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Difference in Phytoestrogen Content in Edamame (*Glycine max.* L. Merr.) Leaf Depending on Species and Cultivation Stages

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Abstract: Soybeans and soy feed ingredients contain bioactive compounds such as phytoestrogen. Phytoestrogen have drawn much attention because of their benefits to human health. They affect the physiology and metabolism of animals and humans that ingest these products. Edamame is a special kind of soybean (Glycine max (L.) Merr.) usually eaten at green stage. In this study, leaves of six local species of Edamame cultivated in the Shonai region of Yamagata prefecture, Japan, have been investigated for bioactive phytoestrogenic constituents and for radical scavenging activity of their polyphenolic fractions. Changes in the concentration of phytoestrogen at the different stages of cultivation were also measured. Among the Edamame species, Murasaki leaf possesses higher amount of phytoestrogens and stronger DPPH radical scavenging activity. Concentration of daidzin and genistin in Muraski leaf decreased at both flowering and harvesting stage but coumestrol showed a significance increase at harvesting stage.

Key words: Edamame-leaf (Glycine max. (L.) Merr.), phytoestrogen, coumestrol, functional food

Introduction

Soybeans and soy feed ingredients contain bioactive compounds that affect the physiology and metabolism of animals and humans that ingest these products (Liu *et al.*, 1999). The most studied of these bioactive compounds are the phytoestrogens. Pytoestrogens are nonsteroidal, estrogen-like compounds produced by plants, particularly the legumes and soy-based foods. There are three main classes of phytoestrogens: isoflavones, coumestans and lignans, which occur in either plants or their seeds (Alice *et al.*, 1998). Isoflavones, especially genistein and daidzein, have drawn much attention because of their benefits to human health. Several studies report health benefits of isoflavones for their estrogenic and antioxidative properties (Nestel *et al.*, 1999; Civitell 1997; Sharma 1979; Piterson *et al.*, 1991; Federal Register, 1999). Coumestrol, the phytoestrogen of coumestan group, has also activities against hormone-related diseases (Kuiper *et al.*, 1998). Although it is not fully understood how phytoestrogens exert their effects, numerous studies have demonstrated their potential for nutritional and pharmaceutical use (Barnes *et al.*, 1995 and Strauss *et al.*, 1998).

Leaves of the Edamame, cultivated in Shonai region of Japan, have not yet been focused so much and are not used as a food material. So it has been thought that if those unutilized leaves would have been found as a source of active polyphenolic compounds that could prevent the oxidative stress and possess estrogenic effect, then it could be used as raw materials for functional food. In this study, the leaves of some local Edamame species were investigated for their major phytoestrogens and for the radical scavenging activity of phenolic fractions. Changes in phytoestrogens during cultivation were also investigated to select the best stage to yield higher amount of phytoestrogens.

Materials and Methods

Chemicals: HPLC grade chemicals were purchased from Wako Chemical industries, ltd., Japan.

Materials: Leaves of six Edamame species (Shirayamadadacha, Murasaki, Tonojima, Bansei, Sapporo midori & Kingpou) were collected from Agricultural farm of Agriculture Faculty, Yamagata University. Leaves were then washed and dried in air at room temperature. These dried leaves have been extracted with MeOH at 30°C for 2-3 days. After filtering with filter unit (DISMIC-13 jp, 0.50 μM, Toyo Roshi Kaisha, Ltd., Japan), the filtrate (polyphenolic extract) was used for the analysis of polyphenolic constituents by HPLC and for DPPH radical scavenging activity measurement.

Five polyphenols standard were used for quantification of the amount of leaf-polyphenols. Polyphenols used as standard were daidzin, genistin, coumestrol, daidzein and genistein. Authentic daidzin, genistin, daidzein, genistein were purchased from Extrasynthese Company, France and coumestrol was obtained from previous work in this laboratory.

HPLC Analyses: The HPLC analysis was performed on a Develosil ODS-UG-5 (4.6x250 mm) packed column

(Nomura Chemical Co., Ltd. Japan) with a Hitachi L- 7405 UV-Vis detector, a Hitachi L-7120 pump. The column temperature was controlled, using a Hitachi column oven L-7300, at 30°C. UV detection was performed at a wavelength of 280 nm. The mobile phase used for analysis was solvent A: 5% MeCN in 1% AcOH; solvent B: 40% MeCN. Flow rate was 0.8 mL/min with a linear gradient of solvent B in solvent A (A 100% at 0 min and B100% at 180 min).

DPPH Radical Scavenging Activity Measurement: DPPH radical scavenging activity of each polyphenolic fraction of the species was measured according to the method of Suda (2000) with a slight modification and expressed as Trolox equivalent. Reagents 'a' (40 μM DPPH solution in Ethanol), 'b' [0.2M Mes (2-morpholinoethanesulphonic acid) buffer (pH 6.0)] and 'c' (50% Ethanol) were mixed at 1:1:1 ratio. A 1.5 ml aliquot of this mixture was poured into each of six test tubes. Then 50% Ethanol was added to the tubes in order of 300, 270, 240, 180, 120 & 60 μl. Sample solution was then added to the tubes in order of 0, 30, 60, 120, 180 & 240 μl with an interval of one minute. Optical Density (OD) was measured at 520 nm after 20 minutes of the addition of sample to each tube. Same experiment was done with Trolox (0.2 mM Trolox in Ethanol) instead of sample for the preparation of the standard curve. Plots were drawn using sample (or Trolox) volumes (0, 30, 60, 120, 180, 240 μl) against respective OD. Trolox equivalent of the sample was calculated using the standard curve of Trolox.

Results and Discussion

Quantification of some Major Phytoestrogens: Major phytoestrogens in the leaves of Edamame species were quantified by HPLC. Among these species Murasaki & Tonojima have been found to have the higher amount of daidzin and genistin as the phytoestrogen isoflavone. Concentraion of daidzein and genistein as aglycone were found almost similar among the species except Kingpou. In Murasaki, the important phytosterogen coumestrol was found in considerable amount compare to other species. Principal soy-isoflavones are mainly present in the glucoside forms genistin and daidzin in plants, e.g. in soybean milk powder, only 4-5% of the isoflavones are in the form of aglycones (Wang and Murphy, 1994). This trend has also been observed in the present study.
The soy isoflavones, genistein and daidzein and their 7-glucosides have recently received much attention because of their potential benefits as phytoestrogens (Price and Fenwick, 1985). Coumestrol has more potent phytoestrogenic effects than lignans, genistein and daidzein and showed the stronger inhibition than genistein in case of the conversion of estrone to 17-beta estradiol. (Makela *et al.*, 1995). The relative oestrogenic potency of coumestrol was 200 times less than oestrone but 30 to 100 times greater than isoflavones (Bickoff *et al.*, 1962). As phytoestrogen concentration was found the highest in Murasaki among the species, we further investigated whether there were any changes in the phytoestrogens at different stage of cultivation of Murasaki. From the quantitative analysis it was observed that amount of daidzin and genistin were highest in the early stage (cotyledon, 17 days after planting), which became lower with the time. It may be due to the cause that isoflavones, biosynthesized at early growing stage, was utilized by plant for growing and/or transferred to seed under being produced. Amount of coumestrol was found highest at harvesting stage and extremely low at early stage, suggesting that coumestrol accumulates only in leaf. Knuckles *et al.* (1976) found only 0.12 mg coumestrol per 100 g whole soybean.

DPPH Radical Scavenging Activity: To evaluate the antioxidative property, DPPH radical scavenging activities were measured for the polyphenolic extracts of species. It was found that Murasaki had the highest activity amongst the species investigated. Shirayama and Tonojima were found to have moderate activity. Recently, there is a considerable interest in the food industry and in preventive medicine in the development of "natural antioxidants" from plant materials such as flavonoids (Vinson and Hontz, 1995). The effects of flavonoids as captors of free radicals (Kovac *et al.*, 1992) and their influence on artheriosclerosis and their anti-inflammatory, antiallergenic and anticarcinogenic properties have been reported (Escribanoet *et al.*, 1992; Kovac *et al.*, 1992 and Hertog *et al.*, 1993). Kaeko *et al.* (2002) reported that flavonoid quercetin, luteolin, and kaemferol had strong radical scavenging activity where as isoflavones genistein and daidzein had no radical scavenging activity though Wei *et al.* (1995) reported the antioxidative effect of genistein.
Polyphenolic extract of Murasaki Edamame leaf possessed higher DPPH radical scavenging activity and contained much amount of flavonoids compare to the other species that was observed from UV absorption both at around 250 and 340-370 nm by photo diod aray HPLC. So it may be considered that stronger DPPH radical scavenging activity was due to higher content of flavonoids. UV-Vis spectra of peaks corresponding to flavonoids that were higher in Murasaki.
Functional food is defined as "functional foods are those, which contain significant level of naturally occurring components and may enhance health by providing a physiological benefits beyond the provision of the basic nutrients in the food". In this study we found Muraskai leaf contained biologically active isoflavones daidzein, genistein, their glycosides, coumestrol and some other flavonoids in a considerable amounts compare to other

species and that coumestrol can be obtained at harvesting stage. These findings may add a new dimension to the list of functional foods since the leaf of Edamame (Murasaki) is not used as food materials. Further studies are needed for the utilization of the Edamame leaves as raw materials of functional food.

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