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The occurrence of soyasaponin I in *Vigna radiata* L. (mungbean) sprouts as determined by fast atom bombardment, liquid secondary ion mass spectrometry, and linked scanning at constant B/E MS/MS

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Abstract. Mungbean (*Vigna radiata* L.) saponins were isolated from 7-day-old plants (Tainan 3, Tainan 5, Chai Ly, and a commercial cultivar), partially purified by 1-butanol extraction, dialysis, and semipreparative HPLC, with the samples that had been dialyzed being used for most of the mass spectrometry studies. Negative fast atom bombardment (FAB) was used to determine the HPLC elution pattern of soyasaponin I. Positive liquid secondary ion mass spectrometry (LSIMS), and linked scan at constant B/E MS/MS were used to determine the chemical structure of isolated soyasaponin I. Mixtures from each cultivar were shown by LSIMS to contain soyasaponin I as the predominant saponin. Linked scan at constant B/E mass spectra were helpful in confirming the soyasaponin I from a partially purified mixture.

Key words: B/E; Dialysis; Fast atom bombardment (FAB); HPLC; Linked scanning; Liquid secondary ion mass spectrometry (LSIMS); Mungbeans; MS/MS; Saponins; Soyasapogenol B; Soyasaponin I; TLC; *Vigna radiata* L.

Introduction

Saponins (of which those of mungbeans are an example) are mixtures of several triterpenoid (C_{30} pentacyclic) glycosides which upon hydrolysis yield pentose(s), hexose(s), uronic acid(s), and the aglycone, which is the non-sugar portion of the saponin. Saponins are biological detergents, and when agitated in water form a soapy lather which gives rise to the name of this group of compounds. This unique ability to cause foaming has been used by mankind throughout the centuries for making cleaning solutions, and indeed it can act as an aid in identification of plant extracts. Some saponins have hemolytic activity, cholesterol-reducing ability, bitterness, and can serve as allelochemicals; these behavioral properties are found in certain saponins rather than in all members of this chemical family. From the biological viewpoint saponins have a diverse group of properties, some deleterious but many beneficial. Their use in plant drugs, folk medicines, etc. has generated great interest in the chemical characterization of these molecules. This has been evident in the Orient (particularly Japan), where the literature on the isolation, purification, separation, structural elucidation, and biological activity of saponins attests to the

skill of natural-products biochemists and chemists (Kitagawa *et al.*, 1988).

Saponins have been reported in nearly 100 plant families (Price *et al.*, 1987). Most of the families are not commonly used for food or animal feed, but the few that are so used are of great importance. Saponin-containing plants used by humans for food, flavoring, or health tonics include soya (*Glycine max*), beans (*Phaseolus* spp., *Vicia* spp., and *Vigna* spp.), oats (*Avena sativa*), potatoes and eggplant (*Solanum* spp.), tomatoes (*Lycopersicon esculentum*), peanuts (*Arachis hypogaea*), red peppers (*Capsicum annuum*), ginseng (*Panex ginseng*), tea (*Camellia* spp.), fenugreek (*Trigonella foenum graecum*), and liquorice (*Glycyrrhiza glabra*). Saponin-containing plants used as animal feed or forage include alfalfa, lucerne (*Medicago* spp.), clover (*Trifolium* spp.), and sweet clover (*Melilotus* spp.).

Price *et al.* (1986) performed TLC on mungbean saponins and suggested that they are both mono- and bidesmodic, with a considerable number not identified. Hydrolysis produced soyasapogenols B and C and at least 5 other aglycones. Fast atom bombardment mass spectrometry was used by Price *et al.* (1988) to detect soyasaponin I and soyasaponin V in the crude saponin mixture of dried mungbeans (no mention was made of which commercial cultivar). Both of these known structures had previously been isolated from soyabeans by Kitagawa *et al.* (1988).

Mass spectrometry has been used extensively for saponin determination and structure confirmation. Sapogenins (saponin aglycones) have been studied by Budzikiewicz *et al.* (1963) using electron impact (EI) ionization. Hostettmann *et al.* (1981) utilized desorption/chemical ionization mass spectrometry for the structure determination of triterpenoid and spirostanol saponins. Schulten and Soldati (1982) applied field desorption mass spectrometry for the confirmation of saponins from *Gleditsia japonica*. Mostad and Doehl (1987) used liquid chromatography mass spectrometry (LC/MS) with chemical ionization (CI) for the characterization of sapogenins from *Gypsophila arrostii*. Massiot *et al.* (1988) used Californium plasma desorption mass spectrometry (^{252}Cf-PDMS) for structure elucidation of alfalfa root saponins. Numerous investigators such as Price *et al.* (1988), have used FAB for saponin determination.

Structure determination of saponins has also been accomplished by studying their fragmentation in the mass spectrometer using MS/MS techniques. Facino *et al.* (1987) studied saponins in raw plant extracts and Chen *et al.* (1987) examined steroidal oligoglycosides, both investigators using collisionally activated decomposition mass-analyzed ion kinetic energy (CADMIKES). Fraisse *et al.* (1986) also utilized this technique combined with FAB for triterpenic saponins with ester-glycosides or glycosylated tertiary alcohols. Crow *et al.* (1986) in addition to Tomer and Gross (1988) also used FAB combined with CAD MS/MS for structure determination of steroid glycosides on a triple analyzer mass spectrometer. Linked scanning at constant B/E has been used by Mil'grom *et al.* (1991) for the determination of metastable ions resulting from fragmentation of steroid sapogenins; however, there are no reports in the literature of the use of this technique on a 2-sector instrument for the structural determination of intact saponins in pure samples or crude mixtures.

Biological activities of mungbean saponins as well as most of their chemical structures are unknown (Fenwick and Oakenfull, 1983; Price *et al.*, 1986, 1987, 1988; Waller *et al.*, 1993a, b; Oleszek and Jurzysta, 1987; and Oleszek *et al.*, 1992) reported that the non-cholesterol-precipitable fraction of alfalfa (*Medicago sativa* L.) root saponins that produced soyasapogenol B upon hydrolysis [probably containing soyasaponin I as did the alfalfa seed saponins (Waller *et al.*, 1993c)], showed a slight inhibition of wheat seedling growth. By comparison, the cholesterol-precipitable fraction (mostly medicagenic acid glycosides) showed a 2-3-fold inhibition of wheat seedling growth. Mungbean allelopathic activity has not otherwise been detected before, but preliminary reports have been given (Waller *et al.*, 1993a b).

This research was designed to improve knowledge and understanding of the chemical structures of naturally occurring mixtures of mungbean saponins with particular emphasis on the predominant saponin. A preliminary report has been published (Waller *et al.*, 1993 a).

Materials and Methods

Mungbean Plant Material

Vigna radiata L (Wilzek) cv. Tainan No. 3, Tainan No. 5, and Chai Ly (local) seeds were obtained from Tainan District Agricultural Improvement Station, Tainan, Taiwan; and the commercial variety was

obtained locally from a Chinese market.

Mungbean Preparation

Mungbean seeds (Tainan No. 3, Tainan No. 5 and Chai Ly) were set to germinate in Pyrex rectangular dishes with distilled water in the laboratory. The vitexins which are continuously produced by young seedlings (Tang and Zhang, 1986) as well as other undesirable compounds were removed by decanting daily, and fresh distilled water was added throughout the seven day incubation to provide mungbean sprouts. Commercial mungbean sprouts were bought locally from a Chinese market.

Drying and Grinding of Mungbean Sprouts

All laboratory grown samples were either air-dried or dried in a 40°C forced-draft oven. The commercial mungbean samples were dried in a 70°C oven. All material was ground to a fine powder with a grinder made by Ye Shin Iron Factory, Taoyuan, Taiwan.

Chemical Reagents

All solvents used were, except where noted, from E. Merck, Darmstadt, Germany and were HPLC grade. The Lieberman-Burchard spray reagent was prepared according to the method of Tschesche *et al.* (1961). A highly purified sample of soyasaponin I was made available through the courtesy of Professor Marian Jurzysta.

Extraction and Partial Purification

Experiments employed distilled water or methanol to extract the saponins by homogenization in a Waring blender (Waring Products Division, New Hartford (CN, USA). The mixtures were refluxed for 4 h and filtered, the filtrate was reduced in volume using a rotary evaporator, extracted three times with 1-butanol saturated with water to remove the saponins, and the 1-butanol fraction rotary evaporated to dryness. This fraction contains crude mungbean saponins, as analyzed by TLC with Lieberman-Burchard spray detection (not shown). This crude fraction was dissolved in 200-300 ml methanol and the saponins were precipitated with 1500 ml ethyl ether or acetone, centrifuged out for 15 min. at 5,000 rpm, partly dissolved in water (turbid solution), and subjected to dialysis for 5 h. using Spectra Por 3,500 and 6,000-8,000 molecular weight cut-off tubing (Spectrum Medical Industries, Inc., Los Angeles, CA, USA) and lyophilized as described by Massiot *et al.* (1991). The crude saponins were dissolved in 60%-90% aqueous methanol, the solution filtered through 0.45-nm Teflon or nylon or polyamide difluoride, (Gelman Science, Inc., Ann Arbor, MI, USA) and placed on a semipreparative HPLC column.

Isolation of Saponins by Semipreparative LC

A modification of the procedure by Oleszek *et al.* (1990) was used. The methanolic solutions or the eluents from the RP-18 column were loaded on a Hyperprep 120 ODS, 12 μm, 250 mm × 20 mm column with a Hyperprep Guard, 15 μm, 50 mm × 20 mm (Keystone Scientific Co., Inc., Bellefonte, PA) at a variable flow rate of 5-9 ml/min, processed by a LDC/Milton Roy Constametric Pump. No. 4100; and a

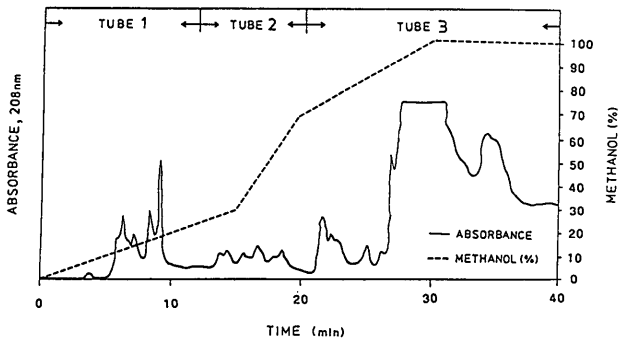

Fig. 1. High Performance Liquid Chromatogram of saponins from *Vigna radiata*, cv Tainan 5 sprouts using the semipreparative column; 15 mg/ml inj. flow rate 5 ml/min; 208 nm.

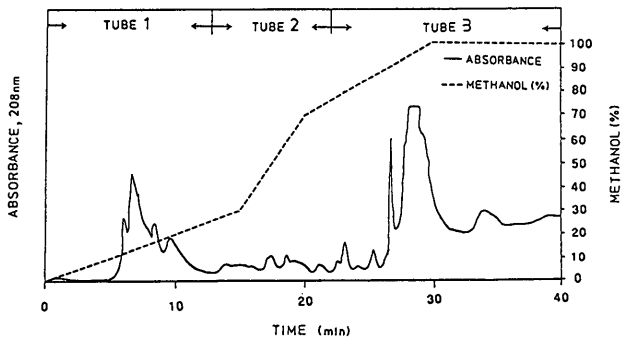

Fig. 2. High Performance Liquid Chromatogram of saponins from *Vigna radiata*, cv Chai Ly sprouts using the semipreparative column; 15 mg/ml inj. flow rate 5 ml/min; 208 nm.

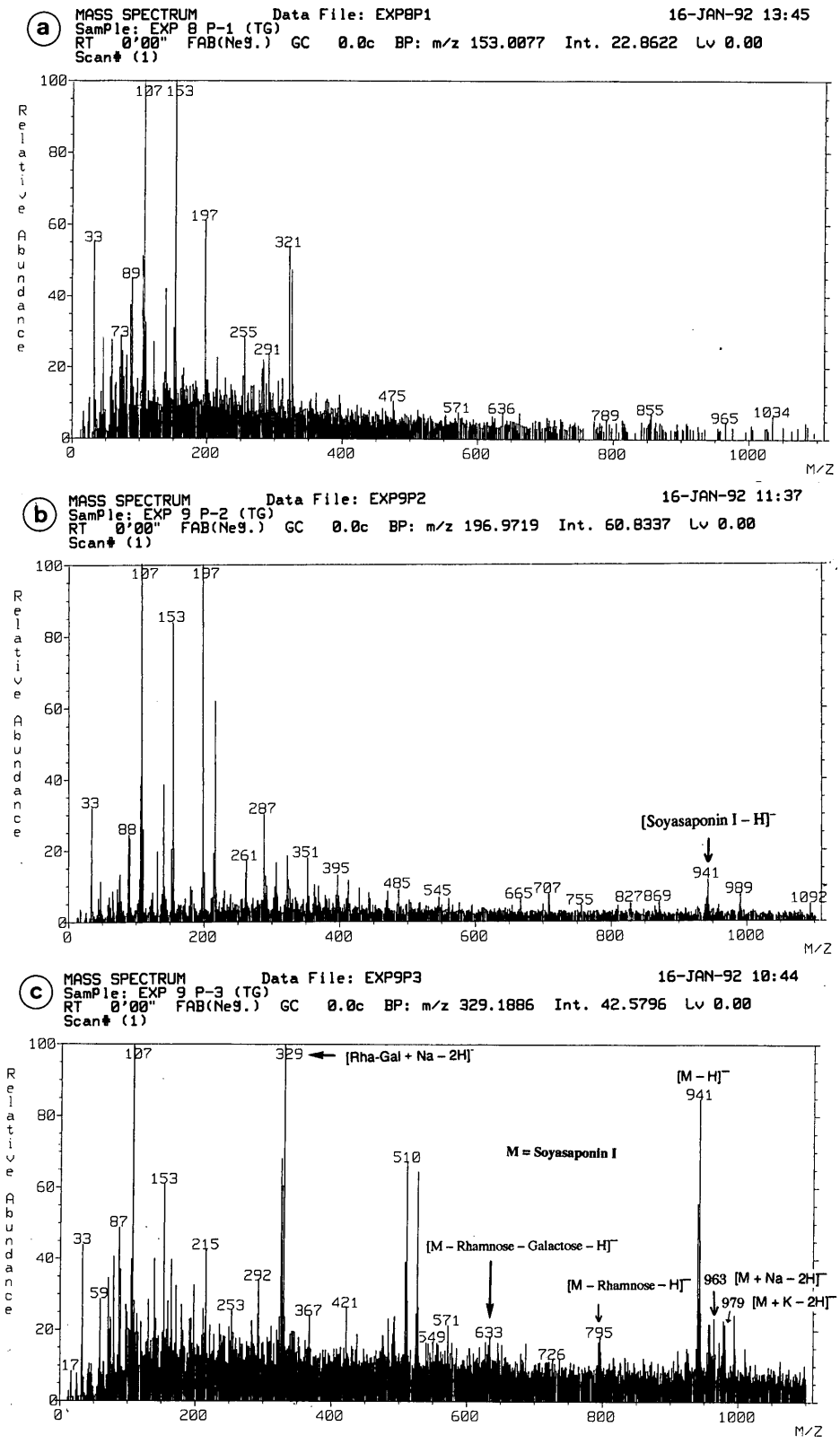

Fig. 3. Negative fast atom bombardment of samples from three tubes collected from the semipreparative HPLC. a) tube 1 (0-11 min), b) tube 2 (12-19 min), and c) tube 3 (20-40 min).

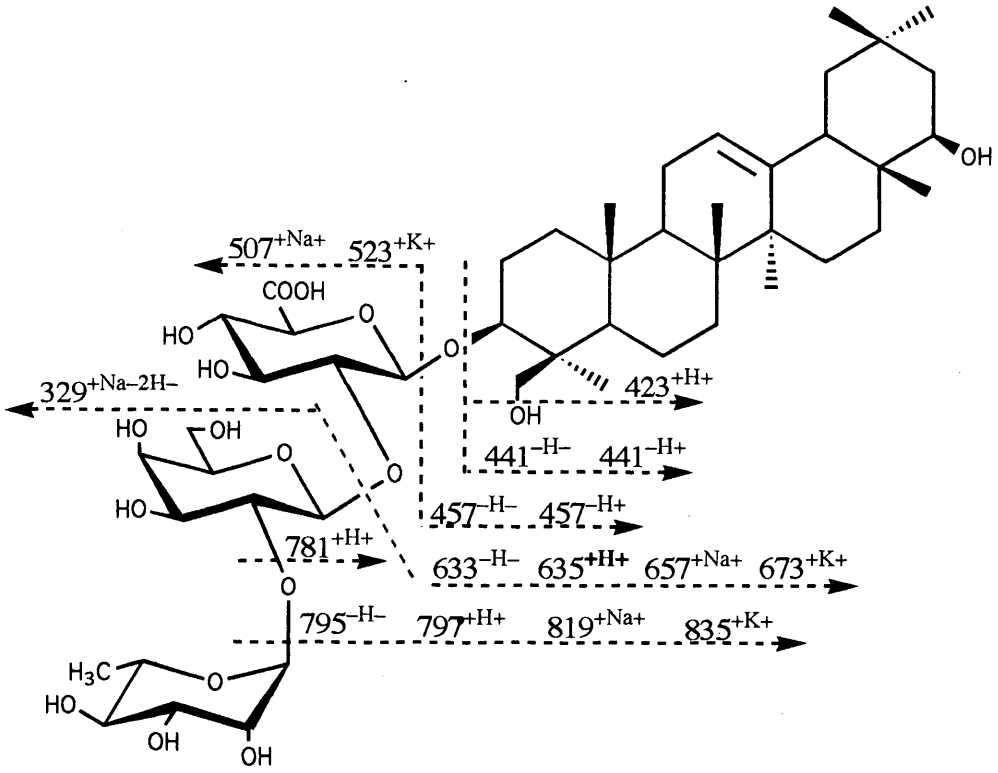

Fig. 4. Fragmentation pattern of soyasaponin I consistent with m/z values observed in FAB and LSI mass spectra. Charge retention is indicated by the direction of the arrow and m/z values for fragments (F) are shown with superscripts indicating adduct and charge as follows: $(F+H)^+ = m/z^{+H+}$; $(F-H)^- = m/z^{-H-}$; $(F-H)^- = m/z^{-H+}$; $(F+Na)^+ = m/z^{+Na+}$; $(F+K)^+ = m/z^{+K+}$; $(F+Na-2H)^- = m/z^{+Na-2H-}$

SpectroMonitor 3000 (Riviera Beach, FL, USA). Programs were developed for a 2-component solvent system (60 - 100% methanol and distilled water), and as the compounds emerged from the column peaks were read at wavelength 208 or 210 nm, and the fractions collected.

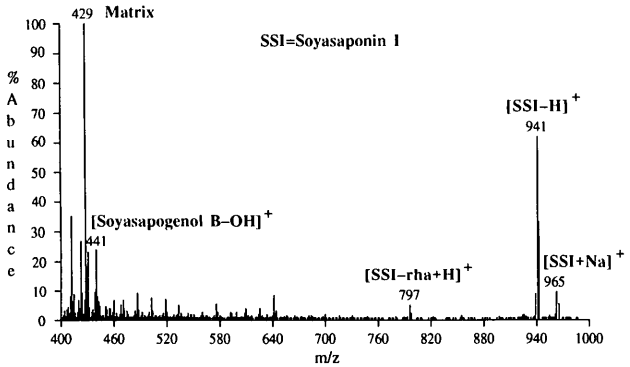

Fig. 5. Liquid secondary ion mass spectrum of pure soyasaponin I.

Mass Spectrometric Analysis

Negative fast atom bombardment (FAB) was performed on a Jeol Model HX-110 mass spectrometer, and 30 μg samples were dissolved in a thioglycerol matrix. Liquid Secondary Ion Mass Spectrometry studies were performed on a VG ZAB2-SE mass spectrometer using a 35-keV Cs^+ primary ion beam to bombard the sample/matrix. Typically, a 20-μg sample was dissolved in either a thioglycerol or 1:1 thioglycerol/glycerol matrix. 0.1M NaCl or 0.1M KCl was added to the matrix when intensification of a particular alkali cation adduct peak was desired.

Linked scan at constant B/E LSIMS/MS spectra were obtained for daughter ions produced by collisionally activated dissociation (CAD) of the selected parent ion, using helium or argon as the collision gas for the $(M+H)^+$ or the $(M+Na)^+$ parent ion, respectively. Collision cell pressure was typically 1×10^{-6} mbar, resulting in approximately a 50% reduction of the parent ion peak intensity. Daughter ion spectra were acquired by the simultaneous computer-

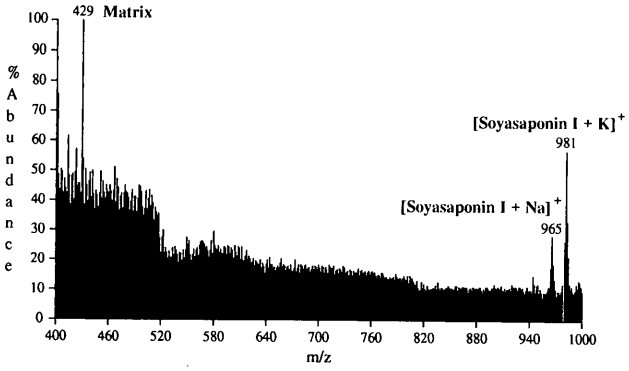

Fig. 6. Liquid secondary ion mass spectrum of *Vigna radiata* (mungbean) cv Tainan 5 sprout saponins after 5-h dialysis.

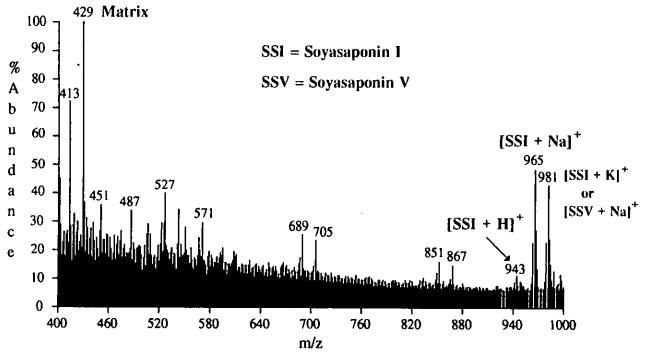

Fig. 7. Liquid secondary ion mass spectrum of *Vigna radiata* (mungbean) cv Chai Ly sprout saponins after 5-h dialysis.

controlled scanning of the magnetic field (B) and the electric sector field (E), while maintaining a constant B/E ratio.

All LSIMS mass spectral figures shown were drawn and labeled on a Macintosh computer using SpectraGraph©, an original shareware program available from West and Mort (1993).

Results and Discussion

A complete analytical process will be described for saponins. The process discussed below is only for mass spectrometry that used the products of the 5 h dialysis as a crude preparation and that of the semipreparative HPLC for determining when the mungbean saponins eluted from the column.

Semipreparative Liquid Chromatography

Figures 1 and 2 are for Tainan 5 and Chai Ly mungbean sprouts saponins processed through the 5-h dialysis procedure. Three fractions were collected: tube 1 (0-11 min), tube 2 (12-19 min), and tube 3 (20-40 min) for analysis. The appearance time of 19-38 min corresponded to the appearance of mungbean saponins according to mass spectrometry. The minute amount of soyasaponin I that appeared in tube 2 was the result of over-loading the column. Tainan 5 and the commercial samples gave similar patterns on HPLC: they were fractionated into 3 tubes with the same retention times.

Negative Fast Atom Bombardment Mass Spectrometry (FAB-MS)

Negative ion FAB mass spectrometry was applied to each of the fractions obtained by HPLC shown in Figs. 1 and 2. The absence of a peak at m/z 941 $[soyasaponin\ I\text{-}H]^-$ in the FAB mass spectrum shown in Figure. 3a indicated that soyasaponin I (MW = 942) was completely absent in fraction I, however a peak at m/z 941 in the spectrum shown in Fig. 3b indicated that a minute amount of soyasaponin I was present in fraction II. Other peaks indicated that some undetermined compounds were also present in fraction II. The FAB mass spectrum shown in Fig. 3c showed a prominent peak at m/z 941 as well as peaks at m/z 963 and 979 indicating the $[M\text{-}H]^-$, $[M+Na\text{-}H]^-$ and $[M+K\text{-}2H]^-$ pseudomolecular ions of soyasaponin I, respectively. Several soyasaponin I fragment ions were also present as labeled on the spectrum. This gave sufficient evidence that fraction III contained soyasaponin I as the predominant mungbean saponin. Figure 4 shows the m/z values observed in various FAB and LSI mass spectra which are consistent with this proposed scheme for the fragmentation of soyasaponin I. Most fragment ions shown in Fig. 4 result from the cleavage of glycosidic bonds with the corresponding cation adduct present as a result of the ionization charge mode and/or presence of sodium or potassium matrix additives. Fraction II and III contained several unknowns that possessed molecular weights above 1050, but these were not studied further. This mass spectrometric investigation showed that as a result of the breeding program for obtaining higher yields and resistance to disease and insect damage at the Asian Vegetable Research and Development Center, the minor saponins may have been lost, however, this had little effect on the occurrence of soyasaponin I. The biological activities of the

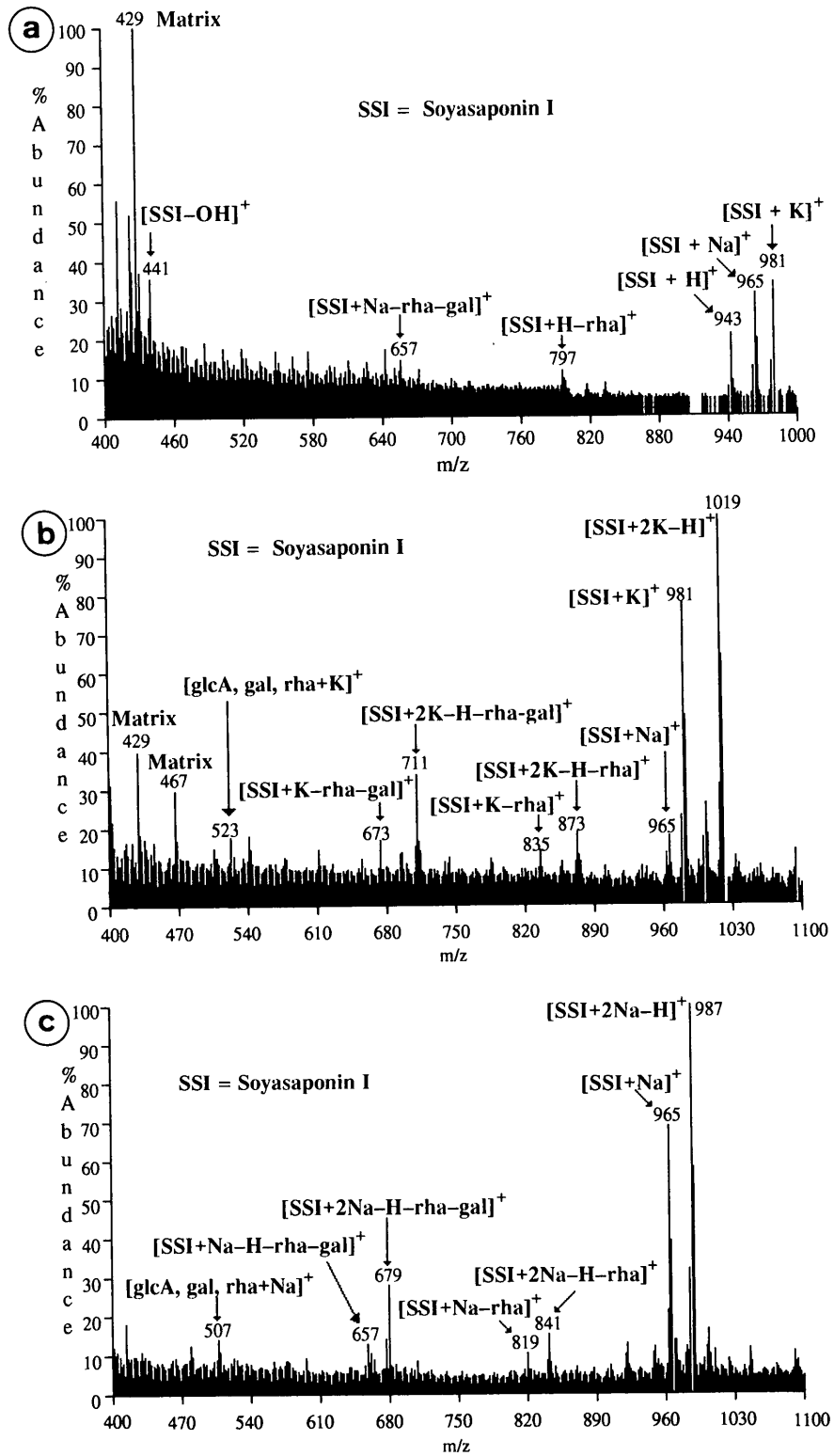

Fig. 8. Liquid secondary ion mass spectrum of *Vigna radiata* commercial cultivar sprout saponins after 5-h dialysis. a) In thioglycerol matrix; b) in thioglycerol and 0.1 N KCl matrix; c) in thioglycerol and 0.1 N NaCl matrix.

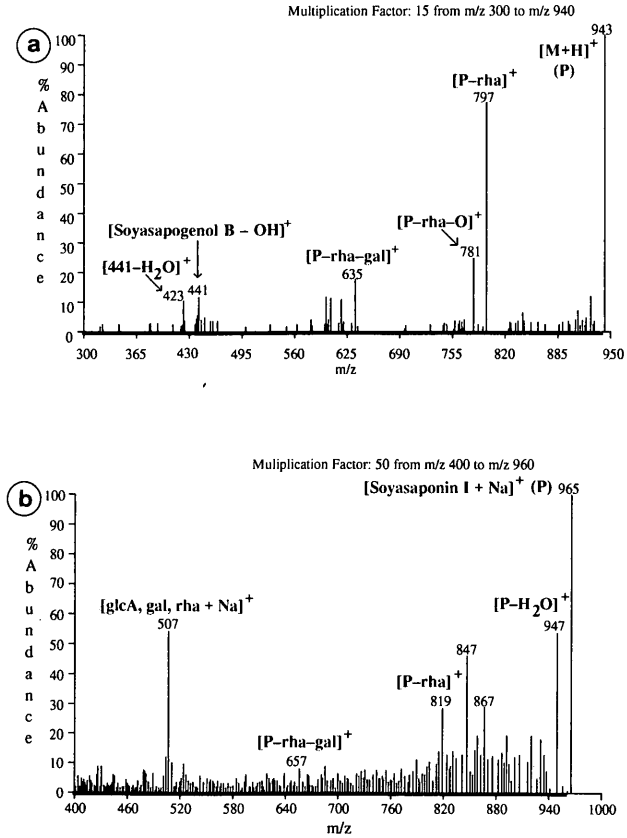

Fig. 9. Linked scan at constant B/E LSIMS/MS spectra of standard soyasaponin I. Daughter ions resulting from a) the fragmentation of the $[M+H]^+$ parent ion (P) and b) the fragmentation of the $[M+Na]^+$ parent ion. Results are consistent with the scheme shown in Fig. 4.

minor saponins are not known.

Liquid Secondary Ion Mass Spectrometry (LSIMS)

The chemical structure of soyasaponin I is 3-O-[α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-soyasapogenol B. It is not possible to determine individual sugar identities, linkages, or the α or β isomers, however the mass spectra are very useful for structure confirmation in conjunction with other methods. The LSIM spectrum of soyasaponin I standard is shown in Figure 5. The peak assignments for soyasaponin I (MW = 942) are as follows: $[M-H]^+ = m/z$ 941; $[M+Na]^+ = m/z$ 965. A peak consistent with the loss of rhamnose from the molecular ion is shown at m/z 797. The peak at m/z 441 corresponds to the loss of OH from aglycone (soyasapogenol B). The base peak at m/z 429 is due to the thioglycerol matrix.

LSIM spectra of the Tainan 5, Chai Ly and commercial preparation saponins, all partially purified by 5-h dialysis are shown in Figures 6-8. All three spectra confirm that soyasaponin I is the predominant saponin in the mixture but also indicate the possible presence of unknown saponins. The peak at m/z 429 in all three figures is due to the thioglycerol matrix. Figure 6 shows the spectrum from Tainan 5, indicating soyasaponin I $[M+Na]^+$ at m/z 965 and $[M+K]^+$ at m/z 981. Figure 7 shows the spectrum of the Chai Ly saponins. Identifiable peaks include soyasaponin I $[M+H]^+$ at m/z 943, $[M+Na]^+$ at m/z 965 and $[M+K]^+$ at m/z 981. The possible presence of soyasaponin V is indicated by the peaks at m/z 981 in Figures 6 and 7, corresponding to soyasaponin V $[M+Na]^+$. Other saponins of higher molecular weights were also observed but these were not studied further.

Soyasaponin I $[M+H]^+$ and $[M+Na]^+$ are shown at m/z 943 and m/z 965 in Figure 8a, the LSIM spectrum for the commercial cultivar. It is also possible to identify some of the peaks consistent with the loss of the sugar moieties from soyasaponin I. Peaks indicating these losses include $[M+H\text{-rhamnose}]^+$ at m/z 797 and $[M+Na\text{-rhamnose-galactose}]^+$ at m/z 657. The peak at m/z 441 corresponds to the loss of OH from the aglycone (soyasapogenol B).

This same sample was dissolved in a thioglycerol matrix containing 0.1 M KCl in order to help identify the peaks resulting from potassium adducts. This spectrum (Fig. 8b) showed much more intense peaks at m/z 981 and m/z 1019, resulting from soyasaponin I $[M+K]^+$ and $[M+2K-H]^+$. Many other structurally significant peaks were also present in the spectrum shown in Figure 8b and are labeled accordingly. Peaks at m/z 429 and m/z 467 are due to the matrix.

The dramatically increased intensity of the m/z 981 peak in Figure 8b, resulting from the addition of K^+, helps to rule out the possibility of the presence of soyasaponin V $[M+Na]^+$ as previously postulated from the spectra shown in Figures 6 and 7. This absence of soyasaponin V is also confirmed by the spectrum of the commercial cultivar shown in Figure 8c, resulting from the addition of Na^+ to the matrix . This spectrum does not show a large increase in intensity for the peak at m/z 981 as would be expected with the presence of soyasaponin V, but does show increased intensity for soyasaponin I $[M+Na]^+$ at m/z 965 and

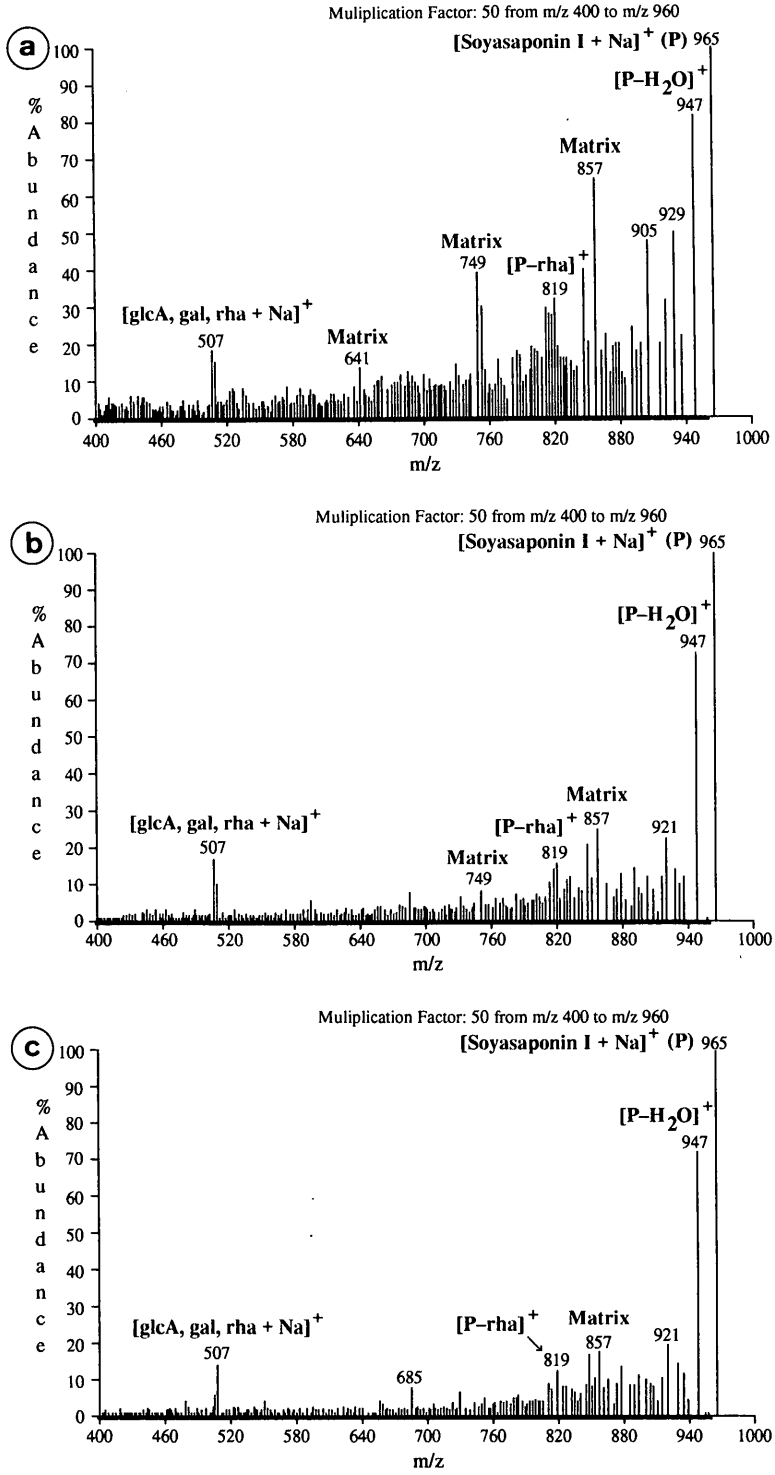

Fig. 10. Linked scan at constant B/E LSIMS/MS spectra of a) *Vigna radiata* (mungbean) cv Tainan 5 sprout, b) *Vigna radiata* cv Chai Ly sprout, and c) *Vigna radiata* commercial cv sprout saponins, each after 5 h dialysis. Daughter ions shown result from fragmentation of the $[M+Na]^+$ parent ion (P) for each cultivar. Results are consistent with the scheme shown in Fig. 4 and with the daughter ion spectrum of soyasaponin I standard in Fig. 9b.

$[M+2Na-H]^+$ at m/z 987. It is clear that LSIMS is very useful for determining the predominance of soyasaponin I in partially purified saponin samples, particularly when specific cation adduct peaks are intensified by the addition of NaCl or KCl to the LSIMS matrix.

Linked Scanning at Constant B/E MS/MS

LSIM spectra of compounds such as saponins typically give limited information about the fragmentation of the molecular species. Some peaks in LSIM spectra can be misleading, seemingly resulting from fragmentation of the molecular species but in reality often resulting from other independent species in the mixture. In order to confirm the presence of soyasaponin-I in mungbean sprouts, additional structural information and confirmation was obtained by performing linked scanning at constant B/E LSIMS/MS. This technique involves obtaining a mass spectrum of daughter ions produced by the collisionally activated dissociation (CAD) of a selected parent ion with a collision gas. CAD occurs in a collision cell in the first field-free region of a reverse geometry (BE) mass spectrometer. A daughter ion spectrum is then obtained by the simultaneous computer-controlled scanning of the magnetic field (B) and the electric sector field (E), while maintaining a constant B/E ratio. Peaks in the daughter ion spectrum result directly from fragmentation of the parent ion, and ions from other species are usually not present. Daughter ions resulting from fragmentation of a matrix ion of the same mass as the parent ion are occasionally present in the spectrum.

The linked scan at constant B/E LSIMS/MS spectrum of standard soyasaponin I is represented in Figures 9a and b. Figure 9a shows the following daughter ions resulting from the fragmentation of the soyasaponin I $[M+H]^+$ parent ion (P) at m/z 943: the loss of rhamnose at m/z 797 and m/z 781, indicating fragmentation on both sides of the oxygen in the glycosidic linkage between rhamnose and galactose; the additional loss of galactose at m/z 635; the aglycone minus a hydroxyl ([soyasapogenol B-OH]$^+$) at m/z 441 and [soyasapogenol B-OH-H_2O]$^+$ at m/z 423. Due to the predominance of sodium adduct peaks in the LSIM spectra, additional linked scan at constant B/E LSIMS/MS experiments were performed on the $[M+Na]^+$ parent ion of the soyasaponin I standard. Figure 9b shows the loss of rhamnose at m/z 819 and the additional loss of galactose at m/z 657. The prominent fragment ion at m/z 507 results from the sodium adduct ion of the entire sugar side chain. This also indicates that the sugar chain is probably the location of the sodium attachment to soyasaponin I.

The linked scan at constant B/E LSIMS/MS daughter spectra of the Tainan 5, Chai Ly, and commercial preparation saponins $[M+Na]^+$ parent ions are shown in Fig. 10a-c. These results closely agree with those shown above for the soyasaponin I standard, but due to the lower intensity of the parent ion compared to that of the standard, daughter ions resulting from fragmentation of the matrix ion at the same m/z (965) as the soyasaponin I parent ion are more evident. The prominent fragment ions are labeled on each spectrum. These results confirm and extend previous unpublished linked scan at constant B/E LSIMS/MS preliminary results obtained by G. R. Waller and J. C. H. Kuei, and N. N. Lai which show the ability of this technique to confirm the presence and structure of soyasaponin I in a saponin sample.

Soyasaponin I was found to be the dominant saponin in Tainan 3, Tainan 5, Chai Ly, and commercial mungbean cultivars. This research establishes that FAB, LSIMS, and linked scan at constant B/E MS/MS are very powerful tools for determining the occurrence of soyasaponin I in partially purified saponins from different cultivars of mungbeans. After more purification, soyasaponin V as well as unknown saponins may exist in high enough concentrations to permit their determination by these mass spectrometric methods.

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以快速原子衝擊，液態次階離子及連續掃描在固定的電磁域及電子域的質譜儀鑑定綠豆芽中之皂苷 Soyasaponin I

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以台南 3 號，台南 5 號，在來及市面上的綠豆生長七天後的綠豆芽，以正丁醇(1-butanol)萃取其皂苷，經透析並以製備性高效能液態色層儀(HPLC)及質譜儀之分析以初步鑑定未純化之皂苷。經以負快速原子衝擊(Negative fast atom bombardment, 簡稱 FAB)分析自 HPLC 分離出的 Soyasaponin I。再以正液態次階離子質譜儀(Positive liquid secondary ion mass spectrometry 簡稱 LSIMS)及以 B/E MS/MS 以判斷並確認綠豆芽萃取出之皂苷 Soyasponin I 之化學構造式。