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Fractionation of soybean functional glycosides from soy-waste based on the chemical reaction of soyasaponin β g

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Abstract

We studied the chemical characteristics of soyasaponin β g with a view to establishing an effective fractionating method for soybean glycoside. Under an acidic condition of below pH 3, soyasaponin β g precipitated with unknown sugar and glycoside compounds of soybean. In addition, soyasaponin β g was hydrolyzed to soyasaponin Bb and 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one moieties under alkaline conditions. 1.0 mM NaOH hydrolyzed soyasaponin β g at the rate of 0.44 μmol/min. Metal ion-binding activity of soyasaponin β g resulted in 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one moiety and 1 mM soyasaponin β g chelated with 1.57 mM Fe^{2+} and 0.2 mM Cu^{2+}. Based on these chemical characteristics of soyasaponin β g, we fractionated soybean saponin from soy-waste after oil extraction (industrial waste) by four steps: (1) precipitation of glycosides under acidic conditions (pH 2), (2) separation of hydrophobic functional compound (isoflavonoid), (3) chelate precipitation of 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one conjugated saponin using $FeCl_2$ and (4) removal of the 2,3-dhydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one and Fe^{2+} complex by an alkaline hydrolysis (pH 12). Finally, 375 mg of group B saponin (84% purity) were obtained from 100 g of soy-waste after oil extraction. This fractionating method is a simple and useful method for producing medicinal foodstuffs from soybean.

Keywords: Saponin; Isoflavonoid; Soybean glycoside; Medicinal foodstuffs

1. Introduction

Soybean glycosides, such as saponin and isoflavonoids have some functional effects in biological systems, hepatoprotective and anticarcinogenic activities, an inhibitory effect on the replication of viruses and in improving the effect on hyper-cholesteremia (Hayashi, Hayashi, Hiraoka, & Ikeshiro, 1997; Kinjo et al., 1999; Oh & Sung, 2001; Ueda, Matsumoto, & Goutani, 1996). Particularly, isoflavonoids have attracted much attention for the activity of dietary phytoestrogen (Adlercrentz, Hamalainen, Gorbach, & Goldin, 1992; Ono & Yamaguchi, 1999). In spite of these health benefits, soybean is still predominantly processed only to extract oil, but no other functional products, such as the saponin (Liu, 1997). In addition, its peculiar taste and flavour reduce soy-food intakes of Western people. Soybean glycoside consumption of Western people (1–3 mg/day) is quite low compared with that of East Asian people (25–100 mg/day) (Liggins et al., 2000;

Abbreviations: DDMP saponin, 2,3-Dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one conjugated saponin.

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Messina, Persky, Setchell, & Barnes, 1994). In the present soybean market, it is necessary to increase consumption of soybean functional compounds rather than soybean or soy-food intake for the utilization of soybean functionality.

Soyasaponin β g is a major saponin of soybean seed and genuine saponin of the group B and E saponins (Fig. 1). Soyasaponin β g was hydrolyzed into soyasaponin Bb and 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) moieties in a fractionation and isolation process, e.g. heating-reflux under alkaline conditions and low grade reagent. Therefore, we reported an accurate analysis and effective isolation method for DDMP saponin under acidic, room temperature and non-metal conditions using chelators, such as ethylenediaminetetraacetic acid (EDTA) (Yoshiki et al., 1995). Soyasaponin β g has important biological activities, such as reactive oxygen-scavenging activity (Yoshiki & Okubo, 1995). However the utilization of soyasaponin β g as a functional compound would be difficult. The high quantity needed would be costly to isolate and preserve due to the unstable structure of soyasaponin β g. Because of the structural stability of the saponin, we anticipate group B and E saponins as the supply source of soybean functionality rather than DDMP saponin.

In this paper, we studied the chemical characteristics of soyasaponin β g. Based on a chemical reaction of soyasaponin β g, we also describe a high quality and simple fractionating method for soybean functional glycosides (isoflavonoid and group B and E saponins) from soy-waste after oil extraction.

2. Materials and methods

2.1. Sample

Soy-waste, after oil extraction, was supplied by Fuji Oil Company (Japan).

2.2. Isolation of soyasaponin β g

Soyasaponin β g was isolated from soybean hypocotyls (*Glycine Max*) by extracting with 70% ethanol containing 0.01% EDTA. The extract was evaporated at 40 °C, and the residue dissolved in H_2O:*n*-BuOH (1:1, v/v). The *n*-BuOH layer was evaporated, and the crude saponin fraction was obtained. The crude saponin fraction was loaded on to a Lobar column (ODS reversed phase column, 25 × 310 nm) using EDTA:MeCN:H_2O:HOAc

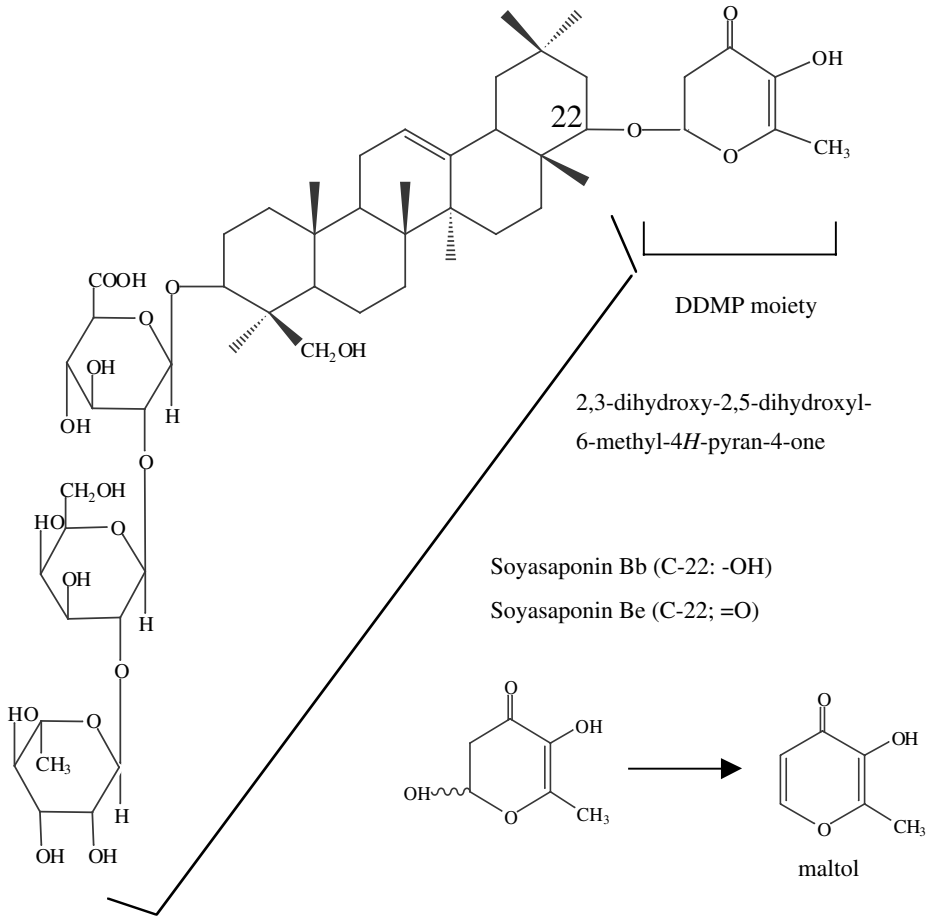

Fig. 1. Structure of soyasaponin β g.

(1:4000:6000:3) as mobile phase and flow rate of 4 ml/min. The eluate absorbing at 292 nm (soyasaponin α g and β g mixture) was further purified by an HPLC (column, ODS reversed phase Lobar column, 10 × 240 mm; mobile phase, MeCN:H_2O:HOAc (4000:6000:3); wavelength, 292 nm; flow rate, 2 ml/min), as described previously (Yoshiki et al., 1995).

2.3. Hydrolysate analysis of soyasaponin β g

Various concentrations of NaOH (2.5–40 mM, 20 μl) were added to an equivalent volume of 1 mM soyasaponin β g. The concentrations of hydrolysate and soyasaponin β g were determined by HPLC. HPLC analysis was carried out on a YMC-packed ODS-AM-303 column (5 μm, 4.5 × 250 mm) using EDTA:MeCN:H_2O:HOAc (1:4000:6000:3) mixture as mobile phase, with a flow rate of 0.9 ml/min. Soyasaponin β g was detected by wavelength at 292 nm and hydrosylate compound (soyasaponin Bb) at 210 nm. The hydrolysis ratio of soyasaponin β g was determined by measuring the reduction in concentration of soyasaponin β g using the HPLC peak of the standard curve.

2.4. Measurement of Fe^{2+}-, Cu^{2+}- and Mn^{2+}-binding activity

Metal ion-binding activity was measured according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992) with minor modifications. Soyasaponin β g solution (2 ml) was added to 2 ml of 10 mM hexamine buffer containing 10 mM KCl and 2 mM $FeCl_2$, 0.1 mM $CuCl_2$ or 0.2 mM $MnCl_2$. After 1 h at room temperature, 0.2 ml of 1 mM tetramethyl murexide (TMM) was added. Metal-binding activity was determined as the decrease in the absorption ratio of A_{485}/A_{350} in the presence of soyasaponin β g (A_{350}, TMM A_{max}; A_{485}, TMM–metal complex A_{max}). Soyasaponin β g and buffer solutions were prepared with distilled water of HPLC grade (Nakarai Tesque, Japan) to avoid reaction with trace metal.

2.5. TLC analysis of soybean glycoside

TLC was conducted on a Kieselgel 60 F-254 plate (Merck Co. Ltd.) using $CHCl_3$:MeOH:H_2O (65:35:10, v/v lower layer). The components on the TLC plate were visualized by spraying with 1% cerium (IV) sulfate in 10% H_2SO_4 and heating at 110 °C for 20 min.

3. Results and discussion

3.1. Acidic precipitation of soyasaponin β g

Soybean glycosides and sugar compounds in the soy-waste after oil extraction were precipitated under acidic conditions of below pH 3. Sugar contents in the precipite analyzed by the Phenol–sulfuric acid and TLC methods suggested that the variation point was between pH 4 and 3 (Fig. 2). This result corresponded with the pH effect on the *n*-BuOH–H_2O partition of saponin (pH 3.3) (Shimoyamada, Okubo, Yoshikikoshi, Yoshiki, & Watanabe, 1995). The complete acidic precipitation of soybean glycosides required a reaction time of up to 12 h and acidic conditions under pH 2.

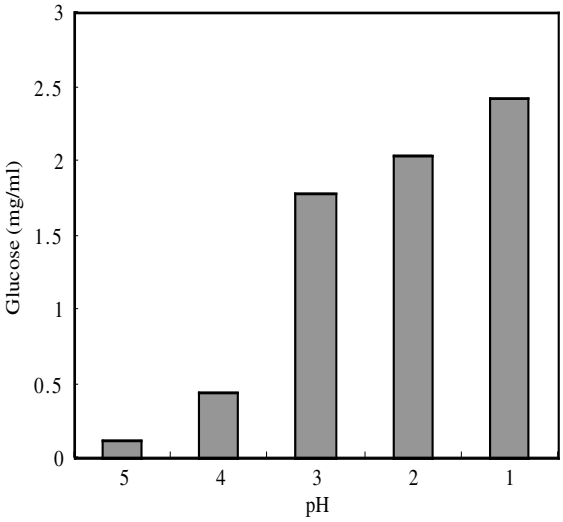

Fig. 2. Sugar contents in acid precipitation of soybean waste after oil extraction. Precipitation made from soybean waste by HCl titration. Total sugar contents was measured by the Phenol–sulfuric acid method in terms of glucose concentration (mg/ml).

3.2. Alkaline hydrolysis of soyasaponin β g

Soyasaponin β g was hydrolyzed into group B saponin and DDMP moieties under alkaline conditions. The content of soyasaponin β g was estimated by HPLC after NaOH hydrolysis (60 min), and the hydrolysis ratio was calculated as the ratio of the soyasaponin β g content to the total soyasaponin β g. Soyasaponin β g (1 mM) was decreased to 50% by 5 mM NaOH and was hydrolyzed to 98% by 20 mM NaOH (Fig. 3). One mM NaOH hydrolyzed soyasaponin β g at the rate of 0.44 μmol/min.

3.3. Metal ion-binding activity of soyasaponin β g

A large decrease of DDMP saponin occurred in the fractionation and isolation process using low grade reagent. Because the reduction of DDMP saponin was inhibited by some chelators, such as ethylenediaminetetraacetic acid (EDTA), metal ion in solvent might be related to the reduction of DDMP saponin. To study the reaction between DDMP saponin and metal ion, metal ion-binding activity of soyasaponin β g was measured.

Tetramethyl murexide (TMM) chelated with metal ion in the order: $Fe^{2+} > Cu^{2+} > Mn^{2+}$. The absorption

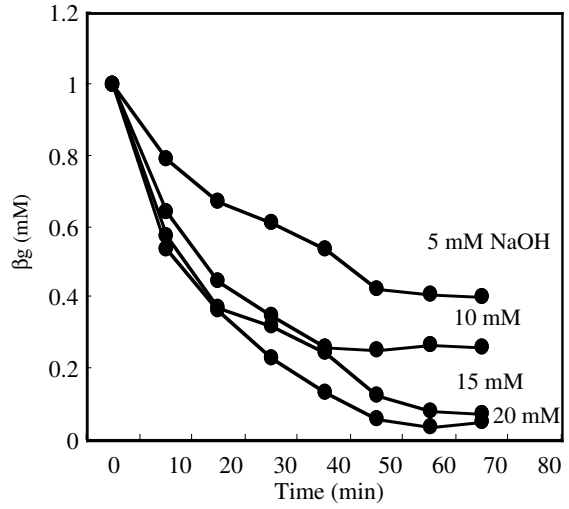

Fig. 3. Concentration dependence and time course of soyasaponin β g contents after NaOH hydrolysis. Soyasaponin β g contents in reaction mixture was analyzed by HPLC. HPLC condition was described in Section 2.3.

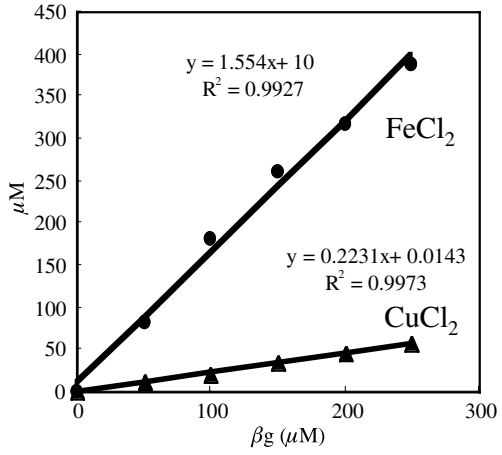

Fig. 4. Fe^{2+}- and Cu^{2+}-binding activities of soyasaponin β g.

ratio (A_{485}/A_{350}) increased linearly, depending on the metal ion concentration. For the metal-binding activity of soyasaponin β g, we used 2 mM $FeCl_2$, 0.1 mM $CuCl_2$ and 0.2 mM $MnCl_2$. Soyasaponin β g showed Fe^{2+}- and Cu^{2+}-binding abilities and increased linearly, depending on the concentration (50–250 μM) of soyasaponin β g (Fig. 4). Soyasaponin β g did not chelate with Mn^{2+}. High concentrations of soyasaponin β g (>150 μM) needed a reaction time to chelate with Cu^{2+} of up to 1 h in room temperature. Metal ion-binding activity of DDMP saponin would contribute to the antioxidative and reactive oxygen-scavenging activities of the soybean saponin fraction observed in vivo and in vitro (Nishida et al., 1993; Yoshiki & Okubo, 1995).

Finnegan, Lutz, Nelson, Smith, and Orvig (1987) reported the formation of a metal ion–maltol complex (aluminium–maltolate). Maltol is the stable form of the DDMP moiety after hydrolysis. Therefore, metal ion-binding activity of soyasaponin β g was considered to result in the DDMP moiety. We compared the metal chelating ratio of soyasaponin β g with that of soyasaponin Bb and maltol to study the partial structure resulting in the metal ion-binding activity of soyasaponin β g. Although soyasaponin Bb did not have any activity, 1 mM maltol chelated to 1.8 mM $FeCl_2$ and 0.8 mM $CuCl_2$. Soyasaponin β g (1 mM) could bind with 1.57 mM $FeCl_2$ and 0.2 mM $CuCl_2$, respectively. Since soyasaponin β g contained one DDMP moiety, the metal binding ratio of the DDMP moiety was lower than that of maltol. Kruck and McLachlan (1989) postulated an aluminium–maltolate complex, shielding the Al^{3+} positive charge with the hydrophilic part of maltol. Soyasaponin β g would form the metal–maltolate complex described by Kruck and McLachlan (1989). Lower metal-binding activity of soyasaponin β g than maltol suggested the prevention of an intramolecular interaction between the DDMP moiety and metal ion by the bulky and hydrophobic structure of soyasaponin β g.

3.4. Fractionation of soybean functional glycosides

Although DDMP saponin has some biological activities, such as reactive oxygen-scavenging acitivity, the unstable chemical structure of DDMP saponin suggests its inadequacy as a supply source of soybean functionality. We studied a high quality and simple fractionating method for soybean functional glycosides, focussed on Group B and E saponins, from DDMP saponin. Based on the chemical character of soyasaponin β g, the fractionating method for glycosides consisted of four steps: (1) precipitation of glycosides (2) separation of isoflavone (3) separation of DDMP saponin and (4) fractionation of group B and E saponins. We arrived at the fractionating procedure in Fig. 5.

Glycosides of soy-waste after oil extraction (industrial waste) were precipitated overnight by the addition of conc. HCl (pH 2). After supernatant removal, 10–20-fold EtOH volumes were added and the precipate was dissolved for about 2 h. The EtOH layer was mixed with 10 g/l of $FeCl_2$ at a ratio of 1:4 (v/v) according to the DDMP saponin contents and allowed to stand overnight. The supernatant was an isoflavone-rich source and precipitate was a DDMP saponin–Fe^{2+} complex-rich source. For the fractionation of isoflavone, *n*-BuOH was added to supernatant (1:1) and dispersed. The *n*-BuOH layer was washed twice with distilled water to remove hydrophilic impurities, such as oligosaccharides. This fraction contained daizin and glycitin at 76.2% purity (peak ratio by HPLC). For the fractionation of saponin, sufficient NaOH was added to the precipitate (light yellow) to reach pH 12–14 for 4 h to remove Fe^{2+} by

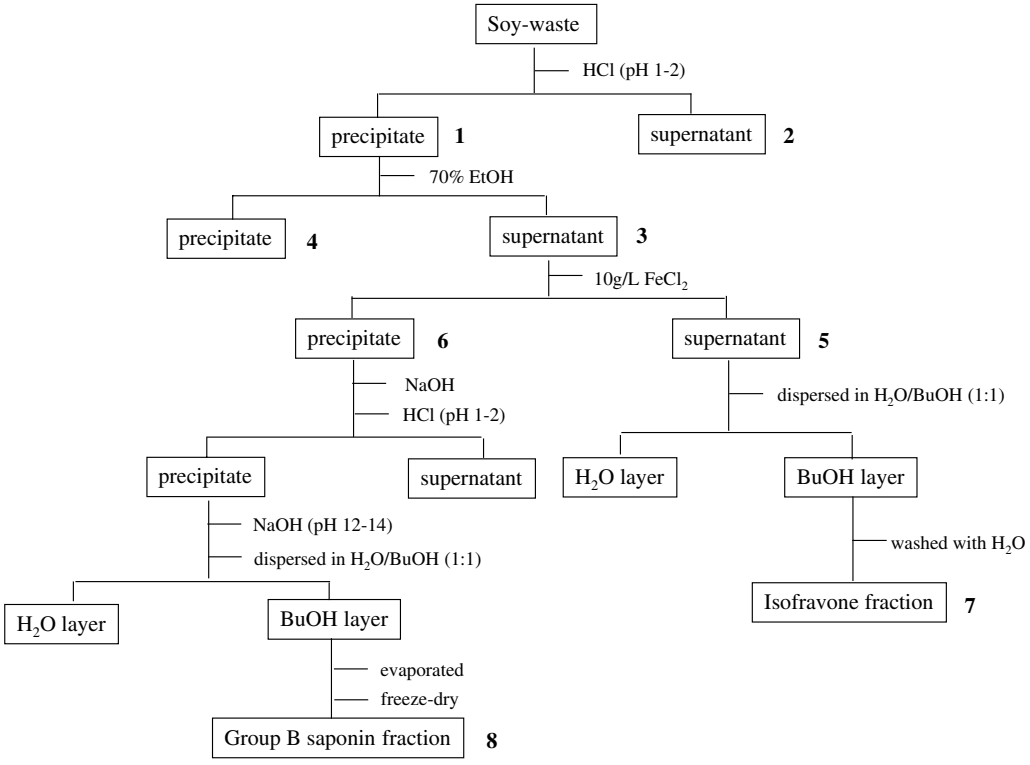

Fig. 5. Fractionating process for soybean functional glycosides based on the chemical characteristic of soyasaponin β g.

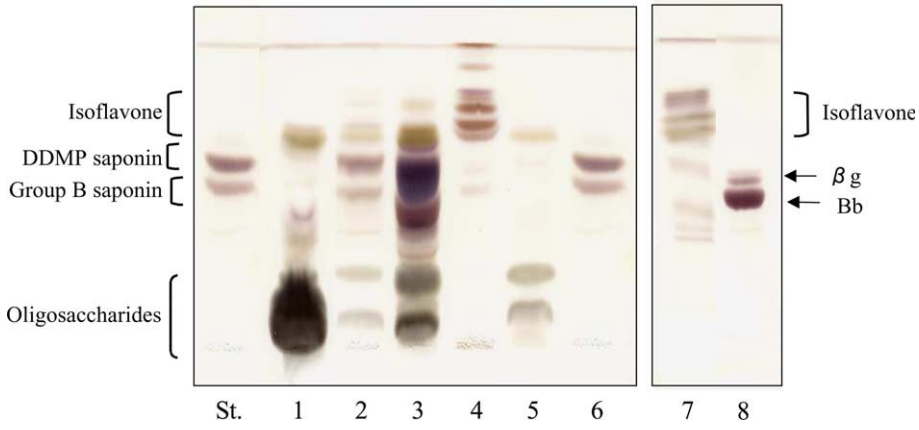

Fig. 6. TLC pattern of each step of fractionating process: St, soyasaponin β g and Bb mixture; 1, precipitate after acid precipitation; 2, supernatant after acid precipitation; 3, supernatant after EtOH addition; 4, precipitate after EtOH addition; 5, supernatant after $FeCl_2$ addition; 6, precipitate after $FeCl_2$ addition; 7, isoflavone fraction; 8, saponin fraction. Number of TLC band is correspondence with number in Fig. 5.

hydrolysis of the DDMP moiety. The alkaline solution of the saponin fraction was precipitated overnight by the addition of conc. HCl. Precipitation was controlled by NaOH at pH 12–14 again to check impurities. When the precipitate turns orange-brown (the colour of impurities) by alkaline conditions, it is necessary to increase the total volume with distilled water for the removal of impurities and to precipitate by HCl again. The HCl precipitate (white or light yellow) was dispersed in H_2O:*n*-BuOH (1:1) and stood overnight. The *n*-BuOH layer was evaporated and freeze-dried to obtain the final fraction of group B (>90%) and E (>10%) saponins. The compounds contained at each step were analyzed by TLC (Fig. 6). Through this fractionating method, 375 mg of the group B and E saponins fraction were obtained from 100 g of soy-waste after oil extraction. HPLC analysis showed 84% purity for group B saponin (Fig. 7).

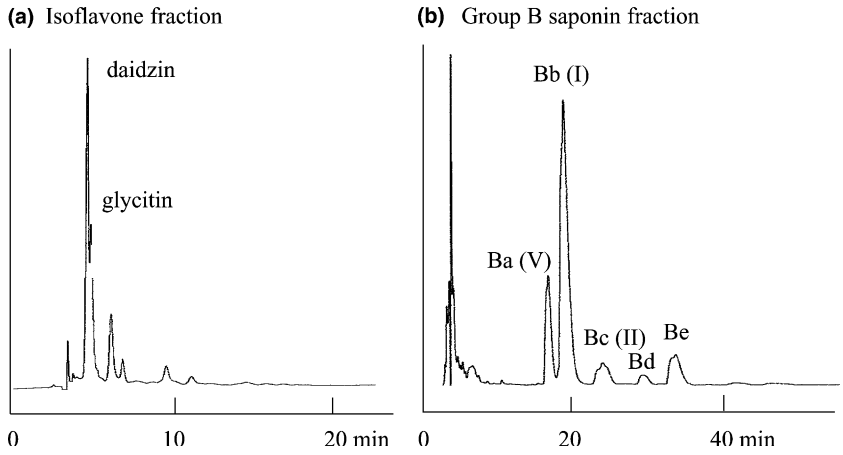

Fig. 7. HPLC patterns with UV detection chromatogram of isoflavone and saponin fractions. Isoflavone analyzed on reversed phase column using MeOH:H_2O:HOAc (4000:6000:3) mixtures as mobil phase, with a flow rate of 0.9 ml/min at 210 nm. Group B and E saponin analysis showed in "hydrolysate analysis of soyasaponin β g". Each compound estimated from retention time compared with that of isolated soybean glycoside. The nomenclature of soybean saponin were according to Okubo et al. and Kitagawa et al. (name in parenthesis) (Fenwick et al., 1991). Soyasaponin Bd and Be is ketone type saponin at C-22 of Ba and Bb, respectively (see Fig. 1).

4. Conclusion

Analytical techniques for soybean glycosides have been established by earlier investigation. However, a complex technique, accompanied with column and solvent selection is needed to fractionate soybean glycosides, due to their high amphiphilic potential. In this paper, we clarified some chemical characteristics of soyasaponin β g: (1) precipitation under an acidic conditions, (2) hydrolysis ratio under alkaline conditions and (3) metal-binding activity. Based on the chemical reaction of soyasaponin β g, we established a high quality and simple fractionating method for soybean functional glycosides consisting of four steps: (1) precipitation of glycosides, (2) separation of isoflavone, (3) chelate precipitation of DDMP saponin and (4) fractionation of group B and E saponins. According to this improved method, we could fractionate isoflavone (76.2% purity) and group B saponin (84% purity) from soy-waste after oil extraction. This fractionating method can be applied at the manufacturing level due to simplification by repetition of precipitation and filtration. In addition, this method would contribute to the reuse of the soybean industrial waste after oil extraction.

Acknowledgement

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