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Purification of Isoflavone from Soybean Hypocotyls using Various Resins

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Abstract : Isoflavone was extracted with various concentration of aqueous methanol using whole hypocotyls as the starting material. Whole hypocotyls were preferred as the raw material because the residue could be easily removed from the solvent after the extraction process. Extraction yield was almost constant at the methanol concentration of 20-80%. Most of the isoflavone was extracted within 1 hr, and the extraction yield remained almost constant thereafter. When the concentration of methanol was 80%, the content of total solid was reduced due to the reduced extraction of contaminating protein as the result of protein insolubilization. Among resins tested, Diaion HP-20, Amberlite XAD-16, and Amberlite IRC-50 showed the highest capacity to absorb the compound. Open column chromatography with Diaion HP-20 showed that 80% aqueous ethanol was most efficient as the eluting solvent with final recovery of the phytochemical being more than 95%. Maximum adsorption of the phytochemical occurred at the acidic pH 2-4. When the spatial velocity was increased to 15 and more, the degree of adsorption was decreased, whereas below spatial velocity of 15, the adsorption capacity of isoflavone to the resin was almost constant. The purity of the isoflavone purified by column chromatography was 78%.

Keywords : methanol, isoflavone, purification, resins

Introduction

For more than two thousand years, Asian people have been consuming soybean as a variety of traditional food products. Until most recently, many of these soybean-based-foods were focused on utilization specific components of the bean such as protein and oil, or the whole bean itself. Minor components of the soybean, such as phytic acid, saponin, and isoflavone were, however, thought usually as unimportant or rather a little harmful to human health. Most of previous researches on these compounds, with isoflavone including, were focused on removing the compounds during soy processing because removal of the compounds improved color and flavor of soybean products.[1,2] However, with the discovery of health-promoting effects of the minor components, efforts are now placed on preventing the loss of these compounds during food processing. Isoflavone especially receive considerable attention due to their anticancer and antiosteoporosis activities.[3,4] Soy isoflavone is referred to as phytoestrogen because it binds to the estrogen receptor (ER) and affect estrogen-mediated processes.[5]

Numerous reports for recovering isoflavone from soy wastes are being reported. Most employ methods using extraction with aqueous ethanol or methanol. Coward *et al.*[6] extracted isoflavone from soybean using 80% aqueous methanol. Zheng *et al.*[7] performed the extraction of isoflavone by adsorption chromatography. Several groups invented the recovery processes of isoflavone from various soybean byproducts such as soy molasses,[8] defatted soybean meal,[9,10] soybean sprouts,[11] and soybean paste.[12] Kitada *et al.*[13] reported that isoflavone could also be isolated from the soybean cooking water, which was produced as a by-product of the miso manufacture.

While small-scale extraction process for isoflavone have been studied intensively, researches on the large-scale production of isoflavone from soybean and its by-products are rather limited. In this paper, feasibility of using soybean hypocotyls, by-product of the soymilk and tofu manufactures, as a source for the isoflavone production was studied.

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Materials and Methods

Materials

Soybean hypocotyls used for extracting isoflavon was obtained from Dr. Chung's Food Co., Ltd. (Cheongju, Korea), a local soymilk manufacturer. HPLC column for isoflavone quantification was ODS A303 (4.6 × 250 mm, YMC, Milford, MA, USA). Amberlites, magnesium silicate, aluminium oxide, and silica gel were purchased from Sigma (St. Louis, MO, U.S.A), and Diaion HP-20 from Mitsubishi Chemical Industries Limited (Tokyo, Japan). Isoflavone standards were obtained from Fujicco (Kobe, Japan).

Quantification of Isoflavone

HPLC analysis was carried out using Agilent system (Agilent 1100 series, Agilent Technologies Inc., Palo Alto, CA, USA). Detector used was UV detector at 254 nm, and the flow rate of the solvent was 1.0 m*l*/min. A linear HPLC gradient was composed of 0.1% glacial acetic acid in H_2O (A) and 0.1% glacial acetic acid in acetonitrile (B). Following injection of 20 μ*l* sample, solvent B was increased from 15 to 35% over 50 min, and held at 35% for 10 min.

Extraction of Isoflavone

Isoflavone was extracted from soybean hypocotyls using aqueous methanol. Briefly, to 2.0 g of ground soybean hypocotyls, 20 m*l* extraction solvent consisting of aqueous methanol (v/v) was added with stirring. After the extraction, the mixture was centrifuged at 3000 × g for 15 min. The solution was filtered through a 0.45-μm membrane filter (Whatman, Rotenburg, Germany) prior to HPLC analysis.

Resin Activation

Crude extract of soy isoflavone was further purified with adsorption resin column chromatography. Resins used for the experiment were Diaion HP-20, Amberlites (XAD-1600, XAD-16, XAD-8, XAD-7, XAD-2, IRC-50), aluminium oxide, magnesium oxide, and silica gel. Prior to use, the adsorbent resins were soaked and swelled in 100% (v/v) methanol for 12 hr to remove impurities. The solvent was removed by washing with excessive amount of distilled water. The washed wet resin was weighed and added to isoflavone solution and degassed so that floatation did not occur at the surface.

Batch Operation

The resins that adsorb the isoflavone was tested using batch resin operation. Briefly, 5 m*l* of activated resin was mixed with 250 m*l* of crude isoflavone solution with stirring for 2 hr at room temperature. After the reaction, the amount of isoflavone remaining in supernatant was measured and the adsorption capacity of resin was calculated by the difference. Initial concentration of isoflavone in the crude solution was 46 mg/*l*. Effect of solution pH on adsorption of isoflavone to the resins were also investigated, likewisely.

Column Chromatography

Glass columns with diameter 15 mm were packed with 50 m*l* of resins selected from batch operations. The pH of sample solution containing crude isoflavone was adjusted to 4.0 before loading on to the column. The column was washed with 2 volume of distilled water before being desorbed with aqueous ethanol with different concentrations. Optimum spatial velocity and recovery efficiency were tested.

Results and Discussion

Chemical Composition of Crude Extract

Major types of isoflavone found in soy hypocotyls were 6"-O-malonyl genistin, 6"-O-malonyl daidzin, and 6"-O-malonyl glycitin, which account for more than 70% of the total phytochemical, while acetylglycoside was present in trace amounts. Isoflavone contents of soybean hypocotyls were 34.07 mg/g.

Effect of Ethanol Concentration on the Extraction of Isoflavone

Isoflavone was extracted with various concentration of aqueous methanol using whole hypocotyls as the starting material. Whole hypocotyls were preferred as the raw material because the residue could be easily removed from the solvent after the extraction process. Fig. 1 shows the effect of different concen-

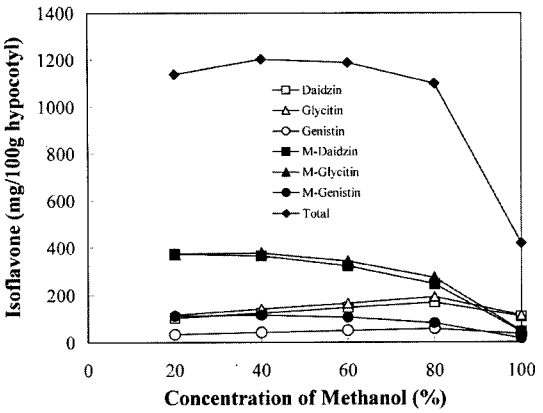

Fig. 1. Effect of methanol concentration on the extraction of isoflavone. Isoflavone was extracted from soybean hypocotyls with 10 volume of aqueous methanol at 60°C for 2 hr.

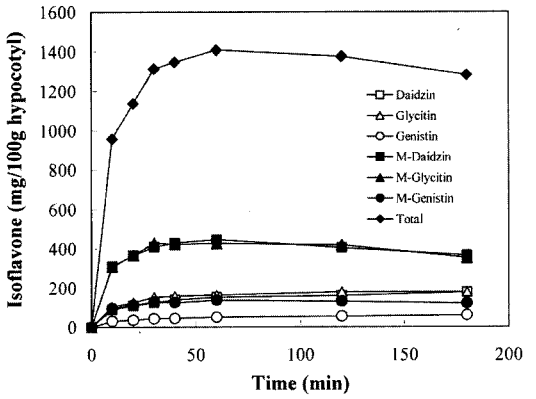

Fig. 2. Extraction of isoflavone from soybean hypocotyls with 60% of aqueous methanol at 60°C.

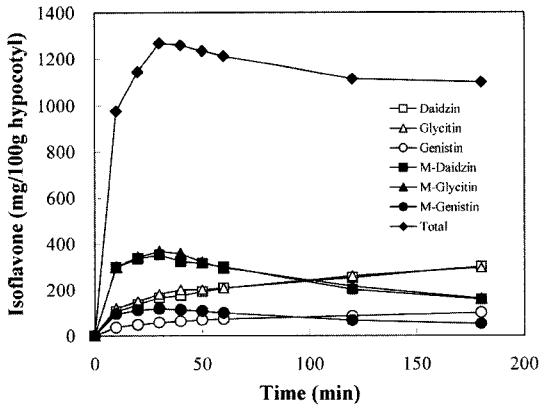

Fig. 3. Extraction of isoflavone from soybean hypocotyls with 60% of aqueous methanol at 80°C.

trations of aqueous methanol on extraction of isoflavone at 60°C. Extraction yield was almost constant at the methanol concentration of 20-80%. Therefore, optimum concentration of methanol for the extraction of isoflavone is 40-60%. For further researches, 60% aqueous methanol was used. Composition of the phytochemicals was affected by methanol concentration. As the concentration of methanol increased, the content of malonylglycoside decreased while glycoside increased.

Effects of solvent temperature on extraction of isoflavone was compared at 60°C and 80°C. For the experiments, 2 g of whole hypocotyl was mixed with 20 m*l* of 60% aqueous methanol and the extraction profile of isoflavone isomers are as shown in Fig. 2 and 3. Most of the isoflavone was extracted within 1 hr, and the extraction yield remained almost constant thereafter. The ester bonds of malonylglycoside was not hydrolyzed under the extraction conditions so that the composition of isoflavone was not varied with time. At 60°C, extraction yield increased with extraction time, reaching maximum within 1hr and then leveled off thereafter. However at 80°C, maximum extraction yield was reached at 30 min, which decreased slightly as extraction time increased. Significant decrease of malonylglycoside was also detected at 80°C. Coward *et al.*[5] proposed that extraction at 4°C for 2-4 hr led to the highest yield of malonylglycoside and room temperature extraction converted the malonylglycoside although the rate is slow. However, in our experiments, the conversion of malonylglycoside was very slow even at 60°C. Our research showed (the data not shown here) that extraction of total solid is affected by the alcohol concentration and extraction temperature. When the concentration of the alcohol was too high, the content of total solid was reduced probably due to the reduced extraction of proteins as the result of protein insolubilization. Thus, for maximum recovery of isoflavone, optimum methanol concentration was 60%.

Batch Operation with Adsorption Resins

Crude extract of soy isoflavone was further purified using some selected adsorption resins (Fig. 4). Adsorption test of isoflavone to the resins were performed as mentioned in methods. Diaion HP-20 is anion exchanger and a polyaromatic resin

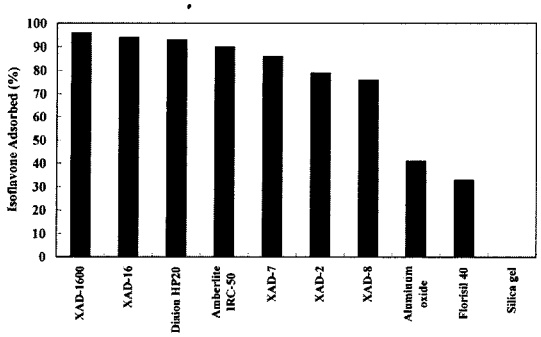

Fig. 4. Adsorption of isoflavone using different resins.

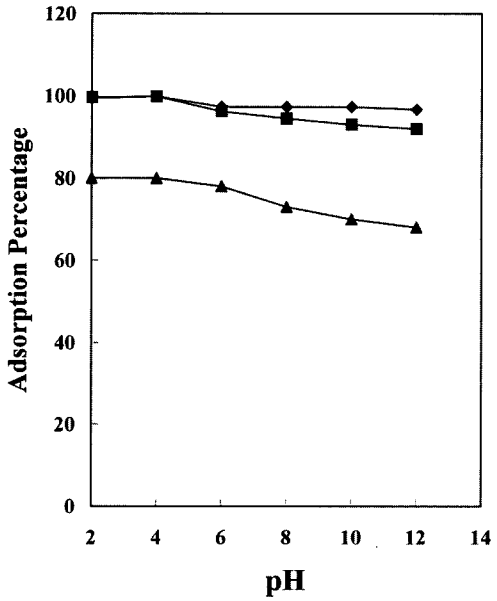

Fig. 5. Effect of pH on adsorption of isoflavone to some selected resins: ◆ Amberlite-XAD-16; ▲ Amberlite IRC-50; ■ Diaion HP-20.

polymerized with polystyrene and divinylbenzene while Amberlite XAD is a non-ionic, hydrophobic and cross-linked polyaromatic resin. These resins have large surface area and fine pore structures inside the particle and were expected to adsorb isoflavone both due to hydrophobic interaction and charge-charge attraction. Magnesium silicate and aluminum oxide adsorbed less amounts of isoflavone, and silica gel did not adsorbed the phytochemical at all. Since Diaion HP-20, Amberlite XAD-16, and Amberlite IRC-50 showed the highest capacity to absorb the compound, these resins were used for the further research (Fig. 4).

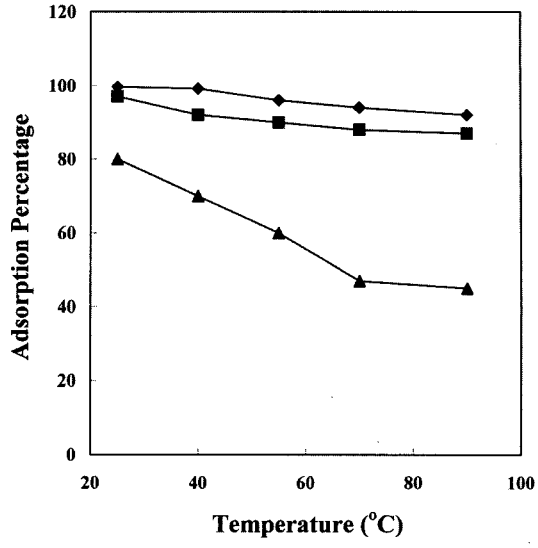

Fig. 6. Effect of temperature on adsorption of isoflavone to some selected resins: ◆ Amberlite-XAD-16; ▲ Amberlite IRC-50; ■ Diaion HP-20.

Maximum adsorption of the phytochemical occurred at the acidic pH 2-4 (Fig. 5). Moreover, adjusting solution pH to 4.0 has additional advantages in that the contaminating protein could be easily removed due to precipitation of the soy protein at the pH.[14,15] Additionally adjusting pH of the operation solution to 4.0 could also suppress microbial contaminations. Fig. 6 shows the effect of temperature on adsorption of isoflavone on to the resins selected. For Diaion HP-20 and Amberlite XAD-16, influence of temperature was insignificant as the adsorption of isoflavone was constant regardless of the temperature tested. Using the results obtained from batch operations, Diaion HP-20 was chosen for further purification of isoflavone using open column chromatography.

Column Operations

Open column chromatography was performed using Diaion HP-20 with the pH and temperature of operation fixed to 4.0 and 20°C. After loading the crude isoflavone extract on to the column, the resin was washed with 2 bed volumes of distilled water to remove the soluble substances such as oligosaccharides, saponin, and protein. Consequently, elution was performed with 60% ethanol. Height-to-diameter ratio of the column was fixed to 10

because our previous research showed that the adsorption of isoflavone was not affected when the spatial velocity was between 5 and 10. Spatial velocity is important in the aspect of productivity and the recovery of target substances could be decreased if the spatial velocity was too high. Therefore the optimization of spatial velocity was necessary. Spatial velocity was defined as the elution rate of solution per liter of resin per hr. As shown in Fig. 7, when the spatial velocity was increased to 15 and more, the degree of adsorption was

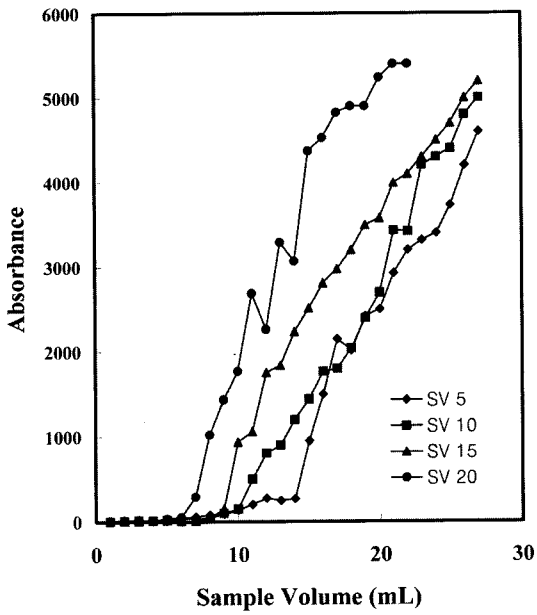

Fig. 7. Effect of spacial velocity on adsorption of isoflavone onto Diaion HP-20 open column.

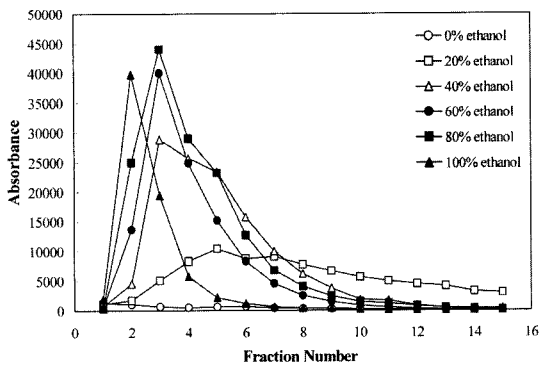

Fig. 8. Desorption of isoflavone using various concentration of aqueous ethanol.

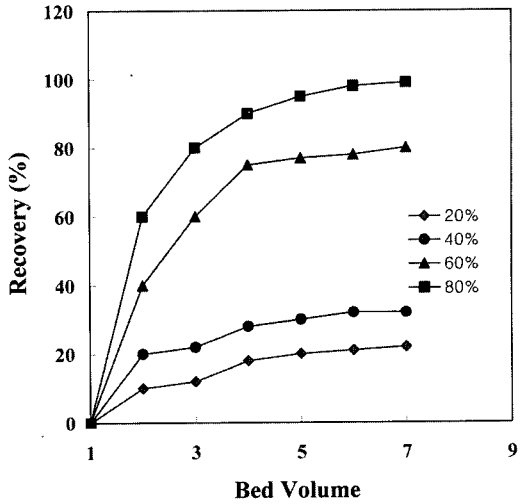

Fig. 9. Recovery of isoflavone when ethanol with different water content was used as the elutio solvent.

decreased, whereas below spatial velocity of 15, the adsorption capacity of isoflavone to the resin was almost constant. Therefore, for further experiments spatial velocity was fixed to 10. Fig. 8 and 9 show recovery of isoflavone from open column with Diaion HP-20, using various aqueous ethanol as the eluent; 80% aqueous ethanol showed the highest percentage of recovery. The purity of isoflavone was checked using HPLC and was found to be 78%.

This result showed that isoflavone found in soy hypocotyls could be effectively purified by simple two steps of methanol extraction and column chromatography using Diaion HP-20.

Conclusions

Major types of isoflavone found in soy hypocotyls were 6"-O-malonyl genistin, 6"-O-malonyl daidzin, and 6"-O-malonyl glycitin, which account for more than 70% of the total phytochemical, while acetylglycoside was present in trace amounts.

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Acknowledgements

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