Effect of Storage and Extraction on Ratio of Soyasaponin I to 2,3-Dihydro-2,5-dihydroxy-6-methyl-4-pyrone-Conjugated Soyasaponin I in Dehulled Peas (*Pisum sativum* L)

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Abstract: The effects of solvent and time on extraction of 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone (DDMP)-conjugated soyasaponin I in dehulled peas (*Pisum sativum* L) were studied. Extraction in 80% ethanol gave a higher and more stable yield than 100% methanol over the time studied. Acetonitrile did not extract any detectable saponins. Both soyasaponin I and the DDMP-conjugated form were present in the extracts of dehulled, dried peas. The proportion of soyasaponin I to the DDMP-conjugate, calculated as the ratio of the molecular ions, increased with increasing extraction time, from an initial 0:24 after 0:5 h to 0:55 after 24 h of extraction in 80% ethanol, showing that the DDMP-conjugate was hydrolysed during the extraction. Storage of dried dehulled peas also increased the proportion of soyasaponin I, from 0:063 in newly harvested peas to 0:34 after 9 months of storage. Peas harvested in 1988 and stored for 7 years had a ratio of 0:78. These results clearly show that the DDMP-conjugate was converted to soyasaponin I both during storage and extraction of the peas. © 1998 Society of Chemical Industry.


Key words: *Pisum sativum*, dehulled seeds, hulls, soyasaponins, DDMP-conjugate, storage, extraction.

INTRODUCTION

Saponins are steroid or triterpenoid glycosides present in a wide variety of plants consumed by humans and animals. The soyasaponins comprise a triterpenoid saponogenol with one or two (mono- or bisdesmosidic) carbohydrate sidechains attached. They have a bitter taste (Price and Fenwick 1984), inhibitory effects against infectivity of the AIDS virus (Okubo et al 1994) as well as possible cholesterol-binding and growth retarding activities (Cheeke 1996). These properties are, however, dependant upon the individual structure of the soyasaponins (Okubo et al 1994). It has been reported that the native saponins in *Pisum sativum* (Tsurumi et al 1992), *Phaseolus coccineus* (Yoshiki et al 1994), *Lupinus angustifolius* (Ruiz et al 1995), *Medicago sativa* and *Glycine max* (Massiot et al 1992) are soyasaponins conjugated with 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone (DDMP) (Fig 1). Traditional methods of extracting, derivatising and analysing soyasaponins have used hot exhaustive extractions. It has been suggested that heating converts DDMP-conjugated soyasaponin I to soyasaponin I (Fig 2) (Kudou et al 1992). Later studies on soyasaponins in legumes using mild (eg room temperature) extractions have shown DDMP-conjugated soyasaponins to be the predominant soyasaponins (Kudou et al 1992; Massiot et al 1992). The DDMP-conjugated soyasaponins have a strong superoxide anion (O₂⁻) scavenging activity, suggesting a preventive role against biomolecular damage due to radical...
Fig 1. MS/MS product ion spectrum of m/z 1069 (DDMP-conjugate soyasaponin I).

attack (Yoshiki and Okubo 1995). It has also been suggested to function as a reductant in root growth regulation (Tsurumi and Tsujino 1995).

In peas, a domestic protein crop in Sweden, only soyasaponin I has been quantified (Curl et al 1985; Price et al 1986; Daveby et al 1997). DDMP-conjugated soyasaponin I was quantified in 7-day-old aetiolated pea seedlings (Tsurumi et al 1992). The latter study reported production of soyasaponin I from DDMP-conjugated soyasaponin I in frozen tissues or from prolonged extraction.

We have investigated the efficiency of different solvents for extraction of DDMP-conjugated soyasaponin I as well as the effect of storage and extraction time on the ratio of soyasaponin I to DDMP-conjugated soyasaponin I in dehulled peas.

**MATERIALS AND METHODS**

**Materials**

Swedish light-coloured and leafless peas (*Pisum sativum* L; subsp hortense; cv Capella) grown during 1988 at Ultuna, Sweden (60°N) and during 1995 at Svalöv, Sweden (56°N) were studied.

The peas were soaked in distilled water at 4°C overnight, dehulled by hand (the germs were included with the dehulled seeds) and the dehulled seeds were freeze-dried. The peas harvested in 1988 were stored dry as whole seeds in room temperature until analysis. Peas harvested in 1995 were stored dehulled in a desiccator until analysis. Prior to analysis samples were ground in a Tecator cyclone sample mill to pass a 0.5 mm sieve. One batch each from the 1995 (in a desiccator) and the 1988 harvest were stored whole and ground (0.5 mm) prior to analysis. All results are based on duplicate analysis with <5% and <8% as highest acceptable differences between duplicate samples for HPLC analyses and LC-MS calculated ratios, respectively.

**Extraction**

To investigate the extraction efficiency, the dehulled peas (0.5 g) were extracted in a shaker at room temperature for 2, 4, 6, 8 and 10 h in 80% aqueous ethanol, 100% acetonitrile or 100% methanol (5 ml). The samples were centrifuged (875 × g, 10 min) and filtered (0.45 μm) prior to analysis by HPLC.

**Ratios of soyasaponin I to DDMP-conjugated soyasaponin I**

The ratios of the area of the molecular ion of soyasapo-
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Fig 2. MS/MS product ion spectrum of m/z 943 (soyasaponin I).

Saponins were analysed by HPLC on a YMC (YMC Inc., Wilmington, NC, USA) J-sphere ODS-L80 S-4 μm (4.6 × 250 mm) steel cartridge. After initial scanning with a photodiode array detector, detection was carried out at 205 and 292 nm. The following equipment was used: Waters 600 E Multisolvent Delivery System (Waters Associates, Milford, CA, USA), LDC spectromonitor 3100 (LDC Analytical, Riviera Beach, FL, USA), Waters 991 Photodiode Array Detector, BioRad AS-100 autosampler (BioRad Inc, Hercules, CA, USA) and SP 4270 integrator (Spectra Physics, San Jose, CA, USA). The column was eluted at a flow rate of 1 ml min⁻¹ starting with 35% CH₃CN followed by a linear gradient to 60% CH₃CN in 40 min; a concentration of 40 mM acetic acid was maintained throughout elution.

For post-column collection for MS/MS experiments the 80% ethanol extract was eluted through a YMC Inc. J-sphere ODS-L80 S-4 μm (10 × 250 mm) steel cartridge with a flow rate of 2.4 ml min⁻¹. All other conditions were the same as above. The fractions (26-71 and 27-37 min) were collected over dry ice and immediately stored in a freezer (−30°C). Prior to analysis the acetonitrile was evaporated under nitrogen, and the sample lyophilised.

HPLC

LC-MS experimental

A Hewlett-Packard Model 1050 LC pump (Palo Alto, CA, USA) was used to provide linear gradients and a constant flow rate of 200 μl min⁻¹. All chromatography was performed on a YMC Inc. J-sphere ODS-L80 LC column (2 × 250 mm). Chromatographic elution for positive ion electrospray analysis started with 18% CH₃CN followed by a linear gradient to 81% CH₃CN in 30 min; a concentration of 40 mM formic acid was maintained throughout elution. Under these conditions soyasaponin I elutes at 20-0 min and DDMP-conjugated soyasaponin I at 22-1 min. A Finnigan
RESULTS AND DISCUSSION

Attempts were made to isolate a standard of DDMP-conjugated soyasaponin I. Owing to degradation during the isolation procedure we were not able to isolate a pure compound which could be used as a standard. Chromatographic runs with a photodiode array detector were compared for a standard of soyasaponin I and the ethanol extract. The soyasaponin I eluted at 26-71 min with a maximum absorbance at 205 nm. The ethanol extract showed two distinct and well-separated peaks at 26-71 and 27-37 min, the latter with maximum absorption at 292 nm and the former with smaller absorption at 205 nm. These data are in very good agreement with earlier reports for soyasaponin I and DDMP-conjugated soyasaponin I (Kudou et al 1992; Massiot et al 1992; Yoshiki et al 1995).

MS/MS product ion spectrum of m/z 943 from the 26-71 min collected fraction gave a molecular ion at m/z 943 and fragment ions at 797, 635, 459, 441 and 423 (Fig 2). These are expected fragment ions for soyasaponin I and verifies the identity of this peak as soyasaponin I. MS/MS product ion spectrum of the 27-37 min collected fraction gave a molecular ion at m/z 1069 and fragment ions at 923, 759, 581, 567, 423, 144 and 126 (Fig 1). These data are in very good agreement with earlier reports on DDMP-conjugated soyasaponin I (Kudou et al 1993; Yoshiki et al 1994). Together with the chromatographic appearance the compound was identified as DDMP-conjugated soyasaponin I.

The yield of DDMP-conjugated soyasaponin I (in relative area units) from the extraction procedures are shown in Fig 3. Extracting with 80% ethanol gave the highest and most stable recovery over the time studied. The amount of saponin extracted increased up to about 4 h. Extraction times from 4 to 32 h gave similar yields. Extracting with 100% methanol gave a very low yield of saponins. One hundred percent acetonitrile did not extract any detectable amounts of DDMP-conjugated saponins.

Both soyasaponin I and DDMP-conjugated soyasaponin I were found in the extracts of mature dehulled peas. However, the ratio of the two saponins changed during storage and with different extraction times. In newly harvested mature dried dehulled peas the ratio of

the areas of the molecular ions of 943 : 1069 after 0-5 h extraction was 0-063. After storage of the dehulled peas in a desiccator for 5 and 7 months, the ratio had increased to 0-19 and 0-24, respectively. When stored whole in a desiccator and ground without dehulling after 9 months the ratio was 0-34. The same cultivar of peas harvested in 1988, stored as whole seeds at room temperature for 7 years and dehulled and ground prior to analysis, had a ratio of 0-76. In peas from the 1988 harvest, stored at room temperature for 7 years and analysed without dehulling, the ratio was 0-78.

The proportion of soyasaponin I to the conjugated form, also increased with increasing extraction time, from an initial ratio of 0-24 after 0-5 h in 80% ethanol to 0-55 after 24 h of extraction (Fig 4). Exclusion of light during extraction had no effect. When extracting for 0-5 h, filtering and then standing at room temperature prior to analysis, the ratio of the areas of the molecular ions increased in a similar manner but at a lower initial rate. These results suggest continued release of DDMP-conjugated soyasaponin I from the pea extract up to approximately 4 h. Prolonged extraction in 80% ethanol seems to enhance the conversion of DDMP-conjugated soyasaponin I to soyasaponin I, the rate after about 4 h being similar with or without the ground peas in the extract.

The half life of DDMP-conjugated soyasaponin I (approximately 150 h) was calculated from the results for the breakdown of DDMP-conjugated soyasaponin I to soyasaponin I at room temperature after 0-5 h extraction. The ratio of [MH]⁺ = 943 : [MH]⁺ = 1069 during the extraction may be determined by the rate of extraction for the compounds, half life of DDMP-conjugated soyasaponin I and the relative composition in the starting material. The fitted line for the ratio of...
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Fig 4. Effect of extraction time on ratio of 

\[ [MH]^+ = 943 : [MH]^+ = 1069 \]

after extraction in 80% ethanol. In Fig 4 represents the ratio obtained by simulation of the course of extraction.

\[ [MH]^+ = 943 : [MH]^+ = 1069 \]

after storage. With the ratio in whole peas being similar to the ratio in dehulled peas, no effect of soaking, dehulling or freeze-drying was found. Neither did exposure to light affect the ratio compared to extraction in darkness.

It is thus suggested that not only heating or prolonged extraction but also storage after harvesting releases soyasaponin I from the DDMP-conjugate form, which could be due to natural enzymatic processes in the cotyledon. Further evaluation of the compositional changes of soyasaponins during storage and extraction is needed.

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