

THE EXTRACTION OF ANTIOXIDANT FROM SOYBEAN

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To my beloved mother, father, my siblings
and friends

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ABSTRACT

In this research, the effect of extraction time and amount of soy powder in solvent extraction from are considered for the study. Extraction is a process to obtaining something from mixture or compound by chemical or physical methods. Solvent extraction is used to extract and purify isoflavones from defatted soy flour. Isoflavones appear to protect against hormone-related disorders such as breast cancer and prostate cancers. Most of natural antioxidants occur as phenols, polyphenols, and flavonoids, three large and related families of antioxidant compounds. Natural has been used since a long time ago for preservation of meat, herb and spices. Natural antioxidant can be found in almost all plant, microorganism, fungi and animal tissue. Flavonoids constitute a large group of naturally occurring plant phenolic compounds. Flavonoids, including flavones, flavonol, isoflavones, flavonones, and chalcones occurs in all type of plant tissue. Flavones and flavonol are found in almost every plant, especially in the leave and petal. Daidzein and Genistein belongs to the isoflavone class of flavonoids that proved be found in soybean. Genistein is the strongest antioxidant followed by daidzein for the second position in soybean.

ABSTRAK

Dalam kajian ini, elemen penting ialah kesan pemisahan terhadap masa dan jumlah tepung soya ditentukan untuk pengekstrakan menggunakan larutan metanol. Proses pemisahan adalah proses untuk mendapatkan sesuatu daripada campuran atau sebatian menggunakan kaedah kimia atau fizikal. Pemisahan larutan digunakan untuk memisahkan dan menuliskan isoflavon daripada tepung soya tanpa lemak. Isoflavon adalah bahan yang digunakan untuk mencegah penyakit yang membabitkan hormone seperti kanser payudara dan kanser prostat. Kebanyakan antioksidan semula jadi ditemui di dalam fenol, polifenol dan kumpulan flavon. Ia merupakan tiga sebatian antioksidan yang berhubungkait dengan kumpulan antioksidan. Kaedah semulajadi telah digunakan sejak zaman dahulu kala, contohnya seperti kaedah pengawetan daging, herba dan rempah ratus. Antioksidan semula jadi ditemui di dalam kebanyakan tumbuhan, mikroorganisma, fungi dan tisu haiwan. Kumpulan flavon banyak terkandung di dalam tumbuhan semula jadi yang mempunyai sebatian fenolik. Contoh kumpulan flavon ialah flavon, flavonones, isoflavon, flavonol dan chalcones yang terdapat di dalam semua jenis tisu tumbuhan. Flavon dan flavonol selalunya didapati pada daun dan kelopak tumbuhan. Daidzen dan genistein adalah kelas isoflavon yang dijumpai di dalam kacang soya. Genistein adalah antioksidan yang paling kuat dan diikuti daidzein yang merupakan kedua terkuat di dalam kacang soya.

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LIST OF SYMBOLS

g - gram

ml - milliliter

kg - kilogram

°C - Degree Celsius

% - percent

μl - micro liter

mm - millimeter

min - minutes

g/ml - gram per milliliter

v/v - volume per volume

ppm - part per million

mAU - mille Absorbance

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CHAPTER 1

INTRODUCTION

1.1 Introduction

An efficient procedure was developed and used to extract and purify isoflavones from defatted soy flour. 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, and 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. Oxidants, commonly known as "free radicals," are introduced through external sources such as exposure to the sun or pollution. Other mediums include stress, as well as things that people put into their bodies, such as alcoholic beverages, unhealthy foods, and cigarette smoke. Free radical molecules are missing the electron in their atomic composition. They attack other molecules and attempt to steal their electrons, thus

creating another free radical. This oxidation process continues until key biological molecules and even genes become permanently damaged from electron loss, lowering the immune system's response to disease and adversely affecting other body systems. Scientists now believe this process is also responsible for the body's decline as we age. This theory, known as "the free radical theory of aging", was first proposed by Dr. Denham Harman in 1954. Over time, if left unchecked, free radicals cause oxidative damage to a wide range of tissues, organs and body systems, slowly damaging more molecules and decreasing immune response. Theoretically, this is why the elderly are so much more fragile and susceptible to disease, much more so than their younger counterparts.

There are different types of antioxidants, and most work better when paired with other antioxidants. This is called synergism. Vitamins can be antioxidants, such as vitamins C and E. The tripeptide glutathione, loosely classified as an amino acid, acts as an antioxidant, along with other true amino acids such as methionine. The raw herbs and vegetables we consume contain natural antioxidants called flavonoids and carotenoids. Together these nutrients protect your cells from electron robbery at the hands of larcenous free radicals, protecting the body from disease and slowing the inevitable signs of aging.

1.1 Objective of This Study

The objectives of this research are to extract isoflavone content from soybean and to study the optimum parameters for the highest yield.

1.2 Scope of the Study

- 1) To determine the optimum amount of soybean powder in solvent extraction
- 2) To determine the optimum time of extraction
- 3) To determine the daidzein and genistein from isoflavone compound of soybean

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

2.1.1 Definition of Soybean

Soybeans are the seeds of the soy plant ('Glycine max'[L.] Merr) a herbaceous plant of the botanical family Leguminosae that grows to a height from a half meter to one meter . The spheroid seeds, or soybeans, are 8 to 10 mm in diameter and grow within a pod similar to that of peas



Figure 2.1: The soybean

2.1.2 Physical Properties of Soybean

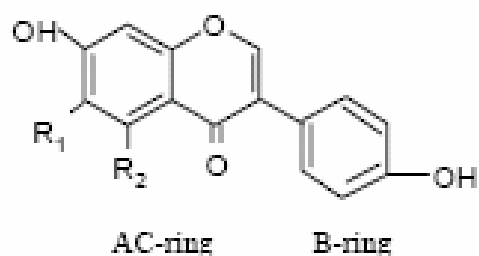
The soybean consists of two cotyledons which represent approximately 90 % of the weight, a seed coat or hull (8% of weight), and two much smaller and lighter structures the hypocotyl and the plumule. The cotyledons contain the proteins and lipids

(oils) that constitute the main nutritional components of the soybean products obtained from soybean. They are also the main storage area for the carbohydrates and various other components of importance, most notably the enzymes (lipoxygenase, urease) and the ANF.

2.1.3 Anticancer Actions

2.1.3.1 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 6 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 isoflavones, 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).



R1	R2	
H	H	Daidzein
OH	H	Genistein
H	OCH ₃	Glycitein

Figure 2.2. Formula structure 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-containing 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 tumor genesis 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 (H₂O₂) production, lipid peroxidation, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation (Wei et al. 2002). In an *in vitro* model, calf thymus 8DNA was used to investigate the inhibition of genistein on UV light and Fenton 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-tetradecanoylphorbol-13-acetate (TPA) and xanthine / xanthine oxidase. Genistein strongly inhibits the H₂O₂ 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 H₂O₂ formation, while daidzein only shows a moderate inhibitory effect to O₂ 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 chemo preventive 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 mic(Mahwah, 1996).

Because free radicals are necessary for life, the body has a number of mechanisms to minimize free radical induced damage and to repair damage which does occur, such as the enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. In addition, antioxidants play a key role in these defense mechanisms. These are often the three vitamins, vitamin A, vitamin C and vitamin E. Further, there is good evidence bilirubin and uric acid can act as antioxidants to help neutralize certain free radicals. Bilirubin comes from the breakdown of red blood cells' contents, while uric acid is a breakdown product of purines. Too much bilirubin, though, can lead to jaundice, which could eventually damage the central nervous system, while too much uric acid causes gout.

Within the human body, millions of processes are occurring at all times. These processes require oxygen. Unfortunately, that same life giving oxygen can create harmful side effects, or oxidant substances, which cause cell damage and lead to chronic disease. Oxidants, commonly known as "free radicals," are also introduced through external sources such as exposure to the sun or pollution. Other mediums include stress, as well as things that people put into their bodies, such as alcoholic beverages, unhealthy foods, and cigarette smoke.

2.1.3.2 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 12 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 dose¹³ 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 deconjugation of estrone in the gut, thus allowing its re-absorption into the peripheral circulation and conversion to estradiol (Harrison et al., 1999)

2.1.3.3 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 isoflavones 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).¹⁸ 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