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The effect of extract from leaves and stalks of *Angelica gigas* on the innate immunity

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Abstract

The dried root of *Angelica gigas* (*A. gigas*) has been traditionally used as an oriental medicine, which is known to improve blood circulation and blood stasis. In the present study, leaves and stalks of *A. gigas* were used to investigate their effects on the innate immunity. The extracts were prepared from leaves and stalks of *A. gigas* and were fed to mice. The numbers of blood cells, total WBCs, neutrophils, lymphocytes, eosinophils and basophils were increased by 50% in mice fed with leaves extract of *A. gigas* compared to control mice. However, the numbers of blood cells were decreased when treated with stalks extract of *A. gigas*. The level of cholesterol and triglyceride in serum was markedly reduced in both mice group fed with leaves extract and stalks extract of *A. gigas* compared to control group ($P<0.01$). There was no significant change in the level of albumin, total protein, phosphate and calcium in serum. Activity of cationic peptide was found to be diffused in the testicles of mice fed with leaves extract of *A. gigas* compared to control group, which might be due to increased lysozyme in testicle. The lysoplate assay and immunohistochemistry assay suggest that the extract of leaves and stalks of *A. gigas* are immunogenic, but the effects might be related with acquired immune response rather than innate immunity.

Key words : *Angelica gigas*, Lysozyme, Innate immunity, Blood assay, Cationic peptide

INTRODUCTION

The innate immunity is evolutionarily a part of protective system in most hosts and functions primarily to protect host from pathogenic microbes. The molecular module of innate immunity has been found both in plants and animals (Hoffmann et al, 1999; Charles and Rulsan, 2002). In 1921, Alexander Fleming discovered a substance from a drop of nasal mucus that can lays bacteria, named as Lysozyme (Fleming, 1922). Lysozyme is a molecule that has antimicrobial effects and its activities in innate immunity have been extensively studied. Lysozyme has more effects like antibacterial, antiviral, antitumor and immune modulatory activities (Vidal et al, 2005). The structure of lysozyme was elucidated by Canfield (1963), which consisting of 129 amino acid residues, including 10 carboxyl and 7 amino groups, 11 arginine residues, 6 tryptophan residues and 4 disulfide bonds. Lysozyme is produced by glandular serous cells, surface epithelial cells and macrophages in the human airway (Ganz, 2004; Konstan et al, 1982; Prager and Jollès, 1996) and is present at high concentration in tears, gastric juice and breast milk (Hankiewicz and Swierczek, 1974). Lysozyme catalyzes the hydrolysis of

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the b (1-4) glycosidic bond of the bacterial peptidoglycan, a major component of the bacterial cell wall (Phillips, 1996).

There are three kinds of *Angelica* species such as *A. gigas* Nakai (Korean dang gui), *A. sinensis* (Chinese dang gui), and *A. aculiloba* (Japanase dang gui). *A. gigas* has dark purple-colored flowers, whereas *A. sinensis* and *A. aculiloba* have white-colored flowers (Ahn et al, 2008). *A. gigas* has been used as a traditional herbal medicine in oriental medicine, and is also one of the most popular herbal medicine in Asian countries, including Korea, Japan and China. The dried form of root of *A. gigas* is used in oriental medicine. It has been reported that *A. gigas* has pharmacological effects such as depression of cardiac contraction, decrease of the carotid arterial pressure and respiration (Chi and Kim, 1970). Recently, some scientists reported that *A. gigas* had anti-inflammatory effects (Shin et al, 2009). Although the root of *A. gigas* has been used in oriental medicine, study on the leaves and stalks of *A. gigas* have not been performed.

In the present study, the extraction was prepared from leaves and stalks of *A. gigas*, and their effects on the innate immunity were investigated.

MATERIALS AND METHODS

Experimental animals

Male ICR mice from Daehan biolink (Eumsung, Korea) were used for this experiment. Body weight of mice was 23～26 g. Each cage housed 5 mice in a room temperature with an alternating 12 hrs light-dark cycle. Food, extracted solution and water were available *ad libitum*. They were divided into 3 groups; control group, experiment 1 group and experiment 2 group. The extraction solution was diluted 2 times for group in this study. We have used an undiluted solution in group 2. A water was supplied to control group. Diluted solution and undiluted solutions were used instead of water for group 1 and group 2.

Preparation of crude extraction from leaves and stalks of *Angelica gigas*

The dried leaves and stalks was cut into thin slices. The extraction method was modified from the article of Choi et al (2003). Crude extract was prepared as follow. 1 kg of sliced leaves and stalks of *A. gigas* was mixed with 5 L of water. The mixture was boiled on 50°C for 3 hrs. The crude extracted solution was filtered through Whatman No 1. filter paper after cooling at room temperature.

Body weight measurement of mice

Body weight measure to watch the mice body weight fluctuation has carried out by group using a electric balance.

Collection of blood and intestinal organs

Mouse was administered intraperitoneally with 3.6% chloral hydrate 0.1 mL per 10 g of body weight for anesthesia. Blood was collected from abdominal vena cava and intestinal organs collection was carried out under the stereoscopic microscope. Blood analysis was performed using HEMAVET (Drew Scientific, USA) for measure of total WBC, lymphocytes, neutrophils, monocytes, eosinophils, basophils after homagenized the tube contained heparin solution for 30 mins. Serum analysis was carried out cholesterol, triglyceride, albumin, total protein, calcium, phosphate by serum analyst (Biochemical system SM 4000, Italy).

Extraction of cationic peptide

Cationic peptide extraction method was based on the method of Cole et al (1999). Extraction process is as follow, It takes 200 mg of organs and put into the 1.5 mL centrifuge tube. They were sonicanized after adding 400 μL of 5% glacial acetic acid into the tube. To obtain the supernatnat after centrifugation at 13,000 rpm, add same volume of Macro-Prep CM cation exchange support (Bio-rad, USA) to the supernatant and mix gently for 3 hrs at room temperature. Removed the super-

natant after centrifugation at 13,000 rpm. White precipitate of Macro-Prep CM cation exchange support was dried completely at room temperature. And then 250 µL of extracting solution (0.1 N HCl+0.1% glacial acetic acid) was added into the tube containing white pellets and it was pipetted gently several times. It was centrifuged at 13,000 rpm for 30 mins. Supernatant was taken by pipette and used to experiment over store at under -20°C.

Cationic peptide quantity

BCA Protein assay kit (Pierce, USA) was used for Cationic peptide quantity that was modified the Lowry's method (Lowry et al, 1951) to the measurement of total protein. The measurement was performed by following the manufacture's mannual. The standard curve was produced first, and then the amount of cationic peptide was calculated using the standard curve after the OD read at 550 nm by the spectrophotometer (Biotek, USA).

Lysoplate assay

This experiment has carried out to measurement of the activity of cationic peptide called the substrate related to innate immunity. Assay method was used modified from method of the Osserman and Lawlor (1966). Briefly, 0.125 g of heat killed *Micrococcus lysodeikticus* (Sigma, USA) put into the 50 mL of 66 mM sodium phosphate buffer (pH 7.0) and homogenized. We called this solution A. We have made solution B that was 5% agarose gel using a low melting agarose (Sigma. USA) and 66 mM sodium phosphate buffer (pH 7.0). Solution B was maintained at 42°C. Assay plate was made in a square petri dish with 13 mm Grid (BD Falcon, USA) by final concentration of 1% was mixed sloution A and solution B. The same amount of cationic peptide put into the hole of solidificated gel and measured the diameter of cationic peptide diffusion after react for 18 hrs.

Immunohistochemistry study

The organs were fixed at 10% neutralized formalin solution. Immunostaining was performed using the histostain-sp kit (Invitrogen, USA). Five-micrometer sections were cut from the paraffin blocks and mounted on charged slides. The sections were deparaffinized, rehydrated, and treated with serum blocking reagent for 10 mins to block endogenous peroxidase activity. Sections then were rinsed and incubated with primary antibodies against lysozyme (Dako, Denmark).

Table 1. A variation of body weight after feeding a mouse on with *A. gigas* extracted solution (g: Mean±SE)

	The first body weight	Control	Exp 1	Exp 2
	24	34	30	30
	25	32	34	29
	24	33	33	32
	23	32	33	34
	26	35	34	33
Average	24.6±0.41	33.2±0.47	32.8±0.59**	31.6±0.74*

*$P>0.05$, **$P>0.05$ (Compared to the control), determined by the student's *t*-test. Exp 1: *A. gigas* extracted solution was diluted 2 times with water. Exp 2: *A. gigas* extracted solution undiluted.

Table 2. White blood cell (WBC) analysis of mice after feed on with *A. gigas* extracted solution (Mean±SE)

Classification	Control	Exp 1	Exp 2	Unit	Results
Total WBCs	5.12±0.35	7.65±0.26*	2.31±0.19*	K/µL	N*
Neutrophils	0.56±0.12	1.38±0.05*	0.69±0.10***	"	N
Lymphocytes	4.30±0.38	7.02±0.16*	2.02±0.25*	"	N
Monocytes	0.18±0.09	0.23±0.05***	0.08±0.03***	"	N
Eosinophils	0.06±0.03	0.52±0.08*	0.02±0.008***	"	N
Basophils	0.03±0.02	0.08±0.007**	0.01±0.006**	"	N

*N: Normal. *$P<0.01$, **$P<0.05$, ***$P>0.05$ (Compared to the control), determined by the student's *t*-test. Exp 1: *A. gigas* extracted solution was diluted 2 times with water. Exp 2: *A. gigas* extracted solution undiluted.

Table 3. Serum analysis of mice after feed on with A. gigas extracted solution (Mean±SE)

Classification	Control	Exp 1	Exp 2
Cholesterol (mg/dL)	76.2±4.54	36±2.33*	55.8±6.34***
Triglyceride (mg/dL)	172.4±4.87	125.8±6.18*	121.6±5.00*
Albumin (g/dL)	2.66±0.25	2.59±0.19***	2.53±0.15***
Total Protein (g/dL)	2.83±0.2	2.76±0.12***	2.72±0.19***
Phosphate (mg/dL)	16.45±0.86	15.76±0.31***	16.78±1.11***
Calcium (mg/dL)	7.3±0.45	7.5±0.28***	8.52±0.26**

$*P<0.01$, $**P<0.05$, $***P>0.05$ (Compared to the control), determined by the student's *t*-test. Exp 1: *A. gigas* extracted solution was diluted 2 times with water. Exp 2: *A. gigas* extracted solution undiluted.

RESULTS

Body weight variation of mouse

The results are shown by Table 1, after feeding a mouse on with *A. gigas* extracted solution for 20 days. Body weight of experimental groups decreased slightly than the control group, but there were no significant variation of body weight in comparition with the control group.

Analysis of White blood cells and serum

The results of differential analysis on white blood cells (WBC) are shown in Table 2. The experiment 1 group increased about 50% more than the control group in the number of total WBCs but the experiment 2 group decreased by more than 50% compared to the control group. Significance of experiment 1 group and experiment 2 group was accepted by *P* value is under 0.01 each. There were significance between experiment 1 group and the control group by *P* valus is under 0.01 but there is no significance between experiment 2 group and the control group because *P* value of experiment 2 group against with the control was more than 0.05 in the number of neutrophils. In the number of lymphocytes, the experiment 1 group increased more than the control group and the experiment 2 group decreased more than the control group. In significance value, they were accepted in the significance of lymphocye number test because the *P* value is under 0.01 each. In the Monocyte, Eosinophil, the signaficance value was not accepted except for the experiment 1 group in the eosinophil. *P* value of experiment 1 group in the neutrophil was unser 0.01. In the basophil, the significance of the experiment 1 group was accepted, but the experiment was not.

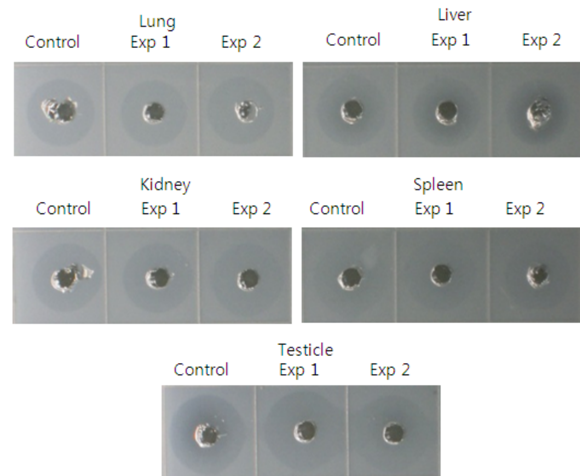

Fig. 1. Results of lysoplate assay to the cationic peptide activity.

Cholesterol level in serum was significantly decreased in the experiment 1 group compared to the experiment 2 group. The significance value of experiment 1 group was accepted but experiment 2 group was not accepted. Triglyceride level was significantly decreased in the experiment 1 group and the experiment 2 group. Significance value also was accepted in each of the experiment groups. In the level of albumin, total protein, phosphate, calcium, there were no difference in the level of the experimental groups with the control group. Calcium level of the experiment 2 group was increased more than the experiment 1 group and the control group (Table 3).

Lysoplate assay

The activity of cationic peptide are shown in Fig. 1. In the results, the cationic peptide activy shows no significant differences in the lung, liver, kidney and spleen

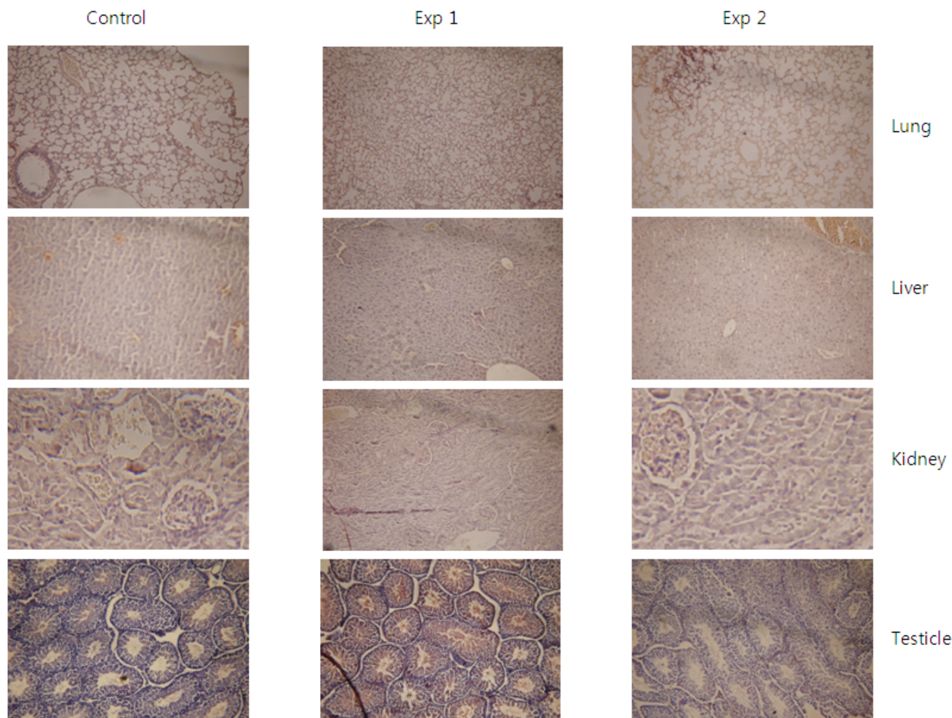

Fig. 2. Distribution of lysozyme in the lung, liver, kidney, testicle through IHC. Lung and liver 100X, kidney and testicle 200X. Exp 1: *A. gigas* extracted solution was diluted 2 times with water. Exp 2: *A. gigas* extracted solution undiluted.

between the control group and the experimental groups. But in the testicle, cationic peitide activity, there was a little more in the experiment 1 group than the control group and experiment 2 group.

Immunohistochemistry study

The results of the immunohistochemistry stain to see the lysozyme are shown in Fig. 2. The existence of lysozyme was no significance change by immunohistochemistry stain in the lung, liver, or the kidney for the control group, experiment 1 group and experiment 2 group. But the lysozyme was strongly detected in the testicle of experiment 1 group, while the lysozyme was not detected in the experiment 2 group.

DISCUSSION

A. gigas has been known to nourish the blood vessel and to improve blood circulation and stasis, and has been used as a herbal medicine in oriental medicine. It has been reported that *A. gigas* has anti-microbial and immune-modulating effects via B cell activation (Kumazawa et al, 1982). *A. gigas* increased IgM and IgG antibody titer against sheep erythrocytes (Kumazawa et al, 1985) and mediated the inflammation in mouse macrophage (Yoon et al, 2007).

In the present study, the effects of the leaves and stalks of *A. gigas* were investigated using animal. The total WBC, lymphocytes, monocytes, eosinophils, basophils were increased in mice fed with the leaves extract of *A. gigas* compared to control group. However, the numbers of these blood cells were decreased in mice fed with the stalks extract of *A. gigas*.

The serum levels of cholesterol and triglyceride were greatly reduced only in mice fed with the leaves extract of *A. gigas* compared to control group, but the change in the level of albumin, total protein, phosphate, and calcium was minimal in both animal groups. These results suggest that leaves extract of *A. gigas* induced more effects in innate immunity than stalks extract of *A. gigas*, which might be due to the disagreement of components between leaves and stalks of *A. gigas*.

The higher activity of cationic peptide was shown in the testicles of mice fed with leaves extract of *A. gigas*. The increase of cationic peptide and absence of lysozyme in testicle might be explained that the lysozyme

was increased at early time in several cells like neutrophils, macrophage, and eosinophil, but be decreased later. These suggest that leaves extract of *A. gigas* could increase innate immunity and might beused as an adjuvant of vaccine.

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REFERENCES

Ahn MJ, Lee MK, Kim YC, Sung SH. 2008. The simultaneous determination of coumarins in *Angelica gigas* root by high performance liquid chromatography-diode array detector coupled with electrospray ionization/mass spectrometry. J Pharm Biomed Anal 46: 258-266.

Canfield RE. 1963. The amino acid sequence of egg white Lysozyme. J Biol Chem 238: 2698-2707.

Charles AJ Jr, Rulsan M. 2002. Innate immune recognition. Annu Rev Immunol 20: 197-216.

Chi HJ, Kim HS. 1970. Studies on the components of umbelliferate plants in Korea: pharmacological study of decursin, decursinol and nodakenin. Korean J Pharmacogn 1: 25-32.

Choi SS, Han KJ, Lee HK, Han EJ, Suh HW. 2003. Antinociceptive profiles of crude extract from roots of *Angelica gigas* Nakai in various pain models. Biol Pharm Bull 26: 1283-1288.

Cole AM, Dewan P, Ganz T. 1999. Innate antimicrobial activity of nasal secretions. Infect Immun 67: 3267-3275.

Fleming A. 1922. On a remarkable bacteriolytic element found in tissue and secretions. Proc R Soc Lond B 93: 306-317.

Ganz T. 2004. Antimicrobial polypeptides. J Leukoc Biol 75: 34-38.

Hankiewicz J, Swierczek E. 1974. Lysozyme in human fluid. Clin Chim Acta 57: 205-209.

Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. 1999. Phylogenetic perspectives in innate immunity. Science 284: 1313-1318.

Konstan MW, Cheng PW, Boat TF. 1982. A comparative study of lysozyme and its secretion by tracheal epithelium. Exp Lung Res 3: 175-181.

Kumazawa Y, Mizunoe K, Otsuka Y. 1982. Immunostimulating polysaccharide seperated from hot water extract of *Angelica acutiloba* Kitakawa (Yamato tohki). Immunology 47: 75-83.

Kumazawa Y, Nakatsuru Y, Fujisawa H, Nishimura C, Mizunoe K, Otsuka Y, Nomoto K. 1985. Lymphocyte activation by a polysaccharide fraction seperated from hot water extracts of *Angelica acutiloba* Kitakawa. J Pharmacobiodyn 8: 417-424.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with folin phenol reagent. J Biol Chem 193: 265-275.

Osserman EF, Lawlor DP. 1966. Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J Exp Med 124: 921-952.

Phillips DC. 1966. The Three-dimensional structure of an enzyme molecule. Sci Am 215: 78-90.

Prager EM, Jollès P. 1996. Animal lysozymes c and g: an overview. EXS 75: 9-31.

Shin S, Jeon JH, Park D, Jang JY, Joo SS, Hwang BY, Choe SY, Kim YB. 2009. Anti-inflammatory effects of an ethanol extract of *Angelica gigas* in a Carrageenan-air pouch inflammation model. Exp Anim 58: 431-436.

Vidal ML, Gautron J, Nys Y. 2005. Development of an ELISA for quantifying lysozyme in hen egg white. J Agric Food Chem 53: 2379-2385.

Yoon T, Cheon MS, Lee DY, Moon BC, Lee HW, Choo BK, Kim HK. 2007. Effects of root extract from *Angelica gigas* and *Angelica acutiloba* on inflammatory mediators in mouse macrophages. J Appl Biol Chem 50: 264-269.