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A fragmentation database of soyasaponins by liquid chromatography with a photodiode array detector and tandem mass spectrometry

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Abstract: Oleanane-type triterpenoids exist as secondary metabolites in various plants. In particular, soyasaponin, an oleanane-type triterpenoid, is abundant in the hypocotyl of soybean, one of the most widely cultivated crops in the world. Depending on their chemical structure, soyasaponins are categorized as group A saponins or group DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) saponins. The different soyasaponin chemical structures present different health functionalities and taste characteristics. However, conventional phenotype screening of soybean requires a substantial amount of time for functionality of soyasaponins. Therefore, we attempted to use liquid chromatography with a photodiode array detector and tandem mass spectrometry (LC-PDA/MS/MS) for accurately predicting the phenotype and chemical structure of soyasaponins in the hypocotyl of five common soybean natural mutants. In this method, the aglycones (soyasapogenol A [SS-A] and soyasapogenol B [SS-B]) were detected after acid hydrolysis. These results indicated that the base peak and fragmentation differ depending on the chemical structure of soyasaponin with aglycone. Thus, a fragmentation database can help predict the chemical structure of soyasaponins in soyfoods and plants.

Key words: triterpenoid saponins, soyasaponins, soybean, LC-PDA/MS/MS

1. Introduction

Soyasaponins are a group of oleanane-type triterpenoids that are widely distributed among legume plants and are particularly abundant in soybean seeds.[1] Soyasaponins are complex molecules consisting of a non-sugar aglycone coupled to sugar chain units,[2] and are characterized by their surfactant properties, which typically yields stable, soap-like foams in aqueous solutions.

Group A saponins are bisdesmosides containing two sugar chains at the C-3 and C-22 hydroxyl positions of the soyasapogenol A (SS-A) glycoside. On the basis of the terminal sugar of the C-22 sugar chain of SS-A, group A saponins are further subdivided into Aa-, Ab-, and A0-series saponins. The A0 series saponins have no sugar at the terminal position of the C-22 sugar chain of SS-A glycoside, whereas the Aa and Ab series saponins have acetyl-xylose and acetyl-glucose, respectively, at the same position.

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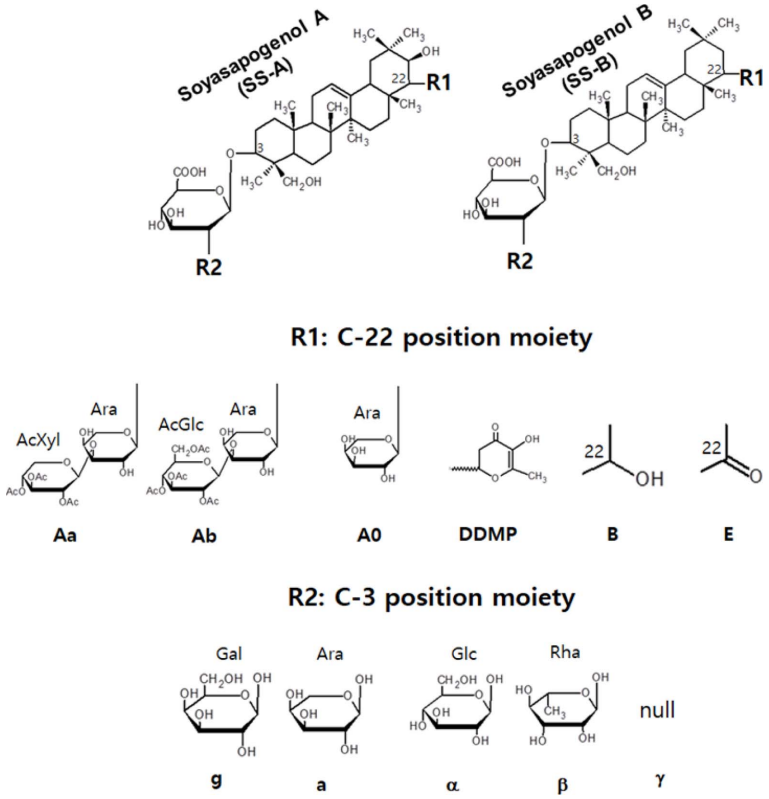

Fig. 1. Nomenclature of soyasaponins. R1: Aa, combination of arabinose (Ara) and acetyl xylose (AcXyl) sugars at the C-22 position of the SS-A glycoside; Ab, combination of Ara and acetyl glucose (AcGlc) sugars at the C-22 position of the SS-A glycoside; A0, only Ara sugar at the C-22 position of the SS-A glycoside; DDMP, 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one moiety; B, hydroxyl at the C-22 position of the SS-B glycoside; E, ketone at the C-22 position of the SS-B glycoside; R2, a combination of galactose (g), arabinose (a), glucose (a) and rhamnose (β) at the C-3 position of the SS-A and SS-B glycosides; null (γ), no sugar combination at the C-3 position of the SS-A and SS-B glycosides.

DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) saponins are monodesmosides with one sugar chain and one DDMP moiety at the C-3 and C-22 hydroxyl positions of the soyasapogenol B (SS-B) glycoside, respectively. DDMP saponins are degraded during most extraction procedures to form group B saponins, which include SS-B and group E saponins with the soyasapogenol E (SS-E) glycoside (*Fig.* 1).[3]

In soybeans, the sugar moieties of the SS-A glycoside in soyasaponins is controlled by combinations of seven alleles (*Sg-1*^{a}, *Sg-1*^{b}, *sg-1*^{0}, *Sg-3*, *sg-3*, *Sg-4* and *sg-4*) at three independent loci (*Sg-1*, *Sg-3*, and *Sg-4*).[4-9] However, the enzymes and genes that produce the acetylated form of group A saponins have not been identified. This acetylated form is known to be responsible for the undesirable taste in soybeans. Although natural mutants of soyasaponins have been used to identify the causative enzymes and genes, natural soyasaponin mutants without the acetyl group have not been found because of a paucity of natural mutants. Furthermore, ultraviolet (UV), infrared (IR) and nuclear magnetic resonance (NMR) analyses require more than 40 mg of purified sample and an extended amount of time to elucidate the chemical structure of soyasaponin.[10,11] Thus, there is a need for a technique that can efficiently predict the chemical structure of soyasaponins with a few soybeans.

In this study, we have shown a fragmentation database based on liquid chromatography with a photodiode array detector and a tandem mass spectrometry (LC-

PDA/MS/MS) can serve as an analytical tool for rapid screening and elucidation of soyasaponin chemical structures.

2. Experimental

2.1. Samples and reagents

We obtained samples of five varieties of common soybean (*Glycine max*), namely, "Enrei," "Ryuho," "Ibarakimame No. 7," "Norin No. 3," and "Kinusayaka." Soybean samples were provided by the National Institute of Crop Science, National Agriculture and Food Research Organization (NARO), Tsukuba, Japan. Acetonitrile (ACN), formic acid (FA), hydrochloric acid (HCl), and methanol (MeOH) were purchased from Sigma Chemical Co. (St Louis, MO, US). All solvents were HPLC and MS grade. HPLC-grade water (H_2O) was produced by a Milli-Q purification system (Millipore, Billerica, MA, US). Soyasaponin Bb ($C_{48}H_{78}O_{18}$, B-βg), soyasapogenol A ($C_{30}H_{50}O_4$, SS-A), and soyasapogenol B ($C_{30}H_{50}O_3$, SS-B) standard reagents were purchased from Sigma-Aldrich, Japan. The standard reagents were dissolved in 80 % MeOH (v/v) before LC–PDA/MS/MS analysis.

2.2. Extraction of soyasaponin from hypocotyls

Hypocotyls and cotyledons were separated from whole seeds. All samples were kept in airtight opaque plastic bottles until extraction. Hypocotyls were extracted with a fifty-fold volume (v/w) aqueous 80 % (v/v) methanol for 24 h at 25 ºC, respectively.

2.3. Hydrolysis of soyasaponin extracts

The soyasaponin extracts (55 μL) were added to 5 μL of HCl solution (final concentration, 1 *N*) for acid hydrolysis at 85 ºC for 3 h. The sample was analyzed by LC-PDA/MS/MS analysis. Then, acid hydrolytic stability of aglycone was evaluated by determining the concentration in the solution.

2.4. LC-PDA/MS/MS analysis

Each sample was applied to an ultrafast liquid chromatography system (Prominence UFLC system; Shimadzu, Kyoto, Japan) equipped with a PDA detector, a C30 reverse-phase column (Develosil C30-UG-3, 2.0 mm I.D. × 150 mm; Nomura Chemical, Seto, Okayama, Japan) at 40 °C, and a tandem mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific, Yokohama, Kanagawa, Japan). Solvent A consisted of acetonitrile containing 0.1 % (v/v) formic acid, and solvent B was a 0.1 % solution of formic acid in water. A linear gradient elution with solvent A was performed at a flow rate of 0.15 mL/min: Solvent A was started at 20 % (80 % solvent B, v/v) and was gradually increased to 65 % (v/v) over the course of 45 min, after which it immediately increased to 100 % (v/v) and was maintained there for 5 min. The eluent composition was then returned to the initial state with 20 % (v/v) solvent A and maintained for 15 min. The eluate from the column was monitored with the PDA detector using ultraviolet (UV) light at 205 nm and a tandem mass spectrometer in positive-ion mode using the electrospray ionization method. We used the automatic full-scan mode across the range of mass-to-charge ratios (m/z) from 300 to 2,000 and the automatic top-three-ion-trap mode with a parent mass list of 110 species of soybean saponins to acquire MS and MS/MS data, respectively. The automatic top-three-ion-trap mode is a dependent MS/MS analysis that is performed with collision-induced dissociation. Among the precursor ions derived from the full-scan mode of the MS, the 36 parent mass ions were first selected and other ions showing the next three highest intensities were used to acquire MS/MS data.

The LC-PDA/MS/MS analysis conditions for samples that had undergone acid hydrolysis were slightly modified. The mobile phases were 0.1 % (v/v) formic acid in water (A) and 0.1 % (v/v) formic acid in acetonitrile (B). The gradient program was as follows: solvent B was initiated at 60 % (v/v), increased to 95 % (v/v) for 35 min, and then increased to 100 % (v/v) for 5 min. The eluent composition was returned to the initial state of 60 % (v/v) solvent B for 15 min. The UV and MS spectra data were recorded and analyzed in Xcalibur software, version 2.1 (Thermo Fisher Scientific, Yokohama, Kanagawa, Japan).

3. Results and Discussion

3.1. Elucidation of soyasaponin chemical structures in seed hypocotyl extracts

The nomenclature of 36 types of soyasaponins was based on the combination of the three aglycone structures and the sugar moiety composition of each aglycone (*Table* 1). The conjugated sugar moiety at the C-3 position differed depending on the composition, and was specifically categorized as the αg, βg, γg, αa, βa, and γa types (*Fig*. 1).

The different soyasaponin chemical structures showed various health functionalities.[13] The soyasaponins of four varieties were classified by LC-PDA/MS/MS analysis. The results of UV detection by PDA at an absorbance of 205 nm are shown in *Fig*. 2(Left). The Aa types (Ryuho and Norin No. 3) mainly included Aa-αg, B-βg, DDMP-αg, and DDMP-βg saponins. The Ab types (Enrei and Ibarakimame No. 7) mainly included Ab-αg, B-βg, DDMP-αg, and DDMP-βg saponins. Furthermore, the A0 types (only Kinusayaka) mainly included A0-αg, B-βg, DDMP-αg, and DDMP-

Table 1. Soyasaponin nomenclature

C-3 position	C-22 position					
	Soyasapogenol A (SS-A)			Soyasapogenol B (SS-B)		
	Aa-series	Ab-series	A0-series	Group DDMP	Group B	Group E
	acetylXyl-	acetylGlc-	H-	-DDMP	-H	=O
β-**D**-glucosyl (1→2)-*O*-β-**D**-galactose-	Aa-αg (Aa*)	Ab-αg (Ab)	A0-αg	DDMP-αg (αg)	B-αg (Ba)	E-αg (Bd)
β-**L**-rhamnosyl-(1→2)-*O*-β-**D**-galactose-	Aa-βg (Au)	Ab-βg (Ac)	A0-βg	DDMP-βg (βg)	B-βg (Bb)	E-βg (Be)
β-**D**-galactose-	Aa-γg (Ae)	Ab-γg (Af)	A0-γg	DDMP-γg (γg)	B-γg (Bb')	E-γg (Be')
β-**D**-glucosyl-(1→2)-*O*-α-**L**-arabinose-	Aa-αa (Ax)	Ab-αa (Ad)	A0-αa	DDMP-αa (αa)	B-αa (Bx)	E-αa (Bf)
β-**L**-rhamnosyl-(1→2)-*O*-α-**L**-arabinose-	Aa-βa (Ay)	Ab-βa (Az)	A0-βa	DDMP-βa (βa)	B-βa (Bc)	E-βa (Bg)
α-**L**-arabinose-	Aa-γa (Ag)	Ab-γa (Ah)	A0-γa	DDMP-γa (γa)	B-γa (Bc')	E-γa (Bg')

*Previously reported soyasaponin nomenclature

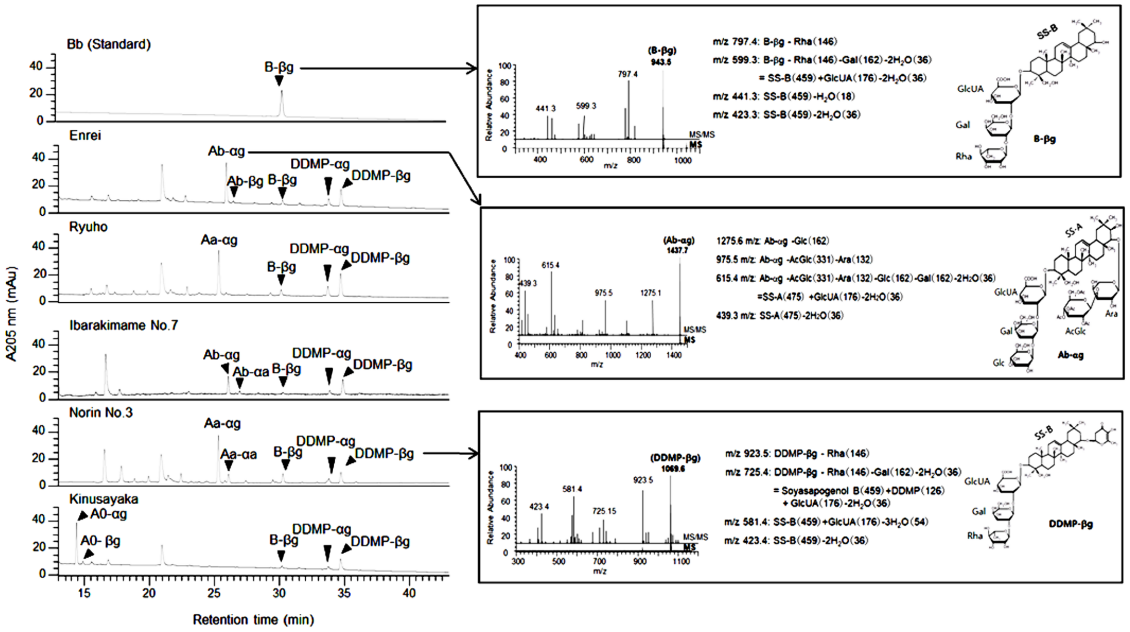

Fig. 2. Comparison of soyasaponins between standard (B-βg) and three samples in the hydrolysis extracts. Left: UV results, Right: soyasaponin chemical structures elucidated by MS/MS fragmentation.

Table 2. Soyasaponin components in the five varieties

Soyasaponin	Common soybean				
	Enrei	Ryuho	Ibarakimame No. 7	Norin No. 3	Kinusayaka
Group A	Ab-αg (26.0*) Ab-βg (26.4)	Aa-αg (25.4) Aa-αa (tr)	Ab-αg (26.0) Ab-αa (27.0)	Aa-αg (25.4) Aa-αa (26.2)	A0-αg (14.4) A0-βg (14.8)
Group B	B-αg (tr) B-βg (30.2)	B-αg (tr) B-βg (30.2)	B-αg (tr) B-βg (30.2)	B-αg (tr) B-βg (30.1)	B-αg (tr) B-βg (30.2)
Group DDMP	DDMP-αg (33.8) DDMP-βg (34.7)	DDMP-αg (33.8) DDMP-βg (34.7)	DDMP-αg (33.8) DDMP-βg (34.7)	DDMP-αg (33.8) DDMP-βg (34.7)	DDMP-αg (33.8) DDMP-βg (34.7)
Group E	E-αg (tr) E-βg (tr)	E-αg (tr) E-βg (tr)	E-αg (tr) E-βg (tr)	E-αg (tr) E-βg (tr)	E-αg (tr) E-βg (tr)

*Retention time
tr: detected by only MS analysis.

βg saponins. However, in all samples, B-αg, E-αg, and E-βg saponins were detected only in MS analysis (*Table* 2).

The MS and MS/MS fragmentation results are shown in *Fig*. 2(Right). In all cases, the mass error, expressed in terms of ppm for precursor ions, ranged from 0 to 3 ppm. The B-βg saponin standard showed an m/z 943.5 peak in MS, which usually contained the m/z 797.4 peak [B-βg – Rha (146)], m/z 599.3 peak [B-βg – Rha (146) – Gal (162) – $2H_2O$ (36) = SS-B (459.3) + GlcUA (176) – $2H_2O$ (36)], and the m/z 441.3 peak [SS-B (459.3) + GlcUA (176) – $2H_2O$ (36)] in MS/MS fragmentation. The Ab-αg saponin showed an m/z 1437.7 peak in MS, which usually contained the m/z 1275.6 peak [Ab-αg – Rha (146)], m/z 959.5 peak [Ab-αg – AcGlc (331) – Ara (132)], m/z 615.3 peak [Ab-αg – AcGlc (331) – Ara (132) – Rha (146) – Gal (162) – $2H_2O$ (36) = SS-A (475.3) + GlcUA (176) – $2H_2O$ (36)], and m/z 439.3 peak [SS-A (475.3) – $2H_2O$ (36)] in MS/MS fragmentation. The DDMP-βg saponin showed an m/z 1069.6 peak in MS, which usually contained the m/z 725.4 peak [DDMP-βg – Gal (162) – $2H_2O$ (36) = SS-B (459.3) + DDMP (126) + GlcUA (176) – $2H_2O$ (36)], m/z 581.3 peak [SS-B (459.3) + GlcUA (176) – $3H_2O$ (54)], and m/z 423.3 peak [SS-B (459.3) – $2H_2O$ (36)] in MS/MS fragmentation. The MS and MS/MS fragmentation results of all soyasaponins are shown in *Table* 3.

The results show that suggested that βg type saponin is detected as a base peak where rhamnose is desorbed from the molecular ion peak, and other saponins are detected by conjugated glucuronic acid at the C-3 position of aglycone. Therefore, each base peak varies depending on the aglycone (*Table* 4).

3.2. Analysis of soyasaponins in seed hypocotyls after acid hydrolysis

Representative samples of the Enrei, Ryuho and Kinusayaka varieties were hydrolyzed. The results of LC-PDA/MS/MS analysis with UV detection by PDA at 205 nm are shown in *Fig*. 3(Left). All three varieties of seed hypocotyls were hydrolyzed to SS-A and SS-B glycosides. Both group DDMP saponins and group E saponins changed to SS-B glycosides by hydrolysis. The effects of acid and base hydrolysis and environmental temperature on the generation of B-βg saponins have been reported previously.[11] The SS-A saponin standard showed an m/z 475.4 peak in MS, which usually contained an m/z 949.7 peak [2SS-A (948) + H(1)], m/z 497.3 peak [SS-A (474) + Na (23)], m/z 457.3 peak [SS-A (475) – H_2O (18)], m/z 439.3 peak [SS-A (475) – $2H_2O$ (36)], and m/z 421.3 peak [SS-A (475) – $3H_2O$ (54)] in MS/MS fragmentation. The SS-B saponin standard showed an m/z 459.4 peak in MS, which usually contained an m/z 949.7 peak [2SS-B (916) + H (1)], m/z 481.3 peak [SS-B (458) + Na (23)], m/z 441.3 peak [SS-B (459) – H_2O (18)], m/z 423.3 peak [SS-B (459) – $2H_2O$ (36)], and m/z 405.3 peak [SS-B (459) – $3H_2O$ (54)] in MS/MS fragmentation (*Fig*. 3, Right). These

Table 3. List of soyasaponins by MS and MS/MS fragmentation information

Soyasaponin	Molecular mass (M)	$[M+H]^+$ (m/z)	Product ion (m/z)	
			Base peak	Fragmentation
Aa-αg	1,364.624865	1,365.63	615.4	1203.7, 1023.5, 847.5, 439.4
Aa-βg	1,348.629950	1,349.64	1203.6	1091.6, 959.5, 813.4, 615.3, 439.3
Aa-γg	1,202.572040	1,203.58	615.4	1023.5, 847.5, 439.3
Aa-αa	1,334.614300	1,335.62	615.4	1173.5, 1023.5, 847.5, 439.3
Aa-βa	1,318.619385	1,319.63	615.4	1173.5, 1023.5, 847.5, 439.3
Aa-γa	1,172.561475	1,173.57	615.4	1023.5, 847.5, 439.3
Ab-αg	1,436.645995	1,437.65	615.4	1275.6, 975.5, 615.4, 439.3
Ab-βg	1,420.651080	1,421.66	1275.6	1095.5, 615.4, 439.3
Ab-γg	1,274.593170	1,275.60	615.4	1095.5, 919.5, 439.3
Ab-αa	1,406.635430	1,407.64	615.4	1245.6, 1095.5, 783.4, 439.3
Ab-βa	1,390.640515	1,391.65	615.4	1245.6, 1061.5, 439.3
Ab-γa	1,244.582605	1,245.59	615.4	1095.5, 915.5, 783.4, 439.3
A0-αg	1,106.550910	1,107.56	615.4	945.5, 439.3
A0-βg	1,090.555995	1,091.56	945.5	765.4, 615.4, 439.3
A0-γg	944.498085	945.51	615.4	765.4, 439.3
A0-αa	1,076.540345	1,077.55	615.4	945.5, 765.4, 439.3
A0-βa	1,060.545430	1,061.55	615.4	929.5, 765.4, 439.3
A0-γa	914.487520	915.49	615.4	783.5, 765.4, 439.3
DDMP-αg	1,084.545430	1,085.55	725.4	923.5, 581.4, 423.4
DDMP-βg	1,068.550515	1,069.56	923.5	725.4, 581.4, 423.4
DDMP-γg	922.492605	923.50	725.4	581.4, 423.4
DDMP-αa	1,054.534865	1,055.54	725.4	893.5, 581.4, 423.4
DDMP-βa	1,038.539950	1,039.55	725.4	893.5, 581.4, 423.4
DDMP-γa	892.482040	893.49	725.4	581.4, 567.4, 423.4
B-αg	958.513735	959.52	599.4	797.5, 423.4
B-βg	942.518820	943.53	797.5	599.4, 441.4, 423.4
B-γg	796.460910	797.47	599.4	617.4, 441.4, 423.4
B-αa	928.503170	929.51	599.4	617.4, 441.4, 423.4
B-βa	912.508255	913.52	599.4	617.4, 441.4, 423.4
B-γa	766.450345	767.46	599.4	617.4, 441.4, 423.4
E-αg	956.498085	957.51	795.5	615.4, 597.3, 439.4, 421.4
E-βg	940.503170	941.51	597.3	615.4, 795.5, 439.4, 421.4
E-γg	794.445260	795.45	795.5	597.3, 439.4, 421.4
E-αa	926.487520	927.49	795.5	615.4, 597.3, 439.4, 421.4
E-βa	910.492605	911.50	795.5	615.4, 597.3, 439.4, 421.4
E-γa	764.434695	765.44	795.5	615.4, 597.3, 439.4, 421.4

Table 4. List of aglycones by MS and MS/MS fragmentation information

Aglycone	Molecular mass (M)	$[M+H]^+$ (m/z)	Product ion (m/z)	
			Base peak	Fragmentation
Soyasapogenol A (SS-A)	474.370910	475.38	457.3	949.7, 497.3, 439.3, 421.3
Soyasapogenol B (SS-B)	458.375995	459.38	441.3	917.7, 481.3, 423.3, 405.3

results show that hydrolysis extracts the R1 residues from the structure without interfering at the C-3 position moiety. SS-B has been reported to possess hepato-protective, antiviral, anti-inflammatory, antimutagenic

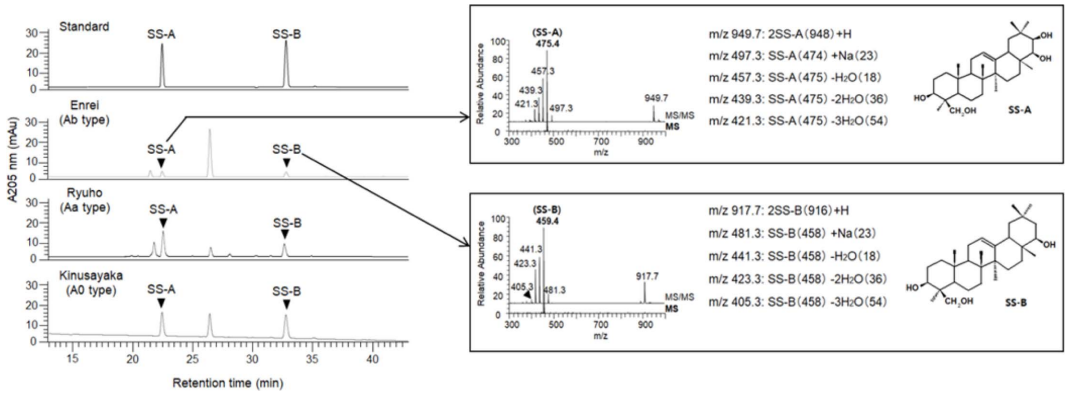

Fig. 3. Comparison of aglycones between standard (SS-A and SS-B) and three samples in the hydrolysis sample. Left: UV results, Right: Elucidated aglycone chemical structures by MS/MS fragmentation.

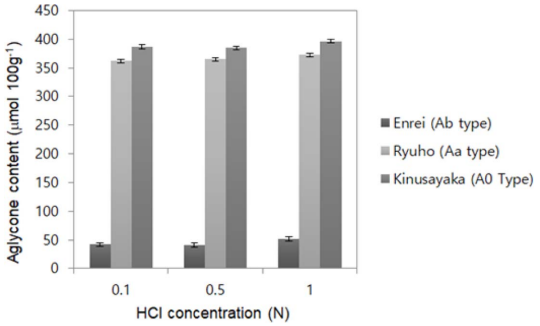

Fig. 4. Changes in the HCl concentration of the acid hydrolysis samples of the Enrei, Ryuho, and Kinusayaka variants, indicating acid hydrolytic stability. Error bars represent the standard deviation of three biological replicates.

and anticancer activities.[15] However, the bioactivities of SS-A have not been reported. The bioactivities of SS-A should be evaluated by purification using acid hydrolysis. We also investigated the hydrolytic stability of aglycones under various hydrolysis conditions ranging from 0.1 *N* HCl to 1 *N* HCl. The aglycone content after hydrolysis is shown in *Fig*. 4. The results indicated no change in the aglycone content in relation to the HCl concentration. In a previous study,[11] the dissociation of soyasaponin B-βg was maximal in hydrochloric acid. Triterpenoid saponins in nature are mainly conjugated with aglycone and sugar. However, their health functions differ depending on the structure of the aglycone. Thus, the aglycone plays an important role and determination of its chemical structure is important.

3.3. Method validation

The standards were diluted with a blank sample to produce standard working solutions of 1, 2, 5, 10, 20, 50, 80, and 100 mg/mL. Recovery analysis was investigated by spiking a standard solution of analytes into samples at concentrations of 50 and 100 mg/mL before processing. The relative standard deviation was determined by analyzing samples (n = 3) on the same day and on different days, which represents the repeatability and stability of the detection method.

4. Conclusions

Recently, natural mutants of common soybeans have been used to identify causative enzymes and genes, but the findings for acetylated group A saponins that cause bitter taste are incomplete. However, the natural mutants do not include soybeans without an acetyl group. So, one previously study[4] generated a mutant library using ethylmethanesulfonate (EMS) treatment. However, amino acid changes may randomly occur because of the EMS treatment. Therefore, the chemical structure of soyasaponins in more than 1000 soybeans needs to be evaluated for screening of phenotype. The fragmentation database used in this study efficiently provides information on the chemical structure of soyasaponins obtained by simple extraction, hydrolysis, and analysis method without time-consuming purification. We also provide a useful method for analysis of soyfoods and plants that are

difficult to purify.

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