Original Article

Journal of Practical Biochemistry and Biophysics
Volume 3 Number 1, January - June 2018

Single Step Purification of Soybean Isoflavones Employing Silica Gel Adsorption Chromatography

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Abstract

The extraction and purification of isoflavones from defatted soy flour (DSF) are attempted by leaching and adsorption chromatography. The isoflavones were extracted in methanol and processed employing silica gel column chromatography. After silica gel column chromatography 0.4 mg/ml of isoflavones content (3.9 mg/g DSF) was obtained which has 16.8 mg of total isoflavones. The process helps to the recovery of the isoflavones along with the removal of contaminants up to 90% which confirmed through HPLC.

Keywords: Concentration; Methanol; Isoflavones; Soybean; Column Chromatography.

Introduction

Soybeans (*Glycine max*) are the rich sources (1.2 - 4.2 mg/g flavonoid) of isoflavones, and are widely available, which are less expensive (Vacek et al., 2008). Isoflavones are often known as phytoestrogens, the group of plant-derived phenolic compounds which shows estrogenic activity (Wildman, 2007). These are commonly found in leguminous plants (peas, beans) and clovers (Saviranta et al., 2008). Isoflavones are known for their potential health benefits. They exhibit antioxidant activity and play an important role in preventing and treating various cancers, osteoporosis and cardiovascular diseases (Achouri et al., 2005). Isoflavones are mainly classified into four groups namely aglycons, glucosides, malonylglucosides and acetyl glucosides. The three isoflavone aglycons, namely, genistein, daidzein and glycitein, are each present in four glucosidic forms in soybeans and soy foods (Griffith and Collison, 2001; Klejdus et al., 2005; Lee et al., 2004).

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Received: May 27, 2018 | **Accepted:** June 14, 2018

Several methods and techniques have been reported for the extraction, isolation, and purification of isoflavones. They can be categorized based on several general principles (or approaches). One is based on extraction followed by precipitation. Another is based on precipitation followed by extraction or separation. The third is based on the use of chromatography or other means either before or after solvent extraction to separate or concentrate isoflavones (Zhang and Schwartz, 2005, Lakshmi *et al*., 2013).

Due to the multiple beneficial effects of soy isoflavones on human health, related products have flooded the market, with unsubstantiated claims and few regulations governing their quality or efficacy. Most products have levels of isoflavones less than their claimed contents, with some containing virtually no detectable isoflavones (Lawton *et al*., 2003). Moreover, many products only contain soy extracts with very little isoflavones (i.e., 0.2%) and abundant unknown impurities. More importantly, isoflavones in many products are in the form of glucosides, which have weaker biological activities and are more difficult to be absorbed by the body than the corresponding aglycones (Zhang *et al*., 2007). Therefore, the quality and efficacy of many isoflavone products are poor, and there is a need to develop products with higher purity and efficacy.

Most reported processes include multiple steps; some require multiple chromatography columns for the production of isoflavones. Adsorption is a potential method for purification of isoflavones with minimum processing steps, allowing the elimination of polar, nonphenolic impurities. Partitioning, using solid phase extraction (SPE), using adsorbant resin is a selective method for completely purifying isoflavones. The main objective of this study was to isolate and purify major isoflavones daidzein, genistein and their glycosylated forms (daidzin, genistin) from defatted soy flour in the single step purification method.

Materials and Methods

Materials

The soybeans *(Glycine max)* of variety JS 335 was procured from National Seed Corporation, India. They were stored at refrigerated temperature (6-8 °C) until use. Adsorbent silica gel was obtained from Sd fine-chem. Ltd, Mumbai, India. HPLC grade solvents, namely, water, ethanol, methanol, acetone and acetonitrile were purchased from Merck, Mumbai, India. All the chemicals used were of analytical grade. The isoflavone standards such as genistein, daidzein, genistin and daidzin were obtained from Sigma Chemical Co. St. Louis, MO, USA.

Methods

Extraction

Isoflavones, genistein and daidzein (aglycones), genistin and daidzin (glycosylated) were purified from defatted soy flour following the method described by Ohta *et al*., (1979) with some modifications. Finely defatted soy flour (25g) was extracted with 250 ml of 80% methanol three times at 80°C for 3 hours. The extract was filtered and the supernatant concentrated under atmospheric pressure first and then under vacuum. A brownish, syrupy liquid was obtained. This was subjected to extraction with two volumes of acetone. The acetone extract was concentrated by vacuum drying. The solid obtained was dissolved in water and subjected to solvent partition using ethylacetate. The extract was partitioned into three layers, uppermost ethylacetate layer, middle solid mass and lower aqueous layer.

Silica gel adsorption chromatography

The ethylacetate extract was subjected to adsorption chromatography on silica gel. The column (30 cm x 2.5 cm) was eluted with 50% water saturated ethylacetate, and 50% water saturated ethylacetate containing 2% ethanol with a flow rate of 1ml/min. Fraction F1 is rich genistein and daidzein, F5 is rich in genistin and F6 is rich in daidzin. Each fraction obtained was rechromatographed on silica gel using the same procedure.

HPLC

The amount of isoflavones in the extracts was analyzed using HPLC (Waters Alliance 2690, Waters, USA) equipped with a photodiode array detector (Waters, USA) and millennium chromatography manager software. A 10 µl sample was loaded onto a C18 column (SGE, 250 x 4.6 mm, 5 µm particle size, SGE, Germany). The mobile phase was composed of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B). The elution was performed in a linear gradient of A against B. The separation was achieved using the following gradient program: 0-5 min, 85% A; 5-36 min, 71% A; 36-44 min, 65% A and 45-60 min, 85% A. The flow rate of the mobile phase was set at 0.6 ml /min and absorption was measured at 260 nm (Murphy *et al*., 1997). The temperature of the column was maintained at 25±1°C. The identity and purity of isoflavones in the samples were confirmed by matching the retention times and areas with the standards.

The purity of isoflavones was determined by HPLC employing a C 18 column (Wang and Murphy, 1994) with gradient elution using acetonitrile water (15 to 35%, in 50 min, flow rate: 1ml/min and detection at 262 nm). The concentration of isoflavones was determined by the standard graph using the method as given by Coward *et al*., (1993).

Calculation of total isoflavones

The concentration of isoflavone glucosides (daidzin, genistin) in a given sample were expressed as aglycon equivalents using the following equation (AOAC, 2005; Lakshmi *et al*., 2013).

$$Ca_e = [MW_a/MW_g] \times C_g \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

where Ca_e = isoflavones aglycon equivalents (µg/g); MW_a = molecular weight of aglycon; MW_g = molecular weight of glucoside; an Cg = concentration of daidzin and genistin (µg/g).

The total isoflavones in microgram aglycon equivalents/g of sample was calculated, by summing the concentrations of daidzein, genistein and adding this total to the sum of aglycon equivalent concentrations of daidzin and genistin as indicated below.

$T_a = C_a$ (daidzein) + C_a (genistein)

$T_{ae} = C_{ae}$ (daidzin) + C_{ae} (genistin)

where T_a = sum of concentrations of aglycons and T_{ae} = sum of aglycon equivalent concentrations of glucosides.

Free Radical Scavenging Activity

The DPPH radical scavenging test was carried out as described as Blois (1958). The extracts (with different dilutions of the extract in 100% methanol, ranging from 0.05 to 0.3 mL), were mixed with 0.5 mM/L DPPH solution. The absorbance was measured at 517 nm immediately and again after 30 min to determine the amount of DPPH scavenged. The free radical scavenging activity of samples was expressed in percentage, and each sample was analyzed in triplicate. The free radical scavenging activity was calculated by using the following equation:

Table 1: The content of isoflavones

Procedure	Isoflavone content		Total Isoflavones (mg)
	mg/ml	mg/g DSF	
Extraction	0.12	1.28	1.2
after purification	0.4	3.9	16.8

** per gram of dry extract.*

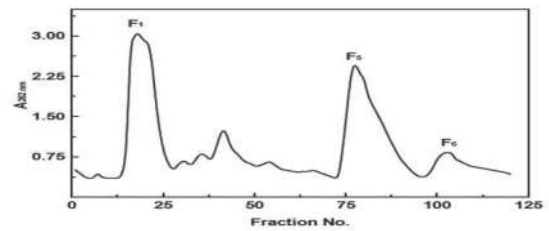

Fig. 1: Elution profile of ethyl acetate extract on silica gel column. F_1 fraction mixture of genistein and daidzein, F_5 and F_6 fraction are rich in genistin and daidzin.

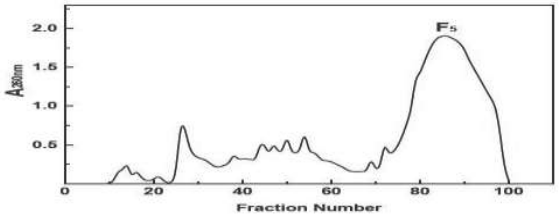

Fig. 2: Rechromatography of fraction F_5

$$\text{Scavenging activity}\,(\%) = \frac{[Aa-(Ab-Ac)]}{Aa} x100$$

where Aa is the absorbance of the control solution of DPPH (without isoflavone extract), Ab is the absorbance of the mixture containing isoflavone extract as well as DPPH, and Ac is the absorbance of the blank solution without DPPH.

Results and Discussion

Extraction using methanol has resulted in 0.12 mg/ml of isoflavones content (1.28 mg/g DSF) which has 1.2 mg of total isoflavones.

The solvent extract (methnolic extract) on silica gel column was resolved into three fractions F1, F5 and F6 as shown in Fig. 1. F1 fraction which was eluted with 50% water saturated ethyl acetate was found to be genistein and daidzein. F5 and F6 fractions which were eluted with 50% water saturated ethyl acetate containing 2% ethanol contained genistin and daidzin respectively. Each fraction obtained was rechromatographed using similar conditions (Fig. 2). The isoflavones genistin, daidzin, genistein, and daidzein purified from defatted soy flour, had a purity of >90% (confirmed by HPLC) as can be seen in Fig. 3.

After silica gel column chromatography it resulted in 0.4 mg/ml of isoflavones content (3.9 mg/g DSF) which has 16.8 mg of total isoflavones. The process helps to the recovery of the isoflavones along with removal of contaminants.

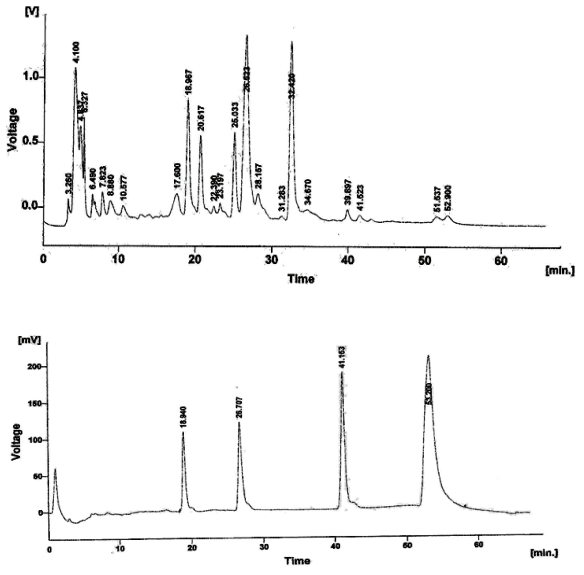

Fig. 3: Chromatograms showing crude and purified isoflavones (RT; 16.946-Daidzein, RT; 26.707-Genistein, RT; 41.163-Daidzin and RT; 53.20-Genistin)

The radical scavenging activity of all the extracts was found to increase with an increase in the concentration (from 0.05 to 0.25 mg) of isoflavones (Fig. 4). Highest antioxidant activity (65.8%) was observed at 0.25 mg concentration of isoflavones compared to other extracts and afterwards, it attained a plateau showing no further change in RSA (%) even with an increase in isoflavone concentration (0.3 mg).

Conclusions

Extraction and purification of isoflavones from defatted soy flour were carried out employing adsorption process resulted in 16.8 mg of isoflavones per gram of defatted soy flour from 1.2 mg/g (DSF) of initial extraction. The silica gel chromatography process successfully used for the purification (>90 %) and recovery of the isoflavones. The purified extract exhibited maximum radical scavenging activity of 65.8% on DPPH at 0.25 mg/g isoflavone concentration.

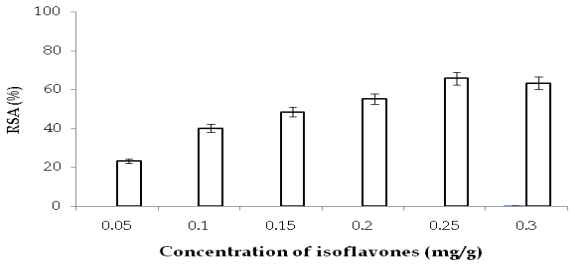

Fig. 4: Radical scavenging activities of isoflavones at different concentrations.

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