Food Sci. Biotechnol. 19(6): 1559-1565 (2010)
DOI 10.1007/s10068-010-0221-9

RESEARCH ARTICLE

Effects of Fermented Ginseng Extracts on Tumor Metastasis in Mice

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Received: 6 July 2010 / Accepted: 23 August 2010 / Published Online: 31 December 2010

Abstract This study was demonstrated that the fermentation of gingseng increases its biological activities, by comparing the anti-tumor and immunostimulating activities of fermented gingseng extracts (FginE) with those of non-fermented ginseng (GinE). In the experimental lung metastasis of colon26-M3.1 carcinoma, the intraperitoneal (i.p.) or peroral (p.o.) administration of FginE showed stronger anti-tumor metastatic activities than those of GinE. When stimulating 2 kinds of extract on macrophages, FginE was shown to have a higher production of interleukin (IL)-12 than GinE. In addition, treatment with FginE induced tumoricidal activity of peritoneal macrophages against colon26-M3.1 cells. When Peyer's patch was co-cultured with FginE, the proliferation of these cells and granulocyte macrophage-colony stimulating factor (GM-CSF) production were induced. In an assay for natural killer (NK) cell activity, an i.p. administration of FginE significantly augmented NK cytotoxicity against Yac-1 tumor cells. Fermented ginseng extracts promotes anti-tumor activities to inhibit tumor metastasis, and its anti-tumor effects are associated with the enhancement of systemic as well as mucosal immune systems.

Keywords: fermented-ginseng, tumor metastasis, innate immunity, mucosal immunity, cytokine

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Introduction

Many efforts have been made to develop and improve immunotherapy strategies for the treatment of various malignancies. The use of biological response modifiers (BRMs) for enhancing host defense responses against tumors is one of the most attractive alternatives to the use of cytotoxic drugs (1). It is well known that the activation of the innate immune system is thought to be a critical role of the defense system against foreign antigens including tumors (2). To stimulate the innate immune system, a number of immunomodulators have been developed including cytokines (3) constituents isolated from microorganisms (4) and herbal plants (5), synthetic adjuvants (6), and oligonucleotides (7), and some of them have been used for clinical therapeutics. It is also well known that the primary mechanisms of the immunostimulating activities by BRMs are due to their ability to activate macrophages or natural killer (NK) cells (2). Activated NK-cells and macrophages can inhibit the growth of a wide variety of tumor cells. Indeed, many experimental studies and clinical trials have shown that natural immunity plays an important role in the blocking of metastasis from primary tumors (4). Among the various immune-related cells, NK cells and macrophages were thought to be the relevant effectors responsible for the natural immunity against tumors (2,3,7). In addition, various cytokines, such as interleukin (IL)-12, tumor

necrosis factor (TNF)-α, or IL-1β from macrophages (8,9), were demonstrated to augment NK cell responses and these proinflammatory cytokines can induce activation of adaptive immunity in part through the stimulation of interferon (IFN)-γ production in NK cells (10).

Ginseng (the root of *Panax ginseng* C.A.),which is one of the best known Asian traditional herbal medicines taken orally in its raw form, has been reported to exhibit various types of biological activity, including anti-inflammatory and anti-tumor effects (11,12). Ginseng ginsenosides (saponines), which are the major components of ginseng, have many various pharmacological activities, and a large number of ginsenoside derivatives have been identified in *Panax ginseng*. These ginsenosides have shown various biological actions (12,13). However, not all of the people can gain from the effective use the ginseng saponins because of the biological differences in each human. Wakabayashi *et al*. (14,15) have proposed the concept that plant glycoside acts as a pro-drug that is metabolized into an active form by intestinal bacterial deglycosylation. Therefore, the study related to the bacteria which ferments the ginseng is in progress for finding productive applications for the functional foods and its ingredients (16).

Therefore, as part of the investigation reported in this paper, the ginseng saponins are fermented with the yeast separated from ginseng soil followed by the comparison of the immune-stimulating activities of the ginseng extracts fermented and not fermented with yeast. For these, we investigated the effects of the systemic or oral administration of the extracts on the experimental tumor metastasis model by using colon26-M3.1 carcinoma cells and analyzing the involvement of the activation of the innate and intestinal immune systems to clarify its anti-metastatic effect against tumors.

Materials and Methods

Preparation of fermented ginseng The root of 4-year cultured *Panax ginseng* C.A., purchased in Ji Lin Province, China was used in this experiment. The root of ginseng which was removed shells grinded in 10 volumes of distilled water and left being stirred for 2 hr at 4°C, and then drying at 55°C. An appropriate amount of dried ginseng extracts was dissolved with phosphate-buffered saline (PBS) and sterilized at 121°C for 20 min (GinE), and store at 4°C until use. In addition, GinE was fermented by the yeast which we designated strain KY17 isolated from ginseng cultured soil sample collected at Yanbian province in China. The KY17 showed belongs to *Streptomyces* sp. according to the result of shape and rDNA sequencing research (unpublished). GinE inoculated with KY17 was cultured on rotary shakers, operating at 110 rap/min at 30°C for 4 days. Finally, the fermentation was sterilized through 0.4 μ pore-size filter, and dried with a freeze dryer (FD8508; IlShin Lab Co., Ltd., Korea).

Mice and cell cultures The experimental protocol was reviewed and approved by the Yuhan University Animal Care Commettee (E2009-002). BALB/c (female, 6 weeks old) were purchased from NARA Biotech., Korea and kept in an animal room in Department of Food and Nutrition in Yuhan University. Water and a diet of pellets were supplied *ad libitum*. A lung metastatic subline of a highly metastatic line of colon 26 carcinoma cells (colon26-M3.1) were maintained as monolayer cultures in Eagle's minimum essential medium (MEM) supplemented with 7.5% fatal bovine serum (FBS), sodium pyruvate, non-essential amino acids, and L-glutamine, which were purchased from Gibco BRL (NY, USA) as described previously (17). YAC-1 lymphomas cell, peritoneal macrophages, and splenocytes taken from the mice were cultured in RPMI-1640 (Gibco BRL) supplemented with 7.5% FBS and L-glutamine.

Experimental lung metastasis Experimental lung metastasis of colon26-M3.1 carcinoma cells (3×10^4 cells) was assessed through the counting of tumor colonies in the lung after intravenous (i.v.) inoculation of tumor cells into BALB/c mice as previously described (17). In the lung metastasis experiment, the ginseng extracts were i.p. administrated to mice at 0.2-20 mg/kg body weight (BW) 2 days before the tumor cell inoculation. Mice were also administered peroral (p.o.) with ginseng extracts (8-80 mg/kg) 7 times for everyday and 6 times for 2 days intervals after tumor inoculation. The mice were euthanized 14 days after the tumor inoculation and their lungs were suspended in Bouin's solution (Sigma-Aldrich, USA). The lung tumor colonies were counted microscopically.

Cytokine assay Peritoneal macrophages were harvested from the mice which were injected intraperitoneal (i.p.) with 5 mg of silica as described previously (18). The cells (1×10^6/mL/well), suspended in the complete RPMI-1640 medium, were plated onto 24-well culture plates. After 2 hr of incubation, the non-adherent cells were removed by being washed with PBS, and the adherent macrophages were stimulated with the indicated doses of ginseng extracts for 24 hr. The concentration of TNF-α and IL-12 in the culture supernatants was determined using enzyme-linked immunosorbent assay (ELISA) kits (BD Pharmingen Co., USA) according to the manufacturer's recommendations.

***In vitro* assay of macrophage-mediated cytotoxicity** In brief, colon26-M3.1 cells (1×10^4) were added to the

macrophage monolayers harvested from the mice given i.p. administration of ginseng extracts (20 mg/kg BW) 3 days before and incubated for 18 hr. This assay was performed in 96-well plates to obtain various macrophage-to-target cell ratios. After centrifugation, the culture supernatants were mixed with an lactate dehydrogenase (LDH) solution (Promega, USA), and the absorbance value of each well was measured at 620 nm (Molecular Device, USA). The percentage of macrophage cytotoxicity was calculated using the following formula: Cytotoxicity (%)= [(experimental release-spontaneous release)/(maximum release spontaneous release)]×100.

Assay of NK-mediated tumor cytotoxicity Three BALB/c mice/group were administered i.p. with 500 mg of FginE (20 mg/kg BW), and their splenocytes were harvested 2 days after the sample treatment. Single cell suspensions of the splenocytes were added to Yac-1 cells (1×10^4/well) to obtain effector-to-target cell ratios (E/T ratio) of 100:1, 50:1, or 25:1 in U-bottomed 96 well plates, and the cultures were incubated for 6 hr (17). After incubation, the plates were centrifuged for 10 min at 900×g. The culture supernatants were admixed with LDH solution (Promega), and the absorbance value of each well was measured at 620 nm. NK cell cytotoxicity was calculated from the radioactivity (count/min) according to the following formula: Cytotoxicity (%)=[(experimental release-spontaneous release)/(maximum release spontaneous release)]×100.

Proliferation assay of bone marrow cells To test the bone marrow cell proliferation activities during the treatment of ginseng extract, bone marrow cells from BALB/c mice were harvested and cultured with the indicated dose of ginseng extract for 5 days (19). Cell proliferation was assayed by a WST based colorimetric assay (EZ-Cytox; Daeil Lab., Korea). Absorbance of each well was monitored at 450 nm.

Induction of GM-CSF from Peyer's patch cells The suspension of Peyer's patch cells was prepared from the small intestine of BALB/c mice, as previously descrived (19). Peyer's patch cell suspension (2×10^5 cell/well) were cultured with FginE for 3 days. Murine granulocyte macrophage-colony stimulating factor (GM-CSF) contents in the cultures of Peyer's patch treated with ginseng extract were measured by an ELISA kit (BD Phaminogen) according to the manufacturer's recommendations.

Statistical analysis The statistically significant differences among PBS-treated control group, GinE-treated group, and FginE-treated group were calculated by applying the Student's 2-tailed *t*-test.

Results and Discussions

Inhibitory effect of ginseng extracts on experimental lung metastasis To investigate the effect of fermented ginseng extracts on anti-metastatic activity, it was compared the effects of fermented vs. non-fermented ginseng extracts on tumor metastasis in experimental lung metastasis of colon26-M3.1. The i.p. administration of FginE induced the dramatic inhibition of lung metastasis of colon26-M3.1 cells in a dose dependent manner. Administration of FginE (2 mg/kg) and GinE (20 mg/kg) inhibited lung metastasis by over 70% compared to the tumor control group (Table 1). We next examined whether the oral administration of FginE can also augment the inhibitory effect against tumor metastasis, since the ginseng has been taken orally in traditional medicine. As a result, the p.o. administration of FginE dramatically inhibited the lung metastasis produced by tumor cells, but the GinE did not (Table 2). The administration of ginseng extracts in those ranges did not show any apparent side effects such as a decrease of body weight or piloelection (data not shown). Taken together, the inhibitory effects of the fermented ginseng extract against metastasis seemed to increase anti-tumor activity compared to that of unfermented ginseng extracts in lung metastasis model by colon26-M3.1 carcinoma cells. Ginseng has been widely believed a miraculous medicine to improve the host defense system in with long-term administration (20). Numerous evidences have showed that ginsenoside is responsible for the pharmacological effects of ginseng and led to the misunderstanding that intact ginsenoside might be the real active principle in the body (21). However, Hasegawa *et al.* (21,22) proposed that ginsenosides acts as prodrugs that are metabolized to the active form by intestinal bacteria deglycosylation and the metabolites stimulate lymphocytes to become cytotoxicity to tumor cells and did not direct affect tumor cell growth *in vitro*. In our experiment, although FginE showed 10 times lower cytotoxicity against colon26-M3.1 tumor cell lines than GinE *in vitro* (data not shown), administration of FginE indeed induced higher anti-metastatic activity than that of GinE (Table 1, 2), which suggests that the yeast, KY17 can convert the ginseng materials to active form to stimulate immune related cell in mice.

Cytokine production from macrophages To investigate the mechanism by which FginE can inhibit the tumor metastasis, we further examined the immune stimulation effect of FginE. It is well known that activated macrophages release various cytokines (8,9), and these cytokines play a role in inducing and modulating immune responses to elicit potent anti-tumor or anti-metastatic activities (7). As shown in Fig. 1, treatment of peritoneal macrophages with FginE in an *in vitro* experiment induced IL-12, and the

Table 1. Effect of systemic administration of fermented ginseng extracts on lung metastasis

Treatment	Dose (mg/mouse)	No. of lung metastases tumor colonies		
		Mean±SD	Range	Inhibition %
Untreated (PBS)	-	92.7±10.9[1)]	79-103	-
Fermented	20	17.6±9.5[a,b]	5-31	81.0
	2	24.7±4.0[a]	19-30	73.9
	0.2	61.0±7.8	51-69	34.0
Non-fermented	20	36.8±7.3[a]	28-48	60.3
	2	47.0±11.8[a]	38-67	49.3
	0.2	86.6±7.8	74-98	-

[1)]Mean±SD (n=5/group); [a]p<0.01, compared with the untreated group; [b]p<0.01, compared with the unfermented group

Table 2. Effect of p.o. administration of fermented ginseng extracts on lung metastasis

Treatment	Dose (mg/mouse)	No. of lung metastases tumor colonies		
		Mean±SD	Range	Inhibition %
Untreated (PBS)	-	105.0±14.7[1)]	95-130	-
Fermented	80	62.6±14.1[a]	48-79	40.4
	8	101.9±9.8	96-121	-
Non-fermented	80	81.6±7.4	70-90	22.3
	8	106.4±11.1	94-124	-

[1)]Mean±SD (n=5/group); [a]p<0.01, compared with the untreated group

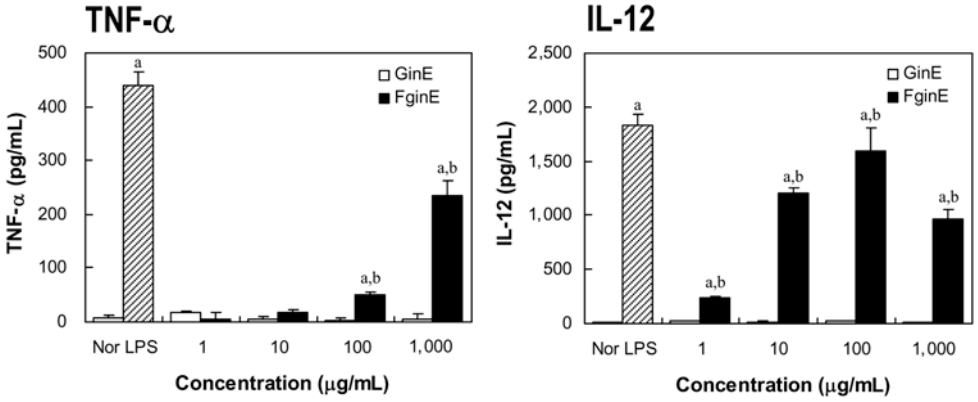

Fig. 1. Effect of fermented ginseng on the induction of cytokines from macrophages. Values are the mean±SD. [a]p<0.01, compared with the untreated group; [b]p<0.01, compared with the unfermented group

inducing activities of FginE were shown to be higher those of GinE. In addition, peritoneal macrophages obtained from FginE-treated mice displayed a higher cytolytic activity against tumor cells than those from the GinE treated and untreated mice (Fig. 2). This suggests that FginE can activate macrophages, and its ability to induce cytokines from macrophages, and the enhancement of macrophage-mediated cytotoxicity against tumor cells (2). The activated macrophages as effector cells, stimulated by IFN-γ and autologous IL-12, can eradiate tumor cells by releasing TNF-α and nitric oxide *in vitro* (23). This suggests that FginE can activate macrophages, and its ability to induce cytokines from macrophages (Fig. 1), and enhance macrophage-mediated cytotoxicity against tumor

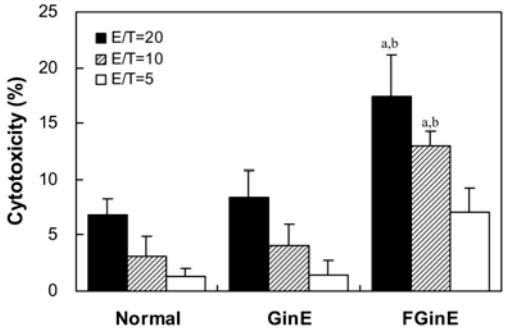

Fig. 2. Effect of fermented ginseng on macrophage-mediated cytotoxicity against tumor cells. Values are the mean±SD. [a]p<0.01, compared with the normal group; [b]p<0.01, compared with the unfermented group

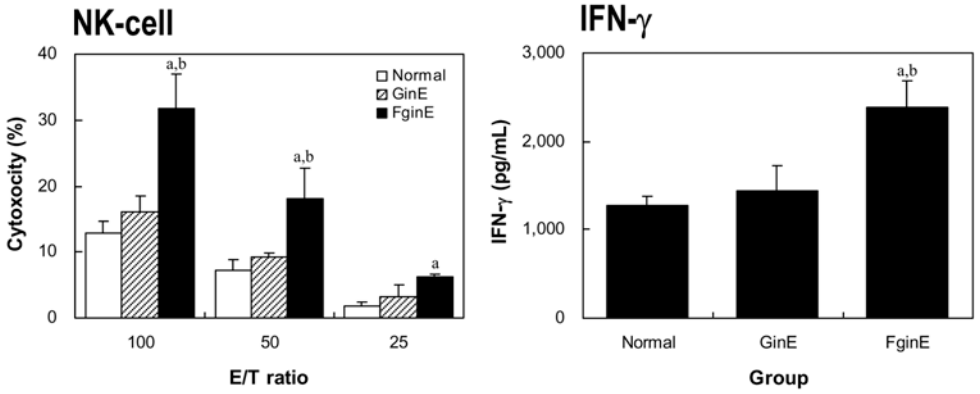

Fig. 3. Effect of fermented ginseng on the enhancement of NK cell activity and IFN-γ production. Values are the mean±SD. [a]$p<0.01$, compared with the normal group; [b]$p<0.01$, compared with the unfermented group

cells (Fig. 2) may be related to its anti-tumor activities (24). It is of particular significance that FginE induced the secretion of IL-12 from macrophages. IL-12 is said to be one of the most essential cytokines to elicit tumor immunity (11). In the present study, the reason of the increase of cytokine production from macrophages by FginE was suggested that the yeast fermentation of ginseng extracts can change the ingredients to activate macrophages (25). In addition, IL-12 has potent anti-tumor growth and metastasis activity through the activation of effector cells against tumor cells, and its effects are most likely through IFN-γ in mainly NK cells (23,26). Actually, many investigators demonstrate that the activation of NK cells by immuno-stimulants led to a reduction of the metastatic colonization of tumors (17,24). Thus, it was also need to analyze the mechanism of the inhibitory effect of FginE on tumor metastasis related to the point of NK cell activation.

Effect of fermented ginseng extract on NK cell activity NK cells play a critical role in immune surveillance against tumors (26). The main functions of NK cells are the lysis of tumors and the production of immunoregulatory cytokines such as IFN-γ (26,27). The effect of FginE on NK cell activity was measured by the cytotoxic activity against Yac-1 cells. As seen in Fig. 3, the splenocytes obtained from mice administered i.p. with FginE 2 days before the assay showed a higher cytotoxicity than those from the GinE treated or untreated mice in an E/T ratio-dependent manner. The cytotoxicity of NK cell is increased in FginE treated mice indicating that FginE can activate these cells. Since IFN-γ production by activated innate cells is an important component of antitumor immunity in tumor-bearing mice (27), IFN-γ secretion by splenocytes of mice treated with FginE is investigated. As shown in Fig. 3, the content of IFN-γ in the culture supernatant of splenocytes from mice treated with FginE with YAC-1 cells showed significantly higher levels (2,378.8±304.1) than in those receiving normal (1,279.8±51.3) or GinE (1,712.3±182.1). Therefore, as shown in Table 1, the ability of FginE to induce various cytokines from macrophages and augment NK-cell mediated cytotoxicity through the production of IFN-γ (26) may be mechanisms related to the enhancement of natural immunity of the host to inhibit tumor metastasis (26,27).

Induction of GM-CSF production from Peyer's patch cells It is generally recognized that immune actions at the mucosal surface are critical for the protection or suppression of antigens present at local sites (28). Indeed, our previous studies showed that the oral administration of herbal medicines significantly inhibited experimental lung metastasis by colon26-M3.1 cells (17,29). In addition, as shown in Table 2, the oral administration of FginE which inhibited the tumor metastasis suggested that several kinds of factors by fermentation may have contributed to the activation of the intestinal immune system in mice. The intestinal immune system modulating Peyer's patchs are generally thought to be the foci of the mucosal immune system. Activated T cells in immune system including Peyer's patch are known to secrete hematopoietins such as IL-3, -4, -5, -6, -13, -17, and the GM-CSF which were known to up-regulate host innate immune responses (19,30). Among these cytokines, GM-CSF stimulates the formation of cell colonies in bone marrow cultures and causes them to differentiate to specific cell lineages such as granulocytes, monocytes, and lymphocytes (31). In order to understand whether FginE enhanced GM-CSF from Peyer's patch cells, Peyer's patch cells from mice were cultured with FginE, and then the levels of GM-CSF in the conditioned medium were examined. As shown in Fig. 4, the secretion of GM-CSF in the cultures of the splenocytes or Peyer's patch cells treated with FginE is significantly higher than in those of the medium alone and GinE, which can lead one to hypothesize that FginE possibly acts to promote the

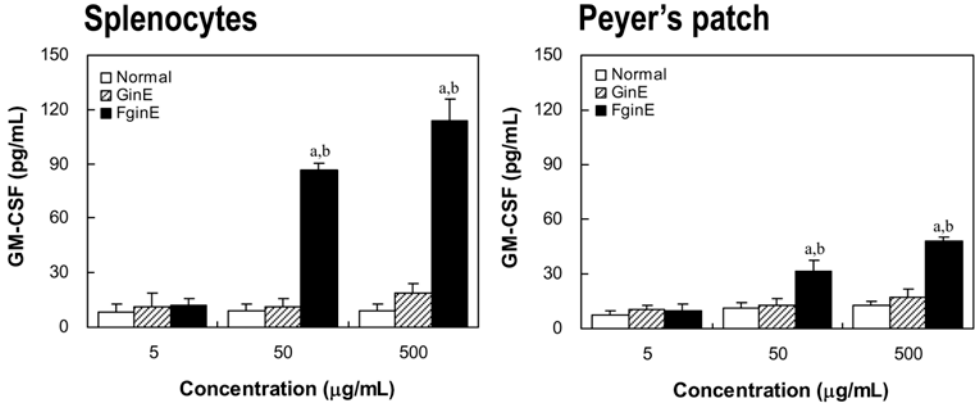

Fig. 4. Effect of fermented gingseng on the production of GM-CSF from Peyer's patch and spleen cells. Values are the mean±SD. [a]$p<0.01$, compared with the normal group; [b]$p<0.01$, compared with the unfermented group

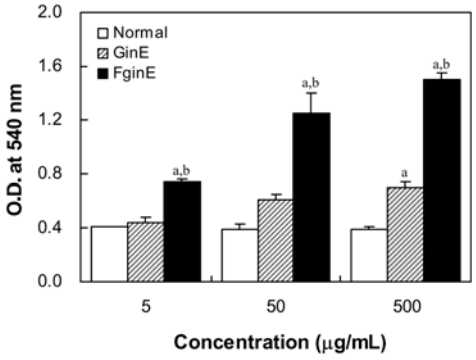

Fig. 5. Effect of fermented gingseng on the Peyer's patch cell mediated proliferation of bone marrow cells. Values are the mean±SD. [a]$p<0.01$, compared with the normal group; [b]$p<0.01$, compared with the unfermented group

activation of bone marrow cells and differentiates them to monocytes or leukocytes in blood (32). Collectively, that FginE could produce GM-CSF suggests that the yeast used in an experiment can change the ginseng saponins into the active metabolite directly producing a cytokines (25).

Bone marrow cell proliferating activities The effect of FginE on Peyer's patch cell-mediated bone marrow cell proliferation assay was investigated. As shown Fig. 5, the proliferating activity of bone marrow cell was shown when the conditioned medium of the Peyer's patch cell stimulated with FginE was added to the bone marrow cell cultures, while the GinE conditioned medium was shown to have lower activity than FginE. This showed that FginE was able to stimulate bone marrow cells to differentiate and expand themselves to monocytes or leukocytes in blood (30,31). Taken together, we concluded that the anti-metastatic activities of FginE by administration were partially due to the activation of immune effector cells against tumors through the stimulation of macrophage, the NK-cells. In addition, the oral administration of FginE could enhance the systemic immunity through Peyer's patch cells activation in the mucosal sites. Further study to elucidate the changes of the composition and structures of ginsenoside during fermentation and the characteristics of the KY17 related to anti-tumor activity of FginE is currently underway in our laboratory.

Acknowledgments This work was supported by the Yuhan University research grants in 2009.

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