제대로 분해하지 못했거나 구조적으로 숨어있던 항원결정기가 노출됨으로써 IgE와 반응한 것으로 생각된다. 반면 분리된 단일 물질과 양성대조군인 프로메타진의 경우에는 항원성과 알레르성을 모두 효율적으로 억제시켰고 분리된 단일 물질은 프로메타진보다 더 낮은 농도에서 높은 활성을 나타냈다.

이상의 결과를 바탕으로 본 논문의 연구 성과들은 학문적으로 식물체에 함유된 미지의 활성본체를 밝혀냈다는데 그 의의가 있고, 활성이 확인된 이들 식물체 추출물, 관련 화합물들을 함유한 알러젠 중화 활성제의 개발 등 산업적으로도 그 활용 가능성이 높다고 판단 되며 계란 알레르기 저감화 소재의 자체 기술의 개발을 위한 기초자료가 될 수 있을 것이다. 이를 위해서 환자의 알레르기 반응에 대한 추가적인 연구가 요구된다.

검색어: 알러젠 중화활성, 계란 알레르기, 오발부민, 비파엽, 항원성, 알레르기성

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