2003

# Purification and antioxidant activities of soybean isoflavones

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# PURIFICATION AND ANTIOXIDANT ACTIVITIES OF SOYBEAN ISOFLAVONES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Food Science

by
Qiju Wu
B.S., Zhejiang University, 1999
August, 2003

# Acknowledgements

I want to begin by thanking my major professor, Dr. J. Samuel Godber, Professor in the Department of Food Science, for his patient guidance, precious advice, and persistent encouragement during the preparation and investigation of this thesis.

Special thanks to Dr. Zhimin Xu, Department of Food Science, he has been directly guiding my research since I came to LSU. Thank you for introducing me into the area of functional food and any patient instruction and help. Your profound insight into problems deeply impressed me. I would also like to express my most sincere appreciation to Dr. Jack N. Losso, Assistant Professor in the Department of Food Science, who has been giving me the most support and the best suggestions. Appreciation is extended to Dr. Grover L. Waldrop, Associate Professor in the Department of Biological Science, for his instruction and serving as a member of my committee and Dr. Vincent L. Wilson, Professor in the Department of Environmental Studies, for his precious time given to me to consult about the problems of my research.

I would like to show my deepest gratitude to my parents for their unconditional and unending love and my brothers and sisters for their persistent support. Without them, I would not have the chance to pursue the cutting edge education in US. Deep gratitude is also given to all my American friends for everything they have done to help me get used to the new culture, so I could start my knowledge absorbing life in US more quickly.

I also wish to thank all the professors and the friendly office staff of the Food Science Department at LSU, whose help with many issues was much appreciated.

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# Abstract

An efficient procedure was developed and used to extract and purify isoflavones from defatted soy flour. The purity of the final product reached 94%. The antioxidant activities of soybean isoflavones (genistein and daidzein purchased from Sigma Chemical Co, and a mixture of isoflavone glycosides extracted from defatted soy flour) were investigated by a cholesterol oxidation model accelerated by 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH). All components exhibited very high antioxidant activities in the inhibition of cholesterol oxidation. Genistein had higher inhibition to the formation of 5,6beta-epoxycholesterol (5,6β-EP) and 7-ketocholesterol (7-keto), while no significant difference was found between the antioxidant activities of daidzein and genistein against cholesterol oxidation to 7beta-hydroperoxycholesterol (7β-OOH) and 5,6alpha-epoxycholesterol (5,6α-EP). The glycosides mixture had characteristics similar to genistein and daidzein in preventing cholesterol oxidation, but the glycosides mixture was found to have lower antioxidant activities compared to aglycones. The biological activities of genistein and daidzein to affect cellular response in SVEC4-10 mouse endothelial cells to t-butyl hydroperoxide-induced oxidative stress were also evaluated. Genistein had higher cytotoxicity than daidzein to SVEC4-10 cells and none exerted discernable antioxidant activities in this cell culture model. The antioxidant activities of soybean isoflavones may contribute to their potential role in reducing the formation of certain types of cancers, so daidzein might be a more appropriate cancer preventive neutraceutical because of its lower cytotoxicity. In view of the high cytotoxicity, this cell culture model might not be suitable for the study of

antioxidant activity of soybean isoflavones. Some modifications or another cell culture model is needed in order to further this study of antioxidant activity. Defatted soy flour is a by-product of oil extraction, so the antioxidant activities of soybean isoflavones could improve the utilization of the by-product and benefit the soy industry.

# Chapter 1

# Introduction

Soy foods have received considerable attention of late for their potential role in reducing the formation and progression of certain types of cancers and some chronic diseases such as cardiovascular disease, Alzheimer's disease, and osteoporosis (Messina, 1999; Messina and Bennink, 1998; Clarkson and Anthony, 1998; Zhao et al., 2002; Anderson and Garner, 1998). Several mechanisms have been proposed, such as lowering oxidative stress, stimulating or inhibiting estrogen receptor, and preventing the proliferation of cells, but the specific mechanism is still under study (Tikkanen et al., 1998; Mitchell et al., 1998; Maggiolini et al., 2001; Hempstock et al., 1999). Oxidative stress has been associated with several cell toxic processes including oxidative damage to protein and DNA, membrane lipid oxidation, enzyme inactivation, and gene mutation that may lead to carcinogenesis, so lowering oxidative stress might be an effective way to prevent the formation of cancers and other chronic diseases (Paulsen et al., 1998). Several anti-carcinogens, including isoflavones, have been identified in soybeans, but soybean isoflavones might be the most likely one because of its antioxidant activity through its phenol structure, which maintains the redox balance of normal cells.

Because more than 70% of isoflavones are left in defatted soy flour, a by-product of oil extraction, defatted soy flour would be an economical resource for isoflavone extraction. Although considerable research has been carried out to investigate soybean isoflavones' antioxidant activities, the underlying mechanisms need to be elucidated. More over, most of the antioxidant activity studies were done with genistein, the major

component of soybean isoflavones. For example, genistein was studied in an oxidation model elicited by UVB irradiation and was found to substantially inhibit a series of oxidative events, including hydrogen peroxide ($H_2O_2$) production, lipid peroxidation, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation (Wei et al., 2002). Genistein was also studied in a low-density lipoprotein (LDL) oxidation model initiated by copper and peroxy radicals and was found to inhibit LDL oxidation (Uesugi et al., 2002). Daidzein is another major component of soybean isoflavones but much less research has been done with it. So the objectives of this research were to explore a procedure to efficiently extract and purify isoflavones from defatted soy flour, to investigate the mechanisms underlying soybean isoflavones' antioxidant activities, and to evaluate and compare the antioxidant activity of daidzein and genistein.

The antioxidant activities of soybean isoflavones have been studied with different models with the LDL oxidation model being the most popular. Cholesterol is an essential molecule for humans as a component of cell membrane and as the precursor of steroid hormones and bile acids, but cholesterol oxidation products (COPs) are considered to be mutagenic and carcinogenic compounds and may cause cardiovascular disease (Panniangvait et al., 1995; Carpenter 2002; Woods and O'Brien, 1998; Wilson et al., 1997; Kumar, 1991). So, an antioxidant activity study using a cholesterol oxidation model would be practical and of great importance as well.

Initiated by free radicals, cholesterol is oxidized to hydroperoxides, peroxides, epoxides, and other degradation products (Smith 1987; Yan and White, 1990; Nam et al., 2001). 7-Hydroperoxycholesterol (7-OOH) is the primary cholesterol oxidation product with 5,6-epoxycholesterol (5,6-EP), 7-ketocholesterol (7-keto), and 7-hydroxycholesterol

(7-OH) as the secondary oxidation products produced after 7-OOH is epoxidized, dehydrated, and reduced. Seven cholesterol oxidation products, 5,6alpha-epoxycholesterol (5,6α-EP), 5,6beta-epoxycholesterol (5,6β-EP), 7-ketocholesterol (7-keto), 7alpha-hydroperoxycholesterol (7α-OOH), 7beta-hydroperoxycholesterol (7β-OOH), 7alpha-hydroxycholesterol (7α-OH), 7beta-hydroxycholesterol (7β-OH), were found during heating of cholesterol (Chien et al., 1998). Similar to lipid oxidation, cholesterol oxidation in the human body is dominated by free radical chain reactions, so the oxidation model with heating may not be appropriate to predict cholesterol oxidation status in the human body. An aqueous free radical reaction initiator, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), has been used to accelerate cholesterol oxidation with a reaction temperature of 37$^{o}$C, which is at relatively similar conditions to the human body (Xu et al., 2001). It is more appropriate to use this model to evaluate the antioxidant activity for compounds of interest against cholesterol oxidation.

Vascular cells, especially endothelial cells are easily attacked by free radicals and the dysfunction of endothelial cells is involved in the progression of athrogenesis. In this research, the effect of soybean isoflavones, genistein or daidzein, on oxidative injury to cultured mouse endothelial cells (SVEC4-10) was examined. Oxidative injury was induced by tert-butyl hydroperoxide (tBOOH), which is an organic hydroperoxide widely used as a model compound to induce oxidative stress (Buc-Calderson et al., 1991; Mirella et al., 1998). SVEC4-10 is an endothelial cell line derived by SV40 transformation of endothelial cells from mouse auxillary lymph node vessels. They could grow infinitely without special addictives and are well differentiated, responding like normal endothelial cells to some interleukins and to extra-cellular matrix signals for tube-like differentiation.

After a latency period of 14 weeks, they would induce spindle tumors with some of the histo-pathologic characteristics of human Kaposi Sarcoma. An understanding of how soybean isoflavones protect SVEC4-10 from oxidative injury *in vitro* would help to clarify how they exert biological activity *in vivo*.

# Chapter 2

# Overview of the Mechanisms for Soybean Isoflavones' Health Improvement Actions

## 2.1 Introduction

More and more evidence indicates that soybean isoflavones may offer protection against a wide range of human conditions, including breast, bowel, prostate and other cancers, cardiovascular disease, brain dysfunction, alcohol abuse, osteoporosis and menopausal symptoms. Several mechanisms for their protective function have been proposed, although the exact mechanisms have not been identified. Soybean isoflavones have antioxidant activity, may influence the production, metabolism and biological function of sex-hormones, may affect the synthesis and activity of intracellular enzymes, may change growth factor action, malignant cell adhesion, proliferation, and differentiation. These factors alone or in some combination contribute protective functionality to human health. The mechanisms will be reviewed here according to their specific actions, such as anti-cancer action, cardiovascular system protective action and anti-osteoporosis action.

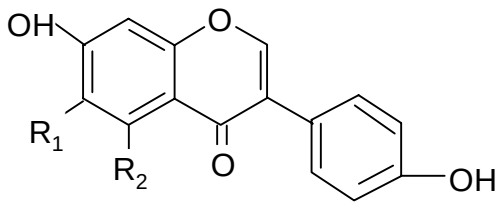
## 2.2 Anticancer Actions

### 2.2.1 Cancer Preventive Function

The anticancer action of soybean isoflavones is focused mainly on their cancer preventive function. Epidemiological evidence and studies in cancer models suggest that isoflavones play an important role in cancer prevention. For example, the consumption of soy products, which contain a mixture of soybean isoflavones, may contribute to the relatively lower rates of breast, colon, and prostate cancer in countries such as Japan and

China (Messina et al., 1994). However, the exact biological mechanisms underlying this effect remain to be fully elucidated.

The most tenable mechanism being postulated for soybean isoflavones' cancer preventive function is that soybean isoflavones have strong antioxidant capacity. The structures of three main soybean isoflaovones, genistein, daidzein, and glycitein are shown in Figure 1. The two pharmacophores that account for soybean isoflavones' antioxidant activity are the 4' hydroxyl group on the B-ring and the hydroxyl groups on the AC-ring. All of them could be hydrogen/electron donators (Heijnen et al., 2002).




AC-ring B-ring

| R1 | R2 | |
|---|---|---|
| H | H | Daidzein |
| OH | H | Genistein |
| H | $OCH_3$ | Glycitein |

Figure 1. Structures of daidzein, genistein, and glycitein.

Free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), could be formed endogenously as the result of normal oxidative metabolic reactions; exogenously as the components of tobacco smoke, diet, drugs, and other environmental pollutants; and indirectly through radiation and metabolism of certain solvents. These reactive compounds will react with and potentially alter the structure and function of several cellular components, such as lipid-contain cell membranes, lipoproteins, RNA and DNA, and cause the formation of a variety of chronic diseases

such as cancer, cardiovascular disease, cataract formation, aging process and some neurological disorders (Rock et al., 1996). As a complicated entity, the human body itself has an antioxidant defense system that prevents free radical formation and removes radicals before oxidative damage can occur, but oxidative damage can still result when the critical balance between free radical generation and antioxidant defenses is unfavorable. Interaction of free radicals with DNA bases can result in the formation of DNA adducts which, during the course of attempted replication, can lead to DNA mutation. Accumulation of DNA mutations (specifically in crucial genes) contributes to the development of neoplastic cells. Indeed, free radicals have been shown to possess many characteristics of a carcinogen (Wiseman and Halliwell, 1996). Antioxidants are thought to decrease the incidence of cancer formation because antioxidants could fight against free radicals directly as free radical scavenger and maintain the normal antioxidant defense system through protecting or reducing endogenous antioxidant enzymes.

As with other antioxidants, soybean isoflavones may exhibit their cancer preventive function through their antioxidant properties. Genistein, the major component of soybean isoflavones, has been demonstrated to inhibit ultraviolet-B (UVB)-induced skin tumorigenesis in hairless mice. The antioxidant properties of genistein may explain the mechanisms of its anti-photocarcinogenic action because through either direct quenching of reactive oxygen species or indirect anti-inflammatory effects, genistein was found to substantially inhibit a series of oxidative events elicited by UVB irradiation, including hydrogen peroxide ($H_2O_2$) production, lipid peroxidation, and 8-hydroxy-2$^{'}$-deoxyguanosine (8-OHdG) formation (Wei et al. 2002). In an *in vitro* model, calf thymus

DNA was used to investigate the inhibition of genistein on UV light and Feton reaction-induced oxidative DNA damage. Genistein potently scavenges both hydrogen peroxide and superoxide anions, which suggests genistein has potential anti-carcinogenic function in photocarcinogenesis through its antioxidant property (Wei et al., 1996).

Soybean isoflavones are also effective in decreasing the oxidative stress caused by other tumor promoters, such as 12-O-tetradecanoyphorbol-13-acetate (TPA) and xanthine/xanthine oxidase. Genistein strongly inhibits the $H_2O_2$ formation induced by TPA both *in vivo* and *in vitro* (Wei et al., 1993). In the model of HL-60 cells, both genistein and daidzein have strong inhibition to TPA induced $H_2O_2$ formation, while daidzein only shows a moderate inhibitory effect to $O_2^-$ generation caused by xanthine/xanthine oxidase. This shows that the antioxidant properties of soybean isoflavones are structurally related and the authors found that the hydroxyl group at position $4^{'}$ is crucial for their antioxidant property (Wei et al., 1995).

Directly preventing oxidative stress is just one of the mechanisms through that soybean isoflavones exhibit their cancer preventive function. Soybean isoflavones' chemopreventive action could also arise from their promotion of endogenous antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase. Dietary administration of genistein (50 and 250 ppm) for 30 days significantly increases the activities of antioxidant enzymes in various organs of SENCAR mice (Mahwah, 1996). Soybean isoflavones extract normalized total hepatic glutathione peroxidase activity, which was suppressed about 17% by phenobartital (PB) (Lee et al., 1995). Soybean isoflavones could act at either the transcription level or the protein level to influence the activities of endogenous antioxidant enzymes, but the specific

mechanism has not been identified. In a model of hepatoma H4IIE cells, daidzein treatment increased catalase mRNA expression. Transfection experiments were performed in order to elucidate the underlying mechanism and the results showed that daidzein could directly activate the rat catalase promoter region (Rohrdanz et al., 2002). Genistein was also found to increase the expression of glutathione peroxidase (GPx)-1 in two prostate-cancer cell lines (Suzuki et al., 2002). On the other hand, Wiseman hypothesized that soybean isoflavones may influence the activity of membrane enzymes by stabilizing the membrane against lipid peroxidation and decreasing the membrane fluidity (Wiseman et al., 1993).

Other mechanisms have been explored to explain soybean isoflavones' cancer preventive function besides their antioxidant activity. For example, it was suggested that isoflavones' cancer preventive function involves the modulation of estrogen synthesis and metabolism because pre-menopausal women with regular soy diets had decreased estrogen synthesis and different metabolism pathways compared to a control group (Xu et al., 1994). Genistein and daidzein may interfere with the expression of SOS response, that is part of cellular stress response and will cause error-prone repair. Genistein decreased both transition and transversion mutation induced by the carcinogen N-ethyl-N-nitrosourea (ENU) via suppressing the SOS response, and both genistein and daidzein suppressed the SOS response in *Salmonella typhimurium* TA1535/pSK1002 against the effect of mutagens, such as of 3-amino-1,4-dimethyl-5H-pyrido[4,3b]indole (Trp-P-1), furylfuramide, and activated Trp-P-1 (Yang and Fix, 2001; Miyazawa et al., 1999). Genistein and daidzein could inhibit the CYP1A1 enzyme activity induced by 2,3,7,8-tetracholorodibenzo-p-dioxin (TCDD; dioxin) (Shertzer et al., 1999). CYP1A1 enzyme

catalyzes the formation of benzo[a]pyrene (BaP) metabolites that will ultimately cause DNA mutation. Soybean isoflavones do not work at the transcription level against the activity of CYP1A1, as daidzein does against catalase. Instead, soybean isoflavones compete non-competitively with the CYP1A1 substrate BaP for microsomal hydroxylation to protect against carcinogenesis caused by BaP. Genistein was also found to significantly inhibit the expression of TPA-induced proto-oncogen (c-fos), which may help prolong tumor latency and decrease tumor multiplicity (Wei et al., 1995). In an animal model of F344 rats, the diet containing genistein and daidzein mixture decreased the development of adenocarcinomas induced by 3, 2'-dimethy-4-aminobiphenyl (DMAB) and testosterone propionate (TP). It was found that the argyrophilic nuclear organizer regions (AgNORs) in adenocarcinomas were significantly inhibited by the soybean isoflavone mixture (Onozawa et al., 1999).

The effective scavenging of reactive radicals and low reactivity of generated secondary antioxidant radicals towards vital intracellular components are two critical requirements for a chain-breaking antioxidant. However, an antioxidant may act as a prooxidant according to the oxidative tension and antioxidant concentration. For example, beta-carotene may help to increase protein oxidation and DNA damage with low oxygen ($O_2$) tension and high concentration (Zhang and Omaye, 2001). Alpha-lipoic acid and didydrolipoic acid, known as antioxidants, may also exert prooxidant properties *in vivo* (Moini et al., 2002). This characteristic may also apply to soybean isoflavones. Daidzein exerted a mild oxidative stress in H4IIE cells, which was recovered by daidzein's promotion to an antioxidant enzyme system (Rohrdanz et al., 2002). Moreover, the antioxidant action may be a shift from one oxidation to another oxidation.

For instance, catechol-containing antioxidant exerted antioxidant activity through shifting lipid oxidation to sulfhydryl arylation (Boots et al., 2002). So there could also be a concern that the antioxidant activity of soybean isoflavones does not necessarily help decrease the incidence of cancer formation.

At present, as a cancer preventive substance, soybean isoflavones are being incorporated into new foods in excess of naturally occurring concentrations, but it should be pointed out that the safety margin of soybean isoflaovens is relatively narrow and toxicity assessment should be carried out. Meanwhile, the cancer preventive effects could be the result of associated dietary or lifestyle factors. The results of Lee et al., 1995, showed that, with a dose of 1,840 μM/kg, soybean isoflavones might have cancer-promoting function in female F344/N rats initiated with diethylnitrosamine (DEN). In Japan, the serum level of daidzein non-metabolizer was examined both in prostate patients and in healthy persons. It was found that the prostate cancer patients had a significantly higher serum level of daidzein non-metabolizer compared to control group, so the authors postulated that daidzein non-metabolizer might be a risk factor for prostate cancer (Akaza et al. 2002).

### 2.2.2 Cancer Inhibitory Function

Cancer inhibitory function of soybean isoflavones comprises the second aspect of their anti-cancer action. Soybean isoflavones could inhibit the growth of most types of hormone-dependent and hormone-independent cancer cells *in vitro*, including breast, prostate, colorectal and colon cancer cells. The reported mechanisms for their cancer inhibitory function are regulation of estrogen-mediated events, inhibition of tyrosine

kinase and DNA topoisomerase activities, synthesis and release of TGF beta and modulation of the differentiation and cell cycle through changing gene expression.

Estrogen plays multiple hormonal roles in human health, among which, mitogenic action of estrogen is the most important to the reproductive system, bones, heart and possibly the brain. There is a common structural characteristic between estrogen and soybean isoflavones, the presence of a phenolic ring, which is the prerequisite for binding to the estrogen receptor. However, soybean isoflavones only have weak estrogenic properties, which enable them to act as anti-estrogens by competing with estrogen for binding to the estrogen receptor and inhibit the mitogenic actions of estrogen, thus exerting the cancer inhibitory function. The binding and activation of estrogen receptor by genistein, daidzein, and glycitein have been studied. The results established that all of them could bind to and activate estrogen receptor, although the binding affinity and activation potency was much lower than that of estradiol (Song et al., 1999). On the other hand, in the test with yeast estrogen screen, only genistein was found to induce an estrogen signal, while no signal was detected for either daidzein or glycitein (De-Boever and Verstraete, 2000). The estrogenic activity of genistein has also been studied in a MCF-7 cell line. The genistein-bound estrogen receptor was processed in the nucleus at about the same rate as the estradiol-bound receptor, but was less effective than estradiol in translocating the cytoplasm estradiol receptor to the nucleus (Wei et al., 2002).

An alternative theory postulated by some scientists indicates that, instead of inhibiting the growth of cancer cells, soybean isoflavones might exert proliferative action to cancer cells through estrogen receptors. The research of Maggiolini et al., 2001, demonstrated that estrogen receptor mediates the proliferative but not the cytotoxic dose-

dependent effects of soybean isoflavones on two breast cancer cells. Still others insist that the inhibitory effect of soybean isoflavones to cancer cells is unrelated to estrogen receptor because soybean isoflavones are equally effective against either estrogen receptor dependent or estrogen receptor independent cancer cells. For example, Genistein was found to induce the differentiation of both MCF-7 and MDA-468 cells, but daidzein did not induce differentiation to either MCF-7 or MDA-46 (Constantinou et al., 1998).

The cancer inhibitory effect of soybean isoflavones might also arise from their influence on the biosynthesis and metabolism of estrogen and the expression of estrogen receptor. Estradiol level of genistein-treated monkeys was higher than that of control group. The reasonable explanation might be that genistein may stimulate the de-conjugation of estrone in the gut, thus allowing its re-absorption into the peripheral circulation and conversion to estradiol (Harrison et al., 1999). In the model of prepubertal rat, pharmacological, but not physiological concentrations of genistein were found to modulate the expression of sex steroid receptors in the uterus, including estrogen receptors (Cotroneo et al., 2001).

There are several other mechanisms through which soybean isoflavones exert their cancer inhibitory effect and inhibiting the activity of protein tyrosine kinase is one of them. Tyrosine kinase is involved in the signal cascade that ultimately leads to cell survival and cell division, so decreased tyrosine kinase activity helps to inhibit the proliferation of cancer cells. In addition, the phosphorylated state of estrogen receptor may also influence the receptor activity, thus decreased tyrosine kinase activity might be associated with soybean isoflavones' hormonal influence on cancer cells (Dayani et al., 1990). However, genistein has been found to inhibit both the serum-stimulated and

epidermal growth factor (EGF)-stimulated growth of human prostate cells and had no significant effect on the EGF receptor tyrosine autophosphorylation. It was suggested that the cancer inhibitory action of genistein was dependent not on the inhibition of EGF receptor autophosphorylation but on a more distal event in the EGF-receptor mediated signal transduction cascade (Peterson and Barns, 1993). In a model of human prostate cancer cells, genistein treatment decreased the expression of mitogen-activated protein kinase 6 (MAPK 6), which is in accordance with the former hypothesis because MAPK is one of the distal events of receptor tyrosine kinase (Suzuki et al., 2002).

Mechanisms other than PTK inhibition have also been postulated to be involved in soybean isoflavones' cancer inhibitory action. In a cell model study, both genistein and TGF beta were found to suppress the epidermal growth factor (EGF) stimulated proliferation of human mammary epithelial (HME) cells and genistein treatment increased the level of TGF beta in a medium of cells through either increased synthesis or decreased degradation. It was suggested that the cancer inhibitory action of soybean isoflavones might involve modulating TGF beta activity (Kim et al. 2001).

The last major mechanism by which soybean isoflavones exert their cancer inhibitory action is by changing the cell's gene expression thus inducing cell differentiation or cell cycle arrest. Four human melanoma cell lines and one B16-B16 mouse melanoma cell line have been used to investigate the growth inhibitory and differentiation inducing action of genistein (Rauth et al., 1997; Yan et al., 1999). Genistein significantly inhibited cell growth and the chemosensitivity might have depended on the content of p53, the cellular tumor suppressor, because higher levels of wild-type p53 expression make cells more resistant to genistein's growth-inhibitory

action. Moreover, in the mouse melanoma cells, genistein was found to increase the expression of p53 and decrease the expression of c-Myc protein. With respect to cell differentiation, genistein induced the cytoskeletal filaments to form a bundle along the direction of elongation of the cells and caused the cells to form dendrite-like structure. The tyrosine phosphorylation levels of cytoskeleton-associated proteins decreased after the genistein treatment. The differentiation inducing effect of genistein is also p53 dependent because cells lacking p53 responded more than cells with p53 through dendrite-like structure formation. The decreased phosphorylation level might arise from the increased tyrosinase activity. It was observed that genistein affected tyrosinase activity at the transcription level because genistein treatment increased the tyrosinase mRNA level. Genistein could cause G2/M arrest in cell cycle progression in various cancer-cell lines, but in contrast to the differentiation effect of genistein, the cell cycle arrest effect was p53 independent. In human hepatocellular Hep G2 and colorectal Colo320 HSR carcinoma cells, genistein strongly increased the expression of p21 protein, a cyclin-dependent kinase (Cdk) inhibitor, in experimental conditions through activating p21 promoter reporter constructs to cause G2/M arrest (Park et al., 2001). In two hormone-sensitive cell lines T47D and ZR75.1 and two hormone-independent cell lines MDAMB-231 and BT20, cell cycle arrest at G2/M phase caused by genistein was coupled with the cellular level of p34 proteins. With the exception of the T47D cell line, expression of protein p34 was not affected, but the inactivated, tyrosine-phosphorylated form of p34, increased after genistein treatment. Protein p62, which is rapidly degraded in cycling cells, was also found to accumulate in all cell lines except T47D. Therefore,

the authors concluded that genistein's cell cycle arrest effect was coupled with more than one protein in all of the studied cell lines (Cappelletti et al., 2000).

Several other mechanisms have been postulated to elucidate the cancer inhibitory function of soybean isoflavones: inducing apoptosis, modulating DNA topoisomerase activity, stabilizing protein-linked DNA strand breakage, and inhibiting the signal transduction pathway. For example, daidzein could induce apoptosis directly without altering cell cycle distribution (Su et al., 2000). However, the concentrations being used in cell culture study are far above the circulating levels of un-conjugated soybean isoflavones in the human body, which points out the limitations of cell culture for the study of the cancer inhibitory action of soybean isoflavones. Also, emerging evidence indicates that the biological activities of soybean isoflavones may arise from modifications to the parent compounds. The hydroxylated and methylated metabolites may be the active forms of genistein in human breast-cancer cells (Peterson et al., 1998). Apple extracts also have tumor proliferation inhibitory activity, while the inhibitory activity might come from the generation of $H_2O_2$ during the interaction of phenolic compounds with cell culture media (Walker and Kanner, 2002). It is obvious that metabolism should be taken into account and the use of reducing agents in cell culture media should also be considered in order to exclude the formation of $H_2O_2$, or other reactive oxygen species when studying the anti-cancer function of soybean isoflavones.

## 2.3 Cardiovascular System Protective Actions

As with the lower incidence of breast and prostate cancers, a lower incidence of heart disease has also been reported in populations consuming large amounts of soy products. Great strides have been taken in the approach to mechanisms for the protective

action of soybean isoflavones in the cardiovascular system. Soybean isoflavones appear to lower serum cholesterol and low-density lipoprotein (LDL) concentrations while increasing the plasma concentration of high-density lipoprotein (HDL). Soybean isoflavones may increase lipoprotein oxidation resistance and decrease plasma F(2)-isoprostane concentrations (a bio-marker for *in vivo* lipid oxidation). They may also inhibit the progression of atherosclerosis in the coronary, iliac and common and internal carotid arteries. Moreover, the cardiovascular benefits of soybean isoflavones appear to be equal for both males and females.

High levels of cholesterol and LDL cholesterol are correlated with cardiovascular diseases, so any substance that decreases the serum concentration of cholesterol would exert cardio-protective action. In a study with 23 healthy postmenopausal women in Japan, a soybean isoflavones mixture was found to dramatically decrease total serum cholesterol and LDL cholesterol levels (Uesugi et al. 2002). In a cholesterol-fed rabbit model, hypo-cholesterol activity of soybean isoflavones was compared to that of estrogen replacement therapy (ERT) and 17 beta-estradiol (E2). The results showed that dietary phytoestrogens significantly reduced aortic cholesterol content with potency comparable to that of ERT and seemed to enhance the anti-atherogenic effect of E2 in this model (Alex Andersen et al., 2001). Potential mechanisms by which soybean isoflaovnes induce lower blood cholesterol concentration include changing thyroid status, bile acid balance and the estrogenic effect (Liechtenstein, 1998). In addition, soybean isoflavones were found to decrease lipogenesis and increase lipolysis in isolated rat adipocytes (Szkudelska et al., 2000).

Increasing evidence indicates that the antioxidant properties of soybean isoflavones may protect human LDL against oxidative modification. For example, genistein could inhibit both copper and peroxy radical mediated LDL oxidation (Kerry and Abbey, 1998; Hwang et al., 2000). This may be of importance because oxidative damage to LDL is considered to be an important stage in the development of atherosclerosis. However, soybean isoflavones have different reactivity to the oxidative stress caused by copper or peroxy radicals, which suggests an antioxidant mechanism other than free radical scavenging.

With glucose-derived oxidants, genistein effectively prevented LDL oxidation, so it may help to prevent the propagation of diabetic complications such as atherosclerosis (Exner et al., 2001). Although only minute amounts of isoflavones could be associated with lipoproteins in vivo, LDL isolated from serum still had reduced susceptibility to oxidation compared to the control LDL. It was suggested that modified LDL particles had been produced in vivo by circulating isoflavones promoting resistance to oxidation *ex vivo* (Tikkanen et al. 1998). Also, it has also been reported that there is no significant difference between the oxidation resistances of isolated LDL compared with control LDL (Kerry and Abbey, 1998). Different esterified soybean isoflavones have been made in order to increase their lipophilicity for incorporation into LDL and oleic acid esters were incorporated more effectively than unesterified isoflavones and stearic acid esters (Meng et al., 1999).

As an anti-atherogenic agent, soybean isoflavones have a number of properties besides their inhibitory activity to LDL oxidation. Genistein is a potent inhibitor of tyrosine kinase activity and thus it is able to block the activation of growth factors, such

as platelet-derived growth factor (PDGF) and basic fibroblast growth factor. PDGF plays an important role in the intimal smooth-muscle cell proliferation that forms part of the atherosclerotic process (Wilcox and Blumenthal, 1995). In addition, genistein may reduce overall thrombosis associated with atherosclerosis by interfering with platelets and thrombin action because genistein could prevent thrombin-induced platelet activation and aggregation by inhibiting tyrosine kinase activity (Asahi et al. 1992). Genistein could inhibit the cell proliferation involved in lesion formation. For example, genistein inhibits the proliferation of many vascular cells, including vascular endothelial cells and also inhibits the atherosclerotically important process of angiogenesis (Raines and Ross, 1995). Furthermore, genistein supplementation was also found to improve endothelial dysfunction induced by ovariectomy in rats as well as estrogen replacement therapy does (Squadrito et al., 2000).

## 2.4 Anti-osteoporosis Actions

Osteoporosis is an increasing problem all over the world, but the use of soy-products has been associated with lower risk of bone fracture in Asian countries. Bone loss is the result of unbalanced bone metabolism: decreased bone formation and increased bone resorption. In order to elucidate the detailed mechanisms, ovariectomized (OVX) rats have been used as an animal model to study the anti-osteoporosis action of soybean isoflavones. One study indicated that genistein is useful for regulating B-lymphopoiesis and restoring bone loss caused by estrogen deficiency, without estrogenic action in the uterus (Ishimi et al., 1999). There are two types of estrogen receptors (ER), ER alpha and ER beta. ER beta predominates in certain estrogen responsive tissues, such as bone and bladder and soybean isoflavones could bind to ER beta almost as well as estrogen does;

although it binds only weakly with ER alpha, which possibly explains genistein's ability to restore bone loss in OVX rats.

However, other research has shown that soybean isoflavones could only reduce bone turnover dose-dependently but does not reverse established osteopenia. The bone loss preventive function of genistein is effective in both orchidectomized male rats that are deficient of androgen and ovariectomized female rats that are deficient of estrogen (Picherit et al., 2001; Ishimi et al., 2002). In the same study with 23 postmenopausal women in Japan, the excretion of bone resorption markers, pyridinoline and deoxypyridinoline, was much lower in the isoflavone group compared to the control group, which suggested that soybean isoflavones could decrease bone resorption to prevent bone loss (Uesugi et al., 2002). Rat osteoclasts have been used as a model to study how genistein suppresses osteoclastic bone resorption. Genistein could inhibit inward rectifier K(+) current (I(Kir)) in rat osteoclasts thus causing membrane depolarization and elevation of Ca(2+) concentration, and finally inhibiting osteoclastic bone resorption (Okamoto et al., 2001). It was also found that genistein could affect osteoclastic differentiation of stromal cells through inhibition of topoisomerase 2 (Topo 2) as other Topo 2 inhibitors do (Yamagishi et al., 2001). In addition, genistein administration and moderate exercise showed a cooperative effect in preventing bone loss in OVX mice (Wu et al., 2001).

## 2.5 Other Applications of Soybean Isoflavones

Soybean isoflavones have been suggested as an alternative to estrogen replacement in postmenopausal women because they could lessen post-menopausal symptoms but not increase the risk of endometrial cancer. Soybean isoflavones

administration might reduce hot flashes and vaginal dryness although it does not work as efficiently as estrogen replacement therapy (Vincent and Fitzpatrick, 2000). In a study with 20 post-menopausal women, soybean isoflavones were found to increase sex hormone-binding globulin (SHBG) significantly in subjects whose SHBG concentrations were in the lower end of the concentration range (Pino-Ana et al., 2000). However, soybean isoflavones do not show estrogenic action on uterus of ovariectomized rat (Ishimi et al., 1999). Never the less, soybean isoflavones have been found to work in the uterus and even support surgically induced endometriosis (Cotroneo and Lamartiniere, 2001). However, the current data are insufficient and more animal studies and pre-clinical experiments are needed regarding isoflavones' anti-post-menopausal symptom actions.

Soybean isoflavones may also have neuroprotective efficacy due to their antioxidant activity because neuronal cell death caused by oxidative stress may contribute to the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. However, in a study with cultured hippocampal neurons, soybean isoflavones were not functional equivalents to endogenously active 17-beta-estradiol or to estrogen replacement formulations, although it did exert some neuroprotective effects. Therefore, it has been suggested that soybean isoflavones would not reduce the risk of Alzheimer's disease or sustain memory function in postmenopausal women (Zhao et al., 2002).

## 2.6 Future Implications of Soybean Isoflavones

The observed health protective actions of soybean isoflavones appear to be mediated by a wide range of mechanisms of which antioxidant activity is probably the most important. Thus, novel products through soybean isoflavones modification to

produce stronger antioxidant activity could be a way to confer even greater protection (Ridgway et al., 1996). Also, as it has been pointed out in this review, the bioavailability and metabolism of soybean isoflavones are of great relevance to their health improvement action because pharmacological and physiological concentrations may exert different functions and the biological activity may arise from their modification. Genistein-piperamine complex (GP), has been synthesized in order to improve genistein's physicochemical properties and the anti-cancer activity of GP was studied and compared to that of genistein with HL-60 cells (Polkowski et al., 2000). Genistein-amine complex did not loose the activity of the parent compound because the measured anti-cancer effects of GP and genistein were qualitatively and quantitatively similar. However, there are considerable individual differences in the bioavailability and metabolism of soybean isoflavones. For example, the conversion by gut micro-flora of daidzein to its isoflavone metabolite equol, which is a more potent estrogen and antioxidant, occurs only in some individual (Wiseman, 2000). So it would be much more practical if the administration of soybean isoflavones could be individually controlled.

# Chapter 3

# Materials and Methods

## 3.1 Extraction and Purification of Soybean Isoflavones

### 3.1.1 Chemicals

HPLC grade hexane, methanol, and ethanol were purchased from Fisher Chemicals (Fair Lawn, NJ).

### 3.1.2 Extraction of Soybean Isoflavones

Soybeans obtained from Delta Grow Seed Company (England, AR) were ground to flour. The flour was defatted using hexane. The mixture of 100 mL of hexane and 50 g of soy flour was shaken at room temperature for 1 hr. The supernatant was removed after the mixture was centrifuged at 1000g for 20 min. The defatted soy flour was dried under a hood overnight.

Methanol was used as solvent to extract isoflavones from the defatted soy flour. The defatted soy flour was mixed with 80 mL of methanol. The extraction conditions and procedure were the same as that in defatting. The supernatant was placed under nitrogen flow to evaporate the solvent to a final volume of approximately 10 mL (Xu et al., 2002).

### 3.1.3 Purification and Quantification of Daidzin, Glycitin, and Genistin

The preparative HPLC system consisted of a Waters (Milford, MA) PrePak RCM base packed with two 25 mm×10 cm Prep Nova-Pak HR C18 (particle size 6 μm) cartridges and a Guard-Pak insert, a Delta Prep 4000 HPLC system, and a 481 LC spectrophotometer detector. Millennium32 Chromatography Manager (Waters) was used to record the chromatogram. The composition of water and methanol in mobile phase was

99:1 (v/v) for the first 20 min, then 50:50 for 10 min, and 0:100 for the last 15 min. The flow rate of mobile phase was 15 mL/min and the wavelength of the detector was set at 254 nm. One mL of isoflavone extract was loaded in the preparative HPLC system each time. The fractions of daidzin, glycitin, and genistin were collected and combined (Xu et al., 2002).

The fractions obtained in the purification were distributed to test tubes in 1 mL aliquots. The test tubes were placed under nitrogen flow to evaporate the mobile phase of the fraction. The residue of one test tube obtained after evaporation was dissolved in 1 mL of methanol and analyzed using an analytical HPLC system. The analytical HPLC system consisted of a Supelco (Bellefonte, PA) Discovery C18 column (id 3 mm × 25 cm), a Waters 2690 separation module, a 996 photodiode array detector, and a Millennium chromatography manager. The mobile phase was a mixture of water and ethanol. The percentage of water in ethanol was from 90% to 50% in 40 min with a constant flow rate of 0.3 mL/min and the detector was set at 254 nm. The residue in the remaining test tubes was combined and stored at – 20$^{o}$C before use.

## 3.2 Antioxidant Activities of Soybean Isoflavones in Preventing Cholesterol Oxidation Accelerated by 2,2'-Azobis(2-methylpropionamidine) Dihydrochloride

### 3.2.1 Chemicals

Cholesterol, genistein, daidzein, AAPH and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade hexane was purchased from Fisher Chemicals (Fair Lawn, NJ), and 2-propanol was from Mallinckrodt Co. (Gibbstown, NJ).

### 3.2.2 Cholesterol Oxidation System

Cholesterol stock solution (12 mM) was prepared by dissolving 0.464 g cholesterol in 60 mL of hexane, 37.2 mL of 0.05 M phosphate buffer (pH 6.9), and 2.8

mL Tween 20 (Xu et al., 2001). AAPH solution (30 mM) was freshly prepared with 0.05 M phosphate buffer. The cholesterol oxidation system was composed of 20 mL cholesterol stock solution and 4 mL AAPH solution with the concentration of cholesterol as 10 mM and the concentration of AAPH as 5 mM.

The reaction solution was added to a 100-mL bottle with a Teflon seal disk cap. The bottle was placed in a 37$^{o}$C water bath and shaken at 200 rotations per minute. A 3 mL aliquot of the reaction solution was sampled at 2hr, 4hr, 6hr, 8hr, 12hr, 24hr, and 48 hr, and centrifuged at 4000 g to separate the hexane layer from the emulsion.

The cholesterol oxidation products in the hexane layer were analyzed using a HPLC method (Xu et al., 2001). The system included an Alltech (Deerfield, IL) CN column (id 4.6 mm × 150 mm), a Waters 2690 separation module, a 996photodiode array detector, and a Millennium chromatography station (Milford, MA). The mobile phase was a mixture of hexane and 2-propanol (99 and 1%) at 1.5 mL/min flow rate. Detection was at 234 nm.

### 3.2.3 Cholesterol Oxidation System Treatment with Soybean Isoflavones

Genistein, daidzein or glycosides mixture was added into the cholesterol oxidation system to form the experimental groups and the concentration in both molar and mass unit of each testing component is listed in Table 1 (average molecular weight was used for glycosides mixture). The control had no test mixture added.

The reaction solutions of the experimental groups and control group were processed as described previously. A 3 mL aliquot of each reaction solution was sampled at 2hr, 4hr, 6hr, 8hr, 12hr, 24hr, and 48hr, and centrifuged at 4000 g to separate the

hexane layer from the emulsion. The cholesterol oxidation products in the hexane layer were analyzed by the same method as in the cholesterol oxidation system.

Table 1. Concentration of each treatment in the reaction system in molar and mass unit

| Treatment | concentration (mg/mL) | concentration (mM) |
|---|---|---|
| Genistein | 0.134 | 0.5 |
| Daidzein | 0.128 | 0.5 |
| Glycosides mixture | 0.128 | 0.3 |

### 3.2.4 Statistical Analysis

The experiment for each test component was repeated three times. The experimental data were analyzed by ANOVA using the General Linear Model procedure (SAS system, SAS Institute Inc., Cary, NC) with significant differences between means separated using Turkey's test at $p<0.05$.

## 3.3 Antioxidant Activities of Soybean Isoflavones on tert-Butyl Hydroperoxide-Induced Oxidative Stress in SVEC4-10 Cells

### 3.3.1 Chemicals

Genistein, daidzein, tBOOH, Hanks' balanced salts and MTT based *in vitro* toxicology assay kit were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Mallinckrodt Co. (Paris, KY). Sodium bicarbonate was from J. T. Baker Chemical Co. (Phillipsburg, NJ).

### 3.3.2 Cell Culture and Cell Growth Curve

Mouse endothelial cells (SVEC4-10) were maintained at the surface of Costar cell culture flasks at 37$^{o}$C under 5% $CO_2$ and 95% air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/L glucose and 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate. The ratio of medium volume

to growth area was 3 and this was applied to both cell culture flasks and plates. Cells were plated out in individual wells of 24-well Costar plates at a density of $8.7\times10^4$ cells per $cm^2$ (666 uL per well) and then the growth of cells was investigated in a series of 6 days by cell counting with Trypan blue exclusion.

3.3.3 Cytotoxicity Study of Genistein and Daidzein

Cells were plated out in individual wells of 96-well Costar plates at a density of $2\times10^5$ cells per $cm^2$ (100 uL per well). On the second day, the culture medium was removed and the cells were re-incubated in Hanks balanced salts solution with or without either genistein or daidzein (dissolved in DMSO with the final concentration of DMSO in Hanks balanced salts solution as 0.7%) at concentrations of 100, 50, 25, 10, 5, 2.5, 1, 0.5, and 0.25 μM for 24 hrs (100 μM solution with 0.7% DMSO was used as stock solution to be diluted by Hanks solution with 0.7% DMSO to obtain different concentrations). Then the soybean isoflavone solution was removed at day three and cell viability was measured using the MTT method and expressed as a percentage of total cellular activity. The MTT method is a means of measuring the activity of living cells via mitochondrial dehydrogenases and it is simple, accurate and yields reproducible results. The key component is (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenol tetrazolium bromide) or MTT. Solutions of MTT, dissolved in balanced salts solution without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals that are insoluble in aqueous solutions. The crystals are dissolved in acidified isopropanol and the resulting purple solution is spectrophotometrically measured at 570 nm. An increase or decrease in cell number

results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material (Mossman, 1983).

### 3.3.4 Cytotoxicity Study of tBOOH

Cells were plated out at the same density and same volume in individual wells of 96-well Costar plates. At day three, the culture medium was substituted with Hanks balanced salts solution with or without different concentration of tBOOH (500, 250, and 100 μM) for 0, 1, 2, 3, 4, and 5 hrs and then cell viability was measured using the MTT method and expressed as a percentage of total cellular activity.

### 3.3.5 Cell Treatment with Soybean Isoflavones

Two approaches were used in this experiment to investigate the antioxidant activity and mechanism of genistein and daidzein. In the first approach (method A), on the third day after cells were plated out in a 96-well plate, cells were re-incubated in Hanks balanced salts solution with the concentration of tBOOH at 500 μM with or without either 1 μM genistein or 1 μM daidzein for 0, 1, 2, 3, 4, and 5 hrs. Then the cell viability was measured using the MTT method and expressed as a percentage of total cellular activity. In the second approach (method B), cell culture media was substituted with Hanks balanced salts solution with or without either 1 μM genistein or 1 μM daidzein for 24 hrs on the second day after the cells were plated out in a 96-well plate. Then the soybean isoflavone solution was discarded on the third day and the cells were washed with Hanks solution three times in order to remove the un-attached soybean isoflavones before the cells were re-incubated in tBOOH solution with a concentration of 500 μM for 0, 1, 2, 3, 4, and 5 hrs. After that the cell viability was measured using the MTT method and expressed as a percentage of total cellular activity.

### 3.3.6 Statistical Analysis

Each experiment was replicated three times and each replication had four repetitions. The mean of the four repetitions was used and the results are presented as mean ± SE (standard error). Statistical analysis was performed using a one-factor analysis of variance (ANOVA). Probability of $p < 0.05$ was considered statistically significant.

# Chapter 4

# Results and Discussion

## 4.1 Extraction and Purification of Soybean Isoflavones

### 4.1.1 Purification and Quantification of Daidzin, Glycitin, and Genistin

Although approximately 25% of soybean isoflavones were lost in the hexane defatting procedure, the lipids of soy flour that could reduce the separation efficiency of preparative scale HPLC were largely removed at the same time. In order to prevent any possibility of enzymatic hydrolysis that could break down the glycoside group of daidzin, glycitin, and genistin and lower their extraction yield, only methanol was used in the soybean isoflavones extraction. Figure 2 is the chromatogram of soybean isoflavones extract in the preparative scale HPLC. The three isoflavone fractions inside the dotted line frame were collected. The chromatogram of the collected fractions in the analytical HPLC system is shown in Figure 3 with the three peaks representing daidzin (peak 1), glycitin (peak 2), and genistin (peak 3), respectively, identified according to standard retention times (Xu et al., 2002). As most impurity compounds were eliminated after the purification in the preparative scale HPLC, the three isoflavones peaks were the major peaks and occupied about 94% of the total peak area in the chromatogram.

## 4.2 Antioxidant Activities of Soybean Isoflavones in Preventing Cholesterol Oxidation Accelerated by 2,2'-Azobis(2-methylpropionamidine) Dihydrochloride

### 4.2.1 Cholesterol Oxidation Products with AAPH Acceleration

To date, more than 80 cholesterol oxidation products (COPs) have been described and different COPs will be generated under different conditions. 7α-OH, 7β-OH and 7-keto were found to be the major COPs in cooked meat (Du et al., 2001), while the most

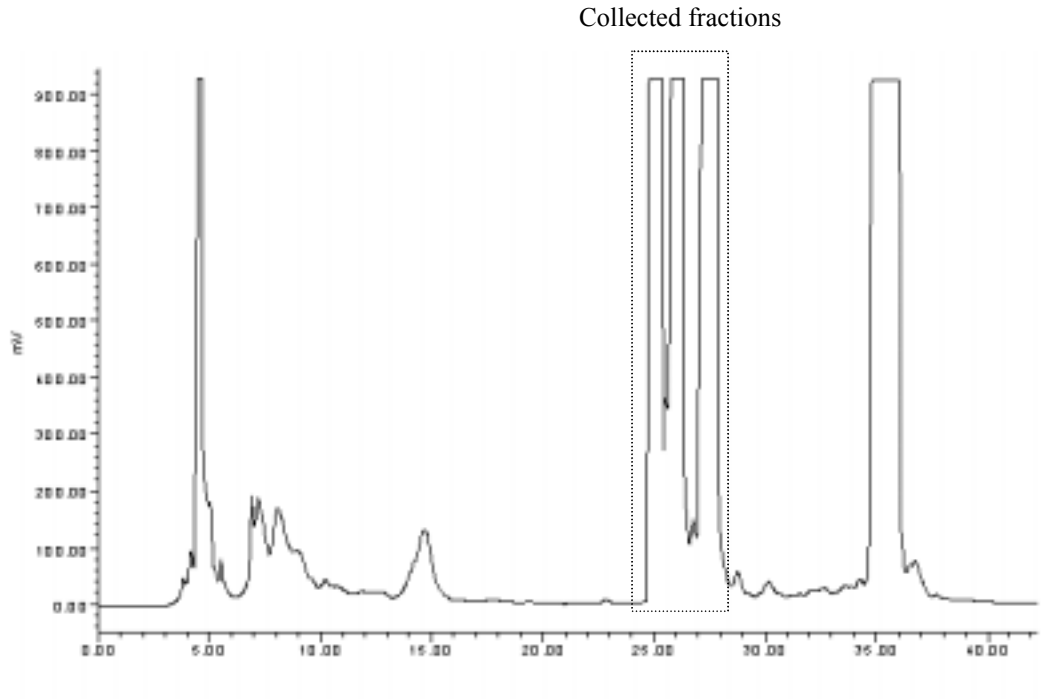



Figure 2. Chromatogram of crude soybean isoflavone extract in the reversed-phase preparative HPLC (Column, C18; mobile phase, water/methanol 99:1 (v/v) for 20 min, 50:50 for 10 min, and 0:100 for 15 min; UV detector, 254 nm).

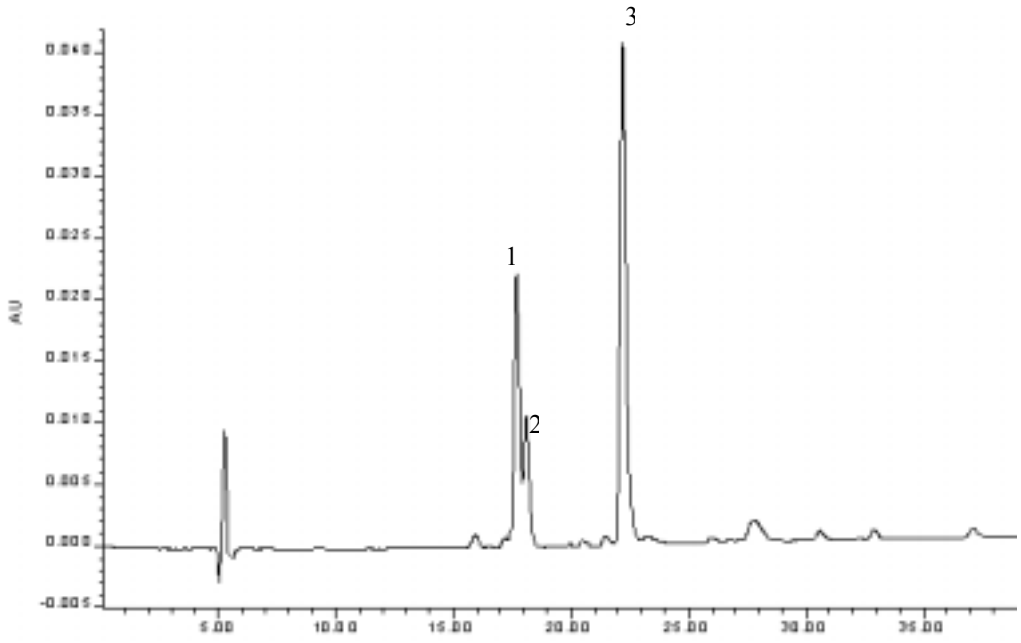



Figure 3. Chromatogram of high-purity soybean isoflavones in the analytical reversed-phase HPLC (Column, C18; mobile phase, water in methanol from 90% to 50% in 40 min; flow rate of 0.3 mL/min; UV detector, 254 nm): (peak 1) daidzin; (peak 2) glycitin; (peak 3) genistin.

abundant COPs found in fried and roasted salmon were 7-keto and cholestanetriol (Echarte et al., 2001). The major pathways of cholesterol oxidation are shown in Figure 4. Cholesterol could be epoxidized to form 5,6-EP or peroxidized to form 7-OOH that is the precursor of 7-OH and 7-keto. Figure 5 is the HPLC chromatogram of cholesterol oxidation initiated with AAPH after 24 hr of reaction. Four peaks were found that represented four major cholesterol oxidation products: 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH. Cholesterol and its degradation components have shorter retention times than these cholesterol oxidation products. Percentage of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH accelerated by AAPH at different oxidation times is shown in Figure 6. The formation of the four COPs, 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH occurred quickly and steadily increased within the 48 hr of reaction. However, the formation rates were different, with about 21percent of the cholesterol being oxidized to 7β-OOH from 24hr to 48hr of reaction, while only 7.03, 2.29, and 2.44 percent of cholesterol was oxidized to 5,6α-EP, 5,6β-EP and 7-keto respectively from 24hr to 48hr of reaction.

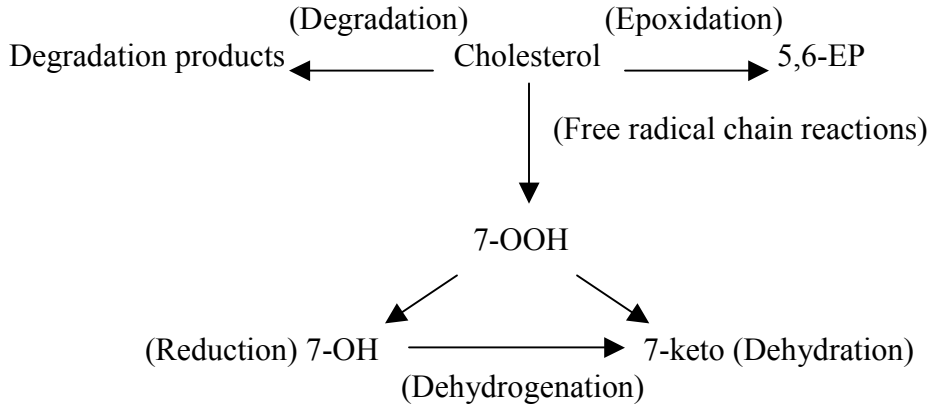



Figure 4. Major pathways of cholesterol oxidation.

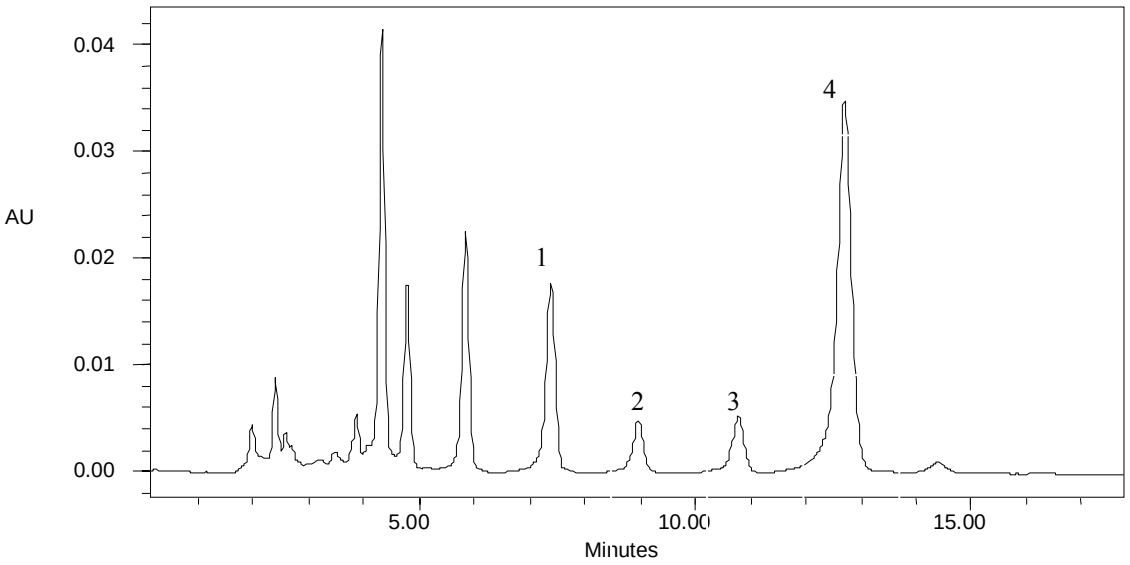



Figure 5. Chromatogram of cholesterol oxidation products after 24 hr of oxidation with 5 mM AAPH and 10 mM cholesterol: (peak 1) 5,6α-EP; (peak 2) 5,6β-EP; (peak 3) 7-keto; (peak 4) 7β-OOH.

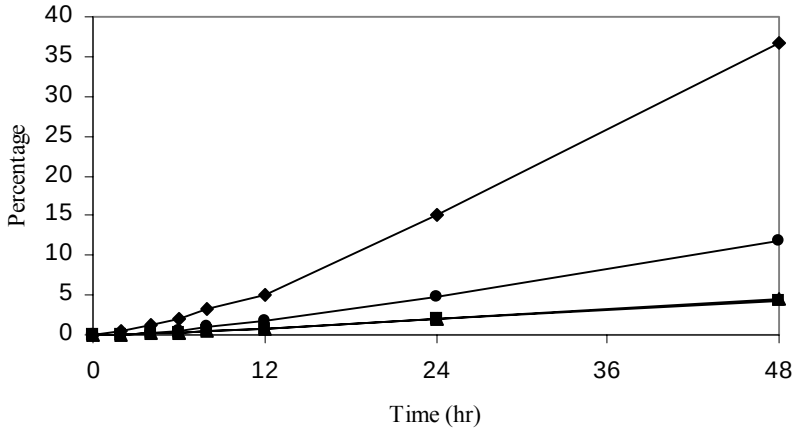



Figure 6. Percentage of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH accelerated by AAPH at different oxidation times: (◆) 7β-OOH; (●) 5,6α-EP; (▲) 7-keto; (■) 5,6β-EP.

### 4.2.2 Antioxidant Activities of Genistein, Daidzein, and Glycosides Mixture from Defatted Soybean Flour

The structure-activity relationships for antioxidant activities of flavonoids have been studied by different groups, with the same conclusion that hydroxyl substitutions on the B-ring and the A-ring are the determinants of antioxidant capacity. Hydroxyl substitutions on the B-ring especially affect the antioxidant potencies of flavonoids. In the case where the B-ring could not contribute to the antioxidant activities of flavonoids, hydroxyl substitutions on the A-ring would become a larger determinant of flavonoid antioxidant activity (Arora et al., 1998; Silva et al., 2002). The two pharmacophores, the 4' hydroxyl group on the B-ring and the hydroxyl groups on the AC-ring (Figure 1), which could be hydrogen/electron donators to scavenge free radicals, account for soybean isoflavones' antioxidant activity (Heijnen et al., 2002). Although genistein and daidzein have the same B-ring structure, because genistein has one more hydroxyl group than daidzein on the AC-ring, genistein might have higher free radical scavenging capacity than daidzein. In a cell culture model with HL-60 cells, genistein was revealed to exert higher antioxidant activities than daidzein to inhibit TPA-induced $H_2O_2$ formation and xanthine/xanthine oxidase-caused $O_2^-$ generation (Wei et al., 1995). Genistein also was shown to prevent glucose mediated LDL oxidation more effectively than daidzein (Exner et al., 2001). However, this is not always true. Neither genistein nor daidzein had influence on LDL oxidation resistance, while both exerted extremely high antioxidant activities against LDL oxidation after being esterified (Meng et al., 1999). The authors postulated that esterification of soybean isoflavones provided them lipophilicity needed for incorporation of soybean isoflavones into LDL. So the similarities between soybean isoflavones and chemicals that need protection is of significant importance relative to

soybean isoflavones' antioxidant activities because the analogous characteristics could enable soybean isoflavones to be closer to their target chemicals thus exerting antioxidant effects more efficiently. In addition, it has been suggested that an antioxidant mechanism other than free radical scavenging reaction may account for soybean isoflavones' antioxidant effects since they exerted different reactivity to the oxidative stress caused by copper or peroxy radicals (Kerry and Abbey, 1998; Hwang, et al., 2000). For example, it was found that both free radical scavenging and iron chelating describe flavonoids' antioxidant activities, although free radical scavenging might play the major role (Acker et al., 1996).

Figure 7 shows the percentage of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH in the genistein, daidzein, or control group after 24 hr or 48 hr of reaction. Both genistein and daidzein exhibited very high antioxidant activity because each group had significantly less COPs than control group after 24 hr or 48 hr of reaction ($p<0.05$). The percentage of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto and 7β-OOH accelerated by AAPH with either 1 mM genistein or 1 mM daidzein treatment after 24 hr or 48 hr of reaction is listed and compared in Table 2. Genistein had higher antioxidant activities against the formation of 5,6β-EP and 7-keto than daidzein ($p=0.0007$ and $p=0.0615$ for 5,6β-EP and 7-keto at 24 hr of reaction; $p=0.0010$ and $p=0.0019$ for 5,6β-EP and 7-keto at 48 hr of reaction). However, the difference between genistein and daidzein to inhibit the formation of 5,6α-EP and 7β-OOH was not significant ($p>0.05$). Soybean isoflavones have different affinity to their substrate and their antioxidant activities are substrate dependent: the higher the affinity that isoflavones have to their substrate, the more efficiently they would exert their antioxidant activities.

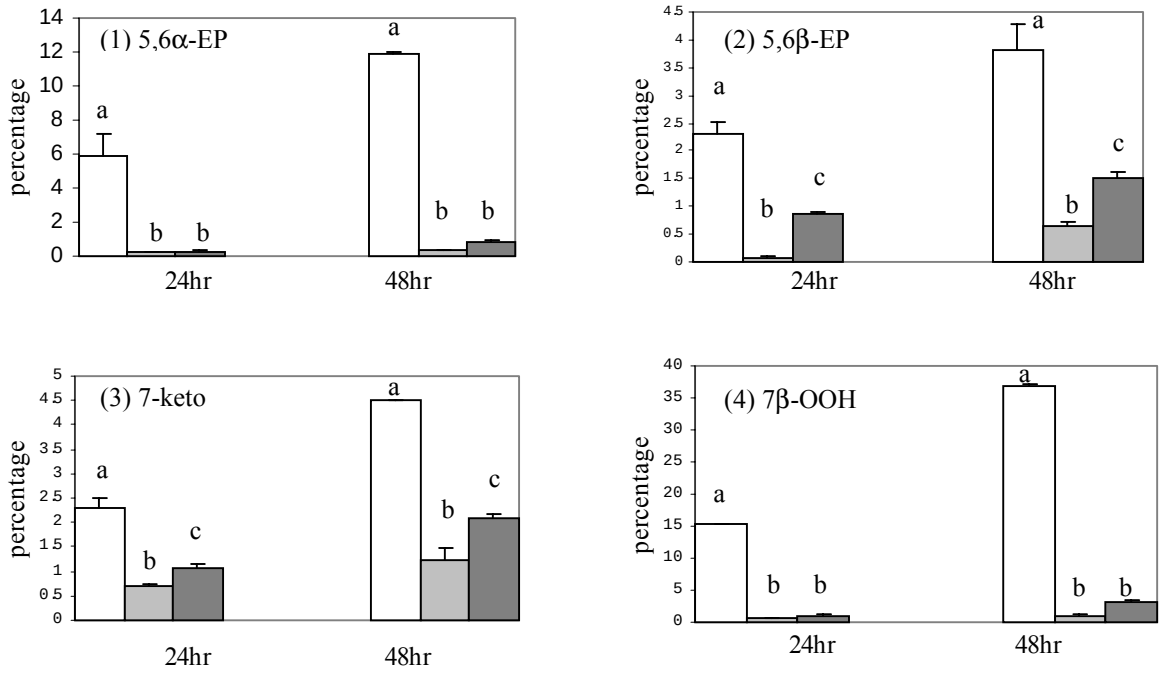



Figure 7. Percentage of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto and 7β-OOH accelerated by AAPH with or without soybean isoflavone treatment after 24 hr or 48 hr of reaction: (bars represent from left to right in each group) control; genistein; daidzein. Significant differences in percentage ($p < 0.05$) are expressed by a different letter at each oxidation time.

Table 2. Comparison of percentages of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto and 7β-OOH accelerated by AAPH with either 1 mM genistein or 1 mM daidzein treatment after 24 hr or 48 hr of reaction (date were represented as mean ± SD)

| | | Genistein (%) | Daidzein (%) | p |
|---|---|---|---|---|
| 24 hr | 5,6α-EP | 0.09±0.02 | 0.13±0.04 | 0.6297 |
| | 5,6β-EP | 0.21±0.03 | 0.44±0.01 | 0.0007 |
| | 7-keto | 0.35±0.02 | 0.54±0.03 | 0.0615 |
| | 7β-OOH | 0.24±0.08 | 0.55±0.12 | 0.1654 |
| 48hr | 5,6α-EP | 0.18±0.01 | 0.39±0.06 | 0.4811 |
| | 5,6β-EP | 0.33±0.03 | 0.75±0.06 | 0.0010 |
| | 7-keto | 0.62±0.13 | 1.05±0.04 | 0.0019 |
| | 7β-OOH | 0.54±0.03 | 1.62±0.10 | 0.2136 |

For example, genistein was found to have higher affinity to liposomal membrane than daidzein and aglycones had higher affinity to liposomal membrane than glycosides (Murota et al., 2002). According to Sigma production information, daidzein has higher lipophility than genistein (daidzein has one less hydroxyl group than genistein), so daidzein might have greater ability to associate with cholesterol in the small droplets of the emulsion. Thus daidzein could exert its antioxidant activity more efficiently than genistein, which would compensate for the fact that daidzein has lower radical quenching capacity and explain the similar antioxidant activity between genistein and daidzein against cholesterol oxidation to 5,6α-EP and 7β-OOH.

As with the formation of different COPs under different conditions, the formation of COPs might have different susceptibility to certain antioxidants. For example, the formation of 7-keto was retarded by BHA, BHT, and a-tocopherol markedly, but the formation of 7α-OH and 7β-OH was reduced to a lesser degree (Csallany et al., 2002). The percentages of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto, or 7β-OOH accelerated by AAPH with or without soybean isoflavone treatment at different reaction times are shown in Figure 8. The rates for the formation of 7β-OOH, 7-keto, 5,6α-EP, and 5,6β-EP were calculated as the slopes of the lines. They all decreased dramatically with either genistein or daidzein treatment compared to control (Table 3). Both genistein and daidzein had significantly stronger inhibition to the formation of 7β-OOH than to 7-keto. 7β-OOH, which could be dehydrated to 7-keto, is the precursor of 7-keto in the cholesterol oxidation reaction initiated by free radicals (Figure 3), so soybean isoflavones might have stronger inhibition to peroxidation of cholesterol, the former step of chain reaction, than to dehydration, the later step of the chain reaction. The formation of 5,6α-

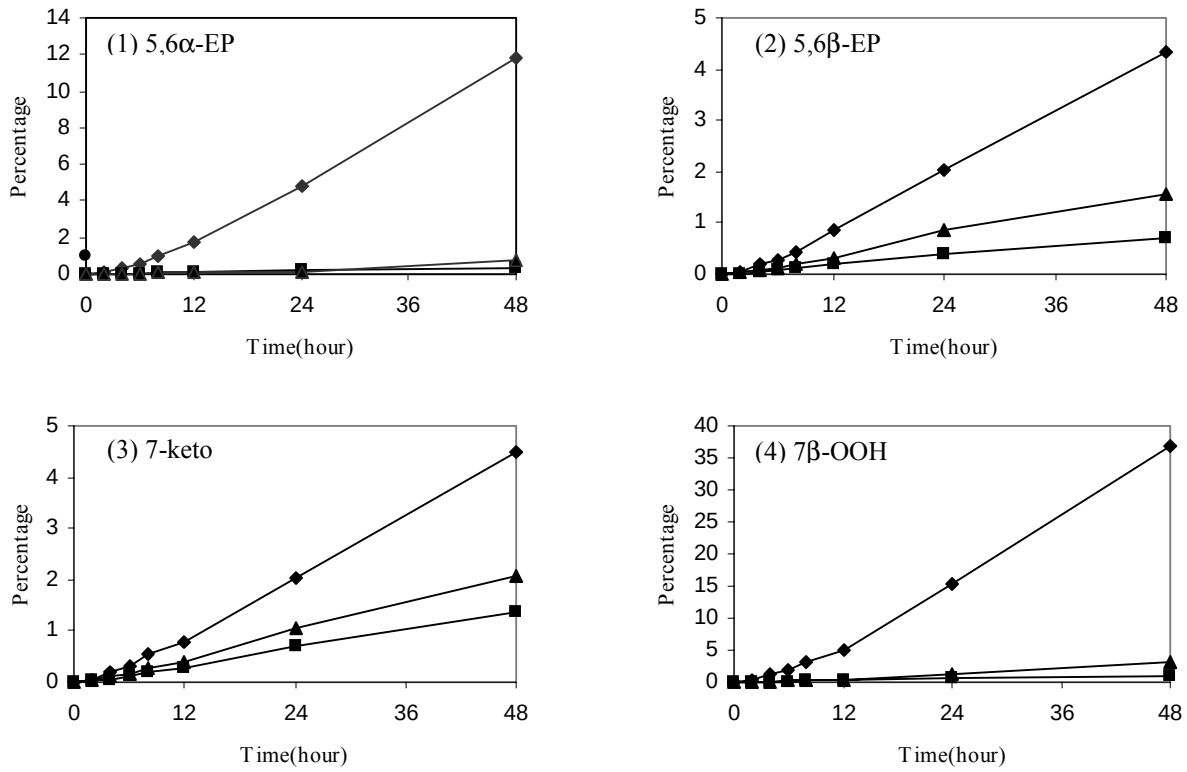



Figure 8. Percentage of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH accelerated by AAPH with or without soybean isoflavone treatment at different reaction times: (◆) control; (▲) daidzein; (■) genistein.

Table 3. Percentage of formation rates for 7β-OOH, 7-keto, 5,6α-EP, and 5,6β-EP decreased by either genistein or daidzein treatment compared to control (unit for formation rate: percentages per hour)

| | 7β-OOH | | 7-keto | |
|---|---|---|---|---|
| Treatment | Formation rate | Decreased percentage | Rate | Decreased percentage |
| Genistein | 0.0254 | 97.24% | 0.0273 | 64.91% |
| Daidzein | 0.0498 | 91.06% | 0.0397 | 48.97% |
| Control | 0.5570 | | 0.0778 | |
| | 5,6α-EP | | 5,6β-EP | |
| Treatment | Formation rate | Decreased percentage | Rate | Decreased percentage |
| Genistein | 0.0065 | 96.02% | 0.0154 | 82.32% |
| Daidzein | 0.0093 | 94.30% | 0.0324 | 62.80% |
| Control | 0.1632 | | 0.0871 | |

EP and 5,6β-EP are usually investigated together because they have nearly the same formation pathway. The formation of 5,6α-EP and 5,6β-EP were studied separately in this study and the inhibition to the formation of 5,6α-EP by either genistein or daidzein treatment was much stronger than to 5,6β-EP, which could arise from differences in stability between 5,6α-EP and 5,6β-EP, or favorable chiral structure of genistein and daidzein to 5,6α-EP. 7β-OOH had a much higher formation rate than 7-keto and the formation rate of 5,6α-EP was also higher than that of 5,6β-EP, so the inhibition by soybean isoflavone might be related to the formation rate of COPs: the higher the formation rate, the stronger the inhibition from soybean isoflavones. These results suggest that different biomarkers should be used in the study of cholesterol oxidation under different conditions and more than one biomarker should be used to study the antioxidant activity of compounds of interest when using a cholesterol oxidation model.

The percentages of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH accelerated by AAPH with or without glycosides mixture treatment at different reaction times are shown in Figure 9. The formation rate calculated as the slope of the lines for each COPs is shown in Table 4. In a manner similar to geinistein and daidzein, the glycosides mixture had much stronger inhibition to the formation of 7β-OOH than to 7-keto and had stronger inhibition to the formation of 5,6α-EP than to 5,6β-EP. In order to compare the antioxidant activities between aglycones and the glycosides mixture, it was assumed that antioxidant activity would be linear within the molar concentration range used in this study. Although genistein, daidzein, and the glycosides mixture had different molar concentrations in the reaction system, according to this assumption, their antioxidant activities could be compared relative to molar concentration and the results

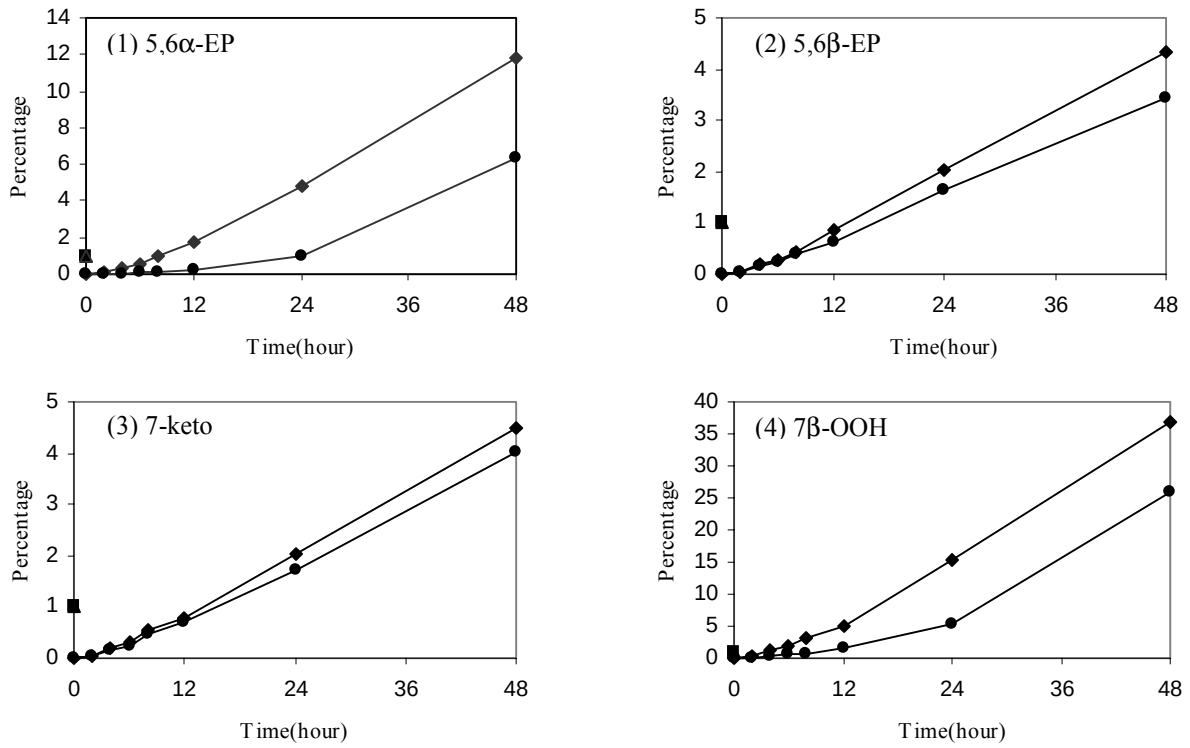



Figure 9. Percentage of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto, and 7β-OOH accelerated by AAPH with or without glycosides mixture treatment at different reaction times: (◆) control; (●) glycosides mixture.

Table 4. Percentage of formation rates for 7β-OOH, 7-keto, 5,6α-EP, and 5,6β-EP as affected by glycosides mixture compared to control (unit for formation rate: percentages per hour)

| | 7β-OOH | | 7-keto | |
|---|---|---|---|---|
| Treatment | Formation rate | Decreased percentage | Rate | Decreased percentage |
| Mixture | 0.1872 | 66.39% | 0.0657 | 15.55% |
| Control | 0.5570 | | 0.0778 | |
| | 5,6α-EP | | 5,6β-EP | |
| Treatment | Formation rate | Decreased percentage | Rate | Decreased percentage |
| Mixture | 0.0330 | 79.78% | 0.0615 | 29.39% |
| Control | 0.1632 | | 0.0871 | |

are shown in Table 5. The glycosides mixture had lower antioxidant activities than the aglycones, genistein and daidzein ($p<0.05$), which might arise from the fact that glycosides have one less hydroxyl group because of the binding of glucose. As the aglycones have higher affinity to liposomal membrane than glycosides (Murota et al., 2002), aglycones might also have higher affinity to cholesterol droplets than glycosides because of their higher lipophilicity, so alycones would exert antioxidant activities against cholesterol oxidation more efficiently than glycosides. Also, the glycosides mixture is composed of genistin and daidzin, together with glycitin that might have lower antioxidant activity because of its methoxyl substitution instead of hydroxyl substitution on the B-ring (Figure 1) (Arora et al., 1998). The study of the different antioxidant activities between glycosides and aglycones would be mute for *in vivo* study because most of the glycosides would be converted to aglycones by intestinal microorganisms during absorption (Adlercreutz, 1995). However, this could have significance *in vitro*. For example, it may be beneficial to treat the soy products in a way that would convert glucosides to aglycone forms in order to increase antioxidant capacity.

Table 5. Comparison of percentages of cholesterol oxidized to 5,6α-EP, 5,6β-EP, 7-keto and 7β-OOH accelerated by AAPH with 1 mM different soybean isoflavone treatment after 24 hr or 48 hr of reaction (date were represented as mean ± SD)

| | | Genistein (%) | Daidzein (%) | Glycosides mixture (%) | p |
|---|---|---|---|---|---|
| 24 hr | 5,6α-EP | 0.09±0.02 | 0.13±0.04 | 0.34±0.06 | 0.0009 |
| | 5,6β-EP | 0.21±0.03 | 0.44±0.01 | 0.60±0.06 | <0.0001 |
| | 7-keto | 0.35±0.02 | 0.54±0.03 | 0.64±0.14 | 0.0120 |
| | 7β-OOH | 0.24±0.08 | 0.55±0.12 | 1.58±0.27 | 0.0002 |
| 48hr | 5,6α-EP | 0.18±0.01 | 0.39±0.06 | 1.69±0.36 | 0.0002 |
| | 5,6β-EP | 0.33±0.03 | 1.75±0.06 | 1.09±0.10 | <0.0001 |
| | 7-keto | 0.62±0.13 | 1.05±0.04 | 1.25±0.06 | 0.0003 |
| | 7β-OOH | 0.54±0.03 | 1.62±0.10 | 7.09±1.19 | <0.0001 |

## 4.3 Antioxidant Activities of Soybean Isoflavones on tert-Butyl Hydroperoxide-Induced Oxidative Stress in SVEC4-10 Cells

### 4.3.1 Growth Curve of SVEC4-10 Cells

SVEC4-10 is an endothelial cell line derived by SV40 transformation of endothelial cells from mouse auxiliary lymph node vessels. They have epithelial morphology and form branching tube-like networks when adhering and growing on the surface of culture plates. The growth curve of SVEC4-10 cells was a sigmoidal line (Figure 10). Cells grew slowly when the density was below $2\times10^5$ cell/cm$^2$ and then divided and grew very fast from the density of $2\times10^5$ cells/cm$^2$ to $5\times10^5$ cells/cm$^2$. After that, the growth of SVEC-4-10 reached a plateau because the growth area had been occupied completely. In order to decrease variance, the plated density of $2\times10^5$ cells/cm$^2$ was applied in each experiment so the density on the third day would be within the lag phase of growth.

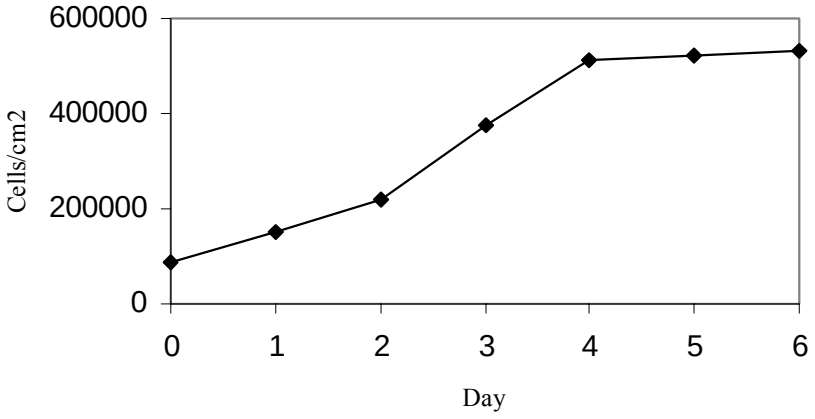



Figure 10. The growth curve of SVEC-4-10 cells with the plated cell density of $8.7\times10^4$ cell/cm$^2$

### 4.3.2 Cytotoxicity of Genistein and Daidzein

According to pharmaceutical studies, plasma concentration of soybean isoflavones could range from 0.55 mM to 0.86 mM after two weeks administration of soy beverage (Barnes et al., 1996). However, in this study, the concentration that did not alter the viability of SVEC4-10 cells over a 24 hr incubation compared to control cells only ranged from 0 μM to 1 μM. Both genistein and daidzein caused cell viability to decrease in a dose dependent manner when the concentration reached above 1 μM (Figure 11).

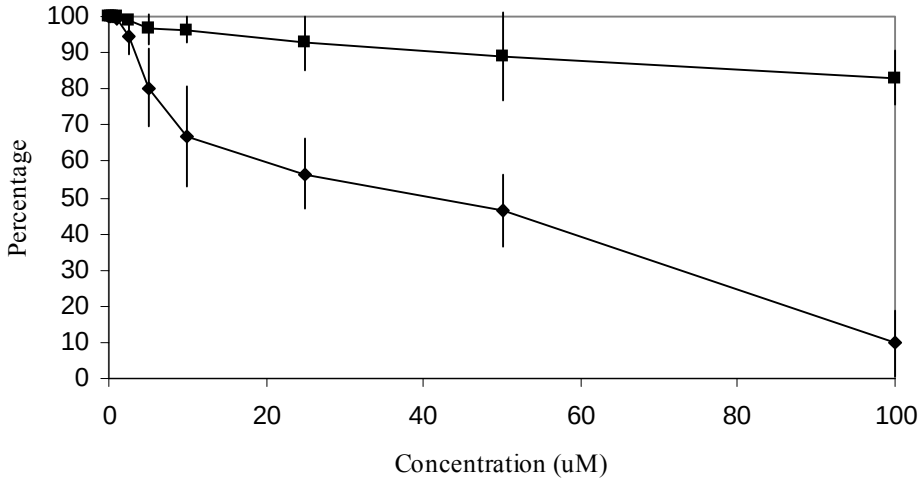



Figure 11. Effect of soybean isoflavones on the viability of SVEC4-10 cells over a concentration ranging from 0 μM to 50 μM for 24 hrs (cell viability was expressed by percentage of total cellular activity): (■) daidzein; (◆) genistein.

Genistein caused a more severe decrease in cell viability than daidzein at the same concentration. Cell viabilities after 24 hr of genistein or daidzein treatment are listed and compared in Table 6. Only at the concentrations of 2.5 μM and 5 μM was there no significant difference between the genistein and daidzein's cytotoxicity (p=0.2105 for 2.5

μM and 0.0782 for 5 μM). Significant differences were found when the concentrations were above 5 μM. For example, at 50 μM, daidzein treatment maintained 88.73 ± 12.11% of cell viability, while genistein treatment maintained only half of the cell viability compared to control. At 100 μM, daidzein treatment maintained 83.10 ± 7.62% of cell viability, while genistein treatment caused the cell viability to decrease to 9.73 ± 8.91%. Obviously, genistein had a higher cytotoxicity than daidzein to SVEC4-10 cells. The anti-proliferation action of soybean isoflavones has been studied with a number of cancer cell lines and many mechanisms have been postulated, such as modulation of estrogen receptor signal and inhibition of tyrosine kinase. However, in a test with yeast estrogen screening, only genistein was found to induce an estrogen signal, while no signal was detected for either daidzein or glycitein (De-Boever and Verstraete, 2000). SVEC4-10 cells have characteristics similar to cancer cells because they could grow indefinitely without special addictives. After a latency period of 14 weeks, they would induce spindle tumors with some of the histo-pathologic characteristics of human Kaposi Sarcoma (O'Connell et al., 1991).

Table 6. Comparison of cell viabilities after different concentrations of genistein or daidzein treatment (data were represented as mean ± SE; probability of $p<0.05$ was considered statistically significant)

| Concentration (μM) | 2.5 | 5 | 10 | 25 |
|---|---|---|---|---|
| Genistein (viability %) | 94.69±5.09 | 80.34±11.00 | 66.64±13.81 | 56.55±9.62 |
| Daidzein (viability %) | 99.11±0.79 | 96.42±4.37 | 96.19±3.57 | 92.59±7.26 |
| P | 0.2105 | 0.0782 | 0.0230 | 0.0066 |
| Concentration (μM) | 50 | 100 | | |
| Genistein (viability %) | 46.52±10.07 | 9.73±8.91 | | |
| Daidzein (viability %) | 88.73±12.11 | 83.10±7.62 | | |
| P | 0.0097 | 0.0004 | | |

In order to avoid any possible cytotoxic effects, the concentration of 1 μM, where daidzein treatment maintained 100± 0% of cell viability and genistein treatment maintained 99.2 ± 1.39%, was chosen to test their antioxidant activity in cells.

4.3.3 Cytotoxicity of tBOOH

Oxidative stress induced by tBOOH could cause DNA damage and membrane integrity loss, ultimately leading to cell death. Figure 12 shows the effect of tBOOH ranging from 100 μM to 500 μM on cell viability of SVEC4-10 cells. A dose-dependent effect was evident. At 100 μM, tBOOH caused the cell viability to decrease to 73.01 ± 15.38% of the control after 5 hr of incubation. A similar decrease was obtained after 3 hr of incubation with 250 μM tBOOH and after 2 hr with 500 μM tBOOH. On the basis of this result, to investigate the antioxidant activity of soybean isoflavones, a concentration of 500 μM was chosen in order to induce a substantial oxidative stress and cause the cell viability to decrease substantially compared to control.

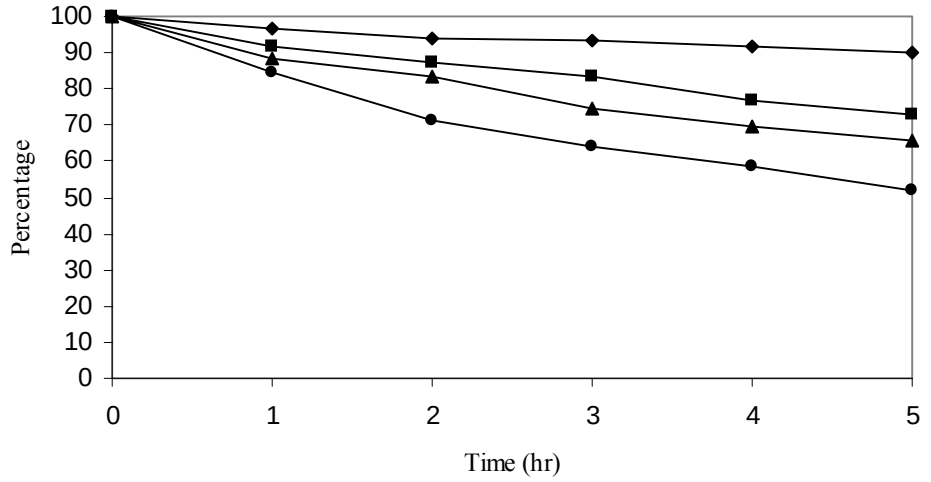



Figure 12. Decreased viability of SVEC4-10 cells in response to increasing concentration of tBOOH (cell viability was expressed by percentage of total cellular activity): (◆) 0 μM; (■) 100 μM; (▲) 250 μM; (●) 500 μM.

### 4.3.4 Antioxidant Activities of Genistein and Daidzein

After being absorbed, soybean isoflavones would either circulate in the blood steam or be incorporated into tissues. Circulating soybean isoflavones might offer protective activity as much as incorporated isoflavones, however, almost all of the previous research that has been done in this area used incorporated isoflavones to study soybean isoflaovnes' antioxidant activities *in vivo* or *in vitro*. In order to elucidate how soybean isoflavones exert their antioxidant activity *in vivo*, two approaches were used here to investigate the protective activity of genistein and daidzein against tBOOH induced oxidative stress in SVEC4-10 endothelial cells. In the first approach (method A), soybean isoflavones were added into the cell culture media together with tBOOH at the same time, which simulated the circulating isoflavones because soybean isoflavones had not been incorporated into SVEC4-10 cells before tBOOH treatment. The result is shown in Figure 13. Different from what had been expected, no significant difference was found between the cell viability of control group and that of experimental groups within 5 hr of tBOOH treatment. In the second approach (method B), which was designed to simulate the incorporated soybean isoflavones, cells were incubated with soybean isoflavone solution for 24 hrs and the non-attached genistein and daidzein was washed away before the cell was treated with tBOOH. Thus, only incorporated soybean isoflavones would be in this experimental model. The results are shown in Figure 14. As with the results from method A, no significant difference was found between the cell viability of control group and that of experimental groups.

The results demonstrated that soybean isoflavones, whether incorporated or non-incorporated, did not exert enough antioxidant activities against tBOOH induced

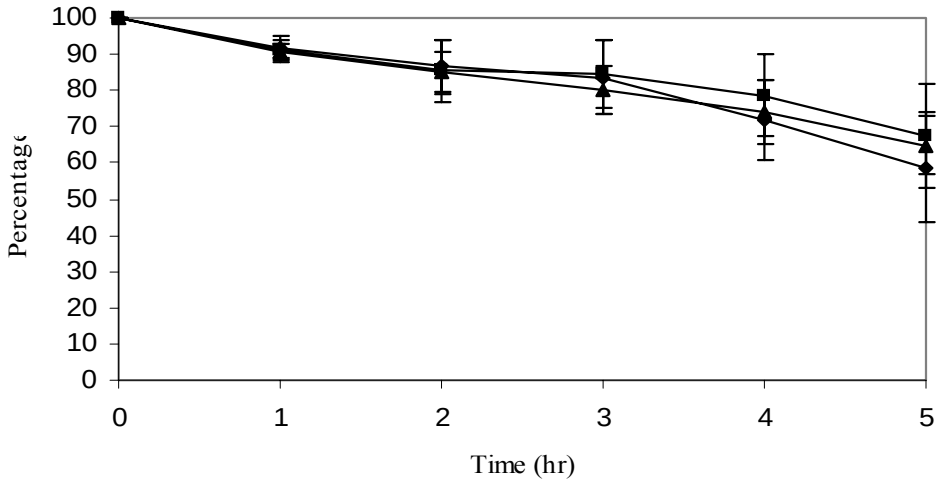



Figure 13. Cellular viability after soybean isoflavone treatment against oxidative stress caused by tBOOH on SVEC4-10 cells (method A) (cell viability was expressed by percentage of total cellular activity): (◆) genistein; (■) daidzein; (▲) control.

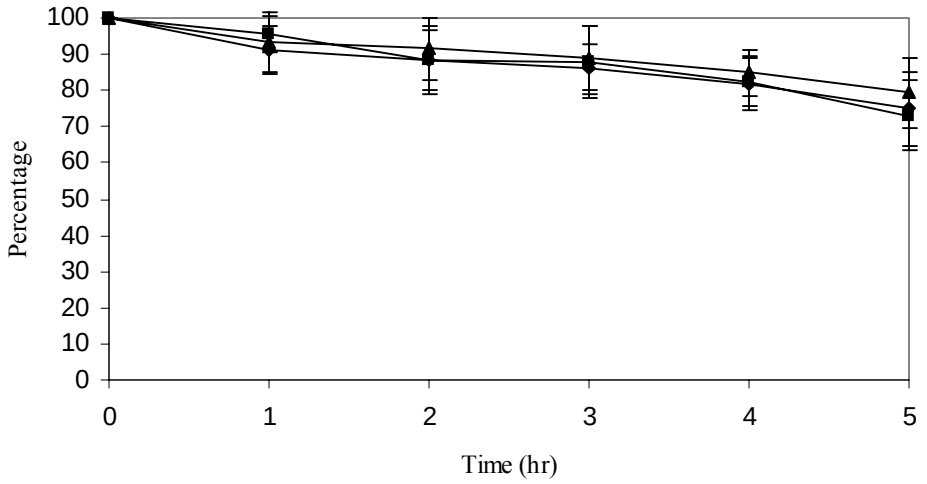



Figure 14. Cellular viability after soybean isoflavone treatment against oxidative stress caused by tBOOH on SVEC4-10 cells (method B) (cell viability was expressed by percentage of total cellular activity): (◆) genistein; (■) daidzein; (▲) control.

oxidative stress in SVEC4-10 cells. The reason might be that the concentration of 1 μM was too low for soybean isoflavones to exhibit any discernable antioxidant activities in method A. The incorporation of soybean isoflavones into LDL has been studied and the incorporation ratio was very low (approximately 3-4%), so copper-mediated oxidation of control LDL and LDL isolated from plasma pre-incubated with genistein was not significantly different (Kerry and Abbey, 1998). Tikkanen et al., (1998) examined the different oxidation resistances between control LDL and LDL pre-incubated with soybean isoflavones but suggested that it was not soybean isoflavones' antioxidant activities directly but modified LDL that was produced by circulating isoflavones that promoted the oxidation resistance of LDL. In this research, the incorporation ratio of soybean isoflavones into cells might be very low due to the low concentration of 1 μM and the limited incubation time of 24 hr may have limited the potential antioxidant activity of soybean isoflavones in method B.

Soybean isoflavones have also been reported to promote endogenous antioxidant enzymes, by which they exhibit their special "antioxidant activity", but the limited incubation time in our research decreased the possible influence that soybean isoflavones might exhibit on the antioxidant system of cells. Moreover, the toxic effects that originated from tBOOH may include S-thiolation of some key proteins thus inhibiting protein synthesis (Latour, et al., 1999), the effects of which could not be recovered by any antioxidant activity alone. These reasons help to explain why treatments had the same cell viabilities as controls in this research. Therefore, some modifications are needed in order to improve this cell culture model, such as increasing the treatment concentration, increasing the treatment duration, or changing the oxidative stress initiator.

Perhaps of even greater importance could be that this cell culture model is not suitable for the study of antioxidant activities, but it might be applied to the study of anti-caner activities of soybean isoflavones that is becoming a more and more popular research area.

The cell viabilities of the 500 μM group (C group) in tBOOH cytotoxicity study and the two control groups in method A (A group) and method B (B group) are listed in Table 5. All three of these groups used 500 μM tBOOH treatment, but both A and B groups had higher cell viability than C at the same treatment time. The only difference among these groups was that there was 0.7% of dimethyl sulfoxide (DMSO) in A and B groups but not in the C group. The cell viability difference might come from DMSO that exerted some antioxidant activity against tBOOH induced oxidative stress, and thus increased the cell viabilities in both A and B groups compared to C group.

Table 7. Comparison of cell viabilities of the 500 μM group (C group) in cytotoxicity study of tBOOH and the control groups in method A (A group) and method B (B group)

| Group/Time (hr) | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| A (%) | 90.58±2.40 | 84.84±5.84 | 79.88±6.60 | 73.84±8.80 | 64.86±7.94 |
| B (%) | 93.3±8.15 | 91.53±8.61 | 89.12±8.79 | 84.81±6.47 | 79.35±9.46 |
| C (%) | 84.66±8.09 | 71.08±13.03 | 64.09±11.79 | 58.75±14.47 | 52.14±18.27 |

# Chapter 5

# Conclusion

Soybean isoflavones, including genistein, daidzein, and glycitein and their derivatives, exist mainly in the glycoside form in the soybean. In order to prevent any possible enzymatic hydrolysis that may break down the glycoside groups, methanol was used as solvent to extract soybean isoflavones from defatted soy flour. A reversed phase HPLC method was developed and used to purify soybean isoflavone extract and the purity of the final product reached 94% based on an analytical HPLC analysis.

Genistein might have higher antioxidant activities than daidzein because it has the same B-ring structure as daidzein yet has one more hydroxyl group on the AC-ring. No significant difference was found between the antioxidant activities of daidzein and genistein against cholesterol oxidation to 5,6α-EP and 7β-OOH, although genistein exerted higher inhibition to the formation of 5,6β-EP and 7-keto than daidzein. The substrate dependent characteristic of soybean isoflavones helps to explain the above result. Because daidzein has higher lipophilicity than genistein (daidzein has one less hydroxyl group than genistein), daidzein might have greater ability to associate with cholesterol in the small droplets of the emulsion, thus exerting antioxidant activity more efficiently than genistein, which could compensate for daidzein's lower radical quenching capacity. The cholesterol oxidation model results also indicated that isoflavone aglycones might have higher antioxidant activities than their glycosides. This would not be important *in vivo* since most of the glycosides would be cleaved to form

aglycones by intestinal microorganisms during their absorption in the GI system but could have significance in cholesterol containing foods.

Cholesterol could be oxidized to form different COPs under different conditions and the formation of COPs might have different susceptibility to certain antioxidants. In this cholesterol oxidation model, all three treatments, genistein, daidzein, and the glycosides mixture, had higher inhibition to the formation of 7β-OOH than to 7-keto and had higher inhibition to the formation of 5,6α-EP than to 5,6β-EP. This suggests that different biomarkers should be used in the study of cholesterol oxidation under different conditions and more than one biomarker should be used when using a cholesterol oxidation model to study antioxidant activity.

Genistein had much higher cytotoxicity than daidzein to SVEC4-10 cells. In order to compare the antioxidant activities of genistein and daidzein at the same level, the concentration of 1 μM was selected. However, contrary to what had been expected, no significant difference was found between the cell viability of control group and that of experimental groups treated with either genistein or daidzein in both methods. This implies that neither genistein nor daidzein exerted discernable antioxidant activities against oxidative stress induced by tBOOH in SVEC4-10 cells. The reasons may be the low concentration of 1 μM and the limited incubation time of 24 hr, or could be due to the inhibition of protein synthesis caused by tBOOH that could not be overcome by antioxidant. Therefore, some modifications are needed in order to improve the cell culture model, such as increasing the treatment concentration, increasing the treatment duration, or changing the oxidative stress initiator. Also, perhaps the cell culture model is

not suitable for antioxidant activities study, but might be utilized in the study of anti-caner activity of soybean isoflavones, which is becoming a popular research area.

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# Vita

The author was born in Zhejiang, China, on December 24, 1977. She earned the degree of Bachelor of Science from the Department of Pharmacology at Zhejiang University in 1999, and then she went to the Graduate School of Zhejiang University majoring in medicinal chemistry. In 2000, she suspended her graduate study in China and set up a private company as a general manager and technical manager. One year later, she came to the United States to continue her graduate study at Louisiana State University.

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