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Characterization of saponins in peas (*Pisum sativum* L.) by HPTLC coupled to mass spectrometry and a hemolysis assay

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ABSTRACT

Peas (*Pisum sativum* L.) contain two kinds of monodesmosidic, triterpene saponins, structurally comparable to those from soybeans — saponin B (soyasaponin I) and DDMP saponin (soyasaponin βg).
A high-performance thin-layer chromatography (HPTLC) method for the fast and reliable estimation of saponins in selected pea cultivars has been developed. Identification of the substances was achieved by coupling HPTLC directly to electrospray ionization mass spectrometry (HPTLC-ESI-MS). Quantitative assessment of saponin B and DDMP saponin was carried out densitometrically after post-chromatographic derivatization with *p*-anisaldehyde sulphuric acid. The thermal stability of DDMP saponin was investigated at 50 °C and 60 °C with a time period of 24 h. Additionally, particular attention was on the hemolytic activity of the saponins analyzed directly on blood-gelatin covered TLC plates.
The presence of DDMP saponin and saponin B in the pea samples was proven by HPTLC-ESI-MS and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Their concentration varied depending on the cultivar, with saponin B being always the more abundant compound, predominantly located in the hulls. The temperature dependent decrease of DDMP saponin concentration was more pronounced at 60 °C than at 50 °C. When treating peas for 24 h, nearly all of the DDMP conjugate was converted to saponin B whereas the total amount of saponins was more or less constant. Neither pea saponins nor those from soybeans possess hemolytic activity. In sum, the results are consistent with previous reports and gave evidence that HPTLC is an adequate methodology facilitating a fast and simultaneous analysis of numerous legume and further samples.

1. Introduction

Grain legumes are cultivated throughout the whole world and have a substantial contribution to a well-balanced and sustainable human nutrition (Duranti, 2006). Particularly in vegetarian or vegan diets, they are considered as valuable nutrient suppliers. With regard to nutritional value, they are two to three times richer in protein than cereals. Nevertheless, they also contain impairing constituents such as protease inhibitors, phytic acid, or saponins (Castell, Guenter, & Igbasan, 1996; Vidal-Valverde et al., 2003).

Especially, saponins are naturally occurring compounds comprising a very heterogeneous group of steroidal or triterpene glycosides (Güçlü-Üstündağ et al., 2007; Lásztity et al., 1998). The main dietary sources of saponins are legumes such as soybeans, peas, and different varieties of beans. But, they are also present in a wide range of vegetables including asparagus, spinach, potatoes, or tomatoes and in numerous herbal remedies for instance soap bark tree (*Quillaja saponaria*), ginseng (*Panax genus*), or licorice (*Glycyrrhiza glabra*) (George, 1999; Shi et al., 2004). Due to adverse effects (bitter taste, astringency) and deleterious properties (membranolytic, hemolytic, piscizide) of some saponins, they were considered as antinutrients, so far (Heng et al., 2006b; Shi et al., 2004). However, recent investigations suggest that dietary saponins might provide some health-beneficial effects including immunostimulatory, hypocholesterolemic, and even cancer preventing properties (Kang, Badger, Ronis, & Wu, 2010; Milgate & Roberts, 1995; Shi et al., 2004).

Peas (*Pisum sativum* L.) contain two kinds of monodesmosidic, triterpene saponins, whose chemical structure is comparable to those of soybean saponins. Primarily, saponin B (soyasaponin I) has been reported to be the main component in green peas. Moreover, 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) saponin (soyasaponin βg) was also identified (Fig. 1). It is also widely distributed in further legumes and is the predominant saponin in soybeans (Heng et al., 2006a). However, detailed investigations regarding saponin composition and content in peas are scarce. The information is inconsistent depending on variety, cultivation and extraction conditions, correspondingly.

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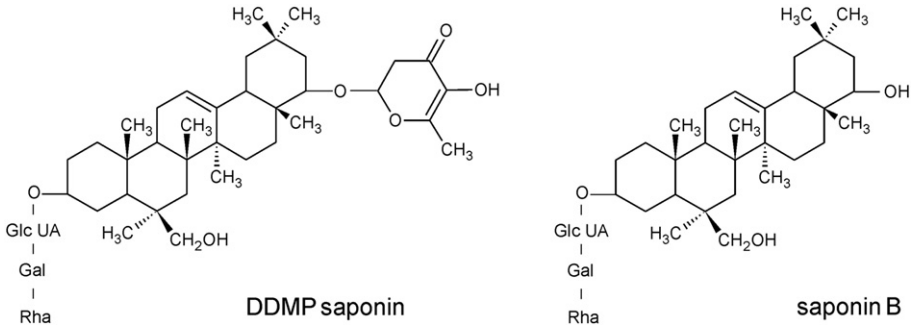

Fig. 1. Chemical structures of DDMP saponin (soyasaponin βg) and saponin B (soyasaponin I).

The objective of this study was to characterize six different pea varieties concerning their saponin profile and content. Thermal stability of the saponins was investigated, as well. Due to its possibilities of evaluating further properties of substances directly and coupling to mass spectrometry, a methodology using high-performance thin-layer chromatography (HPTLC) was established. The haemolytic activity of different saponins was investigated directly on the TLC plates. Further, MALDI-TOF-MS using different matrices was carried out as a reference methodology for the identification of the saponins.

2. Experimental

2.1. Chemicals

As standard digitonin (*Digitalis purpurea*) and soyasaponin I (*Glycine max*, ≥95%) were purchased from AppliChem GmbH (Darmstadt, Germany) and Sigma-Aldrich Chemie GmbH (Steinheim, Germany), respectively. Saponins from soybeans (*Glycine max*) were purchased from Wako Pure Chemical Industries (Japan). Ammonium sulfate was acquired from AppliChem GmbH (Darmstadt, Germany), ammonia (≥25%, p.a.) from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and acetic acid (p.a) and sulphuric acid (≥95%) from TH Geyer GmbH & Co. KG (Renningen, Germany). Hydrochloric acid (37%) and sodium chloride were from Grüssing GmbH (Filsum, Germany) and *p*-anisaldehyde (4-methoxybenzaldehyde) from Merck (Hohenbrunn, Germany), respectively. The matrices 2,6-dihydroxyacetophenone (DHAP), 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (HCCA) for MALDI-TOF-MS analysis were purchased from Bruker Daltonik GmbH (Bremen, Germany). Chemicals for blood gelatin assay amongst others polyisobutylmethacrylate (PIBM), disodium hydrogen phosphate dodecahydrate and sodium dihydrogen phosphate dihydrate were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and Bernd Kraft GmbH (Duisburg, Germany), correspondingly. Chromabond C18 polypropylene columns (500 mg/6 mL) for solid phase extraction were purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany). All solvents were of analytical grade and water was double distilled or of Milli-Q-quality.

2.2. Sample material

Within the scope of a project called LeguAN (*Innovative und ganzheitliche Wertschöpfungskonzepte für funktionelle Lebens- und Futtermittel aus heimischen Körnerleguminosen vom Anbau bis zur Nutzung*, *Innovative functional food and feed products based on regional grain legumes with special consideration of the whole food supply chain* (*engl.*)), which is funded by the Federal Ministry of Food and Agriculture (BMEL) and the Federal Office for Agriculture and Food (BLE), six different pea cultivars 'Salamanca' (Sal), 'Rocket' (Roc), 'Navarro' (Nav), 'Starter' (Sta), 'James' (Jam), and 'Gregor' (Gre) have been investigated. These grain legumes were cultivated by Norddeutsche Pflanzenzucht Hans Georg Lembke KG in Schleswig-Holstein (Holtsee, Germany). Prior to analysis, the hulls and the corresponding peeled seeds were milled to a fine powder (particle size of < 500 μm) by the Institute for Grain Processing (IGV GmbH, Nuthetal, Germany).

2.3. Preparation of samples and standards

Saponin-enriched extracts were prepared following the protocol of Gurfinkel & Rao (2001) with slight modifications. 10 g of milled powder was extracted in methanol (40 mL) for 4 h at 50 °C with gentle agitation in a water bath. Every 30 min, the suspensions were thoroughly mixed. The saponin-containing methanol extracts were filtered and the sample residue was washed with additional methanol, obtaining a final volume of 50 mL. Afterwards, a clean-up procedure was performed to separate the saponins from accompanying compounds (e.g. sugar, proteins). To this end, a 10 mL aliquot of the methanol extract was mixed in a 1:2 ratio with 0.4 M ammonium sulfate and the solution was left on a shaker for at least 20 h. The resulting precipitate containing the non-saponin constituents was removed by centrifugation (3.225 ×*g*, 30 min) at room temperature. The precipitate was washed with a 1:2 (v/v) mixture of methanol and 0.4 M ammonium sulfate, centrifuged and the supernatants were combined (total volume 20 mL). Subsequently, the saponin-rich extracts were evaporated to dryness under a stream of gaseous nitrogen. The dried residue was dissolved in 5 mL of double distilled water (ddH_2O) and mixed thoroughly. To get as pure saponin fractions as possible further sample preparation by solid phase extraction (SPE) was required. For this purpose Chromabond C18 polypropylene columns (500 mg adsorbent, 6 mL, Macherey–Nagel GmbH & Co. KG) were pre-conditioned with methanol (6 mL) and equilibrated with ddH_2O (6 mL). The sample (5 mL) was slowly passed through the column, whereby the saponins are retained by the adsorbent. The cartridge was washed with a mixture of ddH_2O and methanol (95:5, v/v, 3 mL). The purification and fractionation of saponins was carried out in five steps (fractions I–V, 3 mL) with a methanol–water gradient with an alkaline pH by adding 17% ammonia solution. The different fractions collected were evaporated under a stream of nitrogen and brought to a defined volume depending on the saponin concentration in each fraction and the type of sample (hulls or peeled seeds). Dissolving the sample residues was performed with the corresponding eluent as previously used for the SPE. In this regard, fractions I, II, and III extracted from hulls were dissolved in 0.5 mL whereas fractions IV and V were dissolved in 0.2 mL. In contrast, the first three fractions from the peeled peas were resolved in 0.25 mL and the last two in 0.125 mL eluent, respectively. In accordance with preliminary investigations only fractions II, III, and IV contained the DDMP saponin and the saponin B.

Soyasaponin I from soybean (*Glycine max*, Sigma-Aldrich) has been used as a standard for quantification. A stock solution was prepared by dissolving 1 mg soyasaponin I in 1 mL of ethanol. Equidistant standard solutions were prepared by dissolving the stock solution with ethanol over a concentration range of c = 0.125–0.625 mg/mL.