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Studies on *Medicago lupulina* saponins. 5. Isolation and chemical characterization of blossom saponins

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Abstract

The separation of saponins derived from *Medicago lupulina* L. flowers yielded two saponin fractions. The first one, made up of crystalline saponins, readily precipitable from water solution, was a mixture of three glycosides of soyasapogenol B. Acid hydrolysis of these saponins yielded soyasapogenol B and its three artifacts: soyasapogenols C, D and F. Xylose, rhamnose, galactose, glucose and glucuronic acid were found as sugar constituents. The second fraction obtained by cholesterol precipitation consisted of seven haemolytically active medicagenic acid glycosides. Their hydrolysis furnished medicagenic acid and glucose, xylose, rhamnose and traces of glucuronic acid.

Key words: *Medicago lupulina*, *blossom*, *saponins*, *isolation*

INTRODUCTION

In our previous investigation of saponin occurrence in black medic trefoil (*Medicago lupulina* L.) the plant tops (Górski et al. 1984a, b, c, d) and seeds (Jurzysta 1973) were examined. It has been fully documented that both tops and seeds contain medicagenic acid and soyasapogenol B glycosides. In continuation of these studies the saponins from *M. lupulina* flowers were isolated and characterized.

MATERIAL AND METHODS

ISOLATION OF SAPONINS

Flowers of black medic trefoil c.v. Renata were collected from field growing plants. They were picked by hand and immediately immersed

in 96% ethanol, 220 g fresh flowers were extracted five times with 2 dm^3 of ethanol by boiling for two hours under reflux. Then, alcohol was removed in vacuo and the dry residue suspended in water and extracted with methylene chloride in a separatory funnel until the solvent was completely colourless. The organic fraction was discarded and the water solution (200 cm^3) stored during a 24 h period in a cooler at a temperature of 6°C. The precipitate obtained during storage was separated from the water solution by centrifugation, washed with water and crystallized four times from aqueous ethanol, furnishing 540 mg of crystalline saponins — CS (mp. 238°C). To the water solution left after centrifugation 5 g of cholesterol were added and saponins were separated as previously described (Górski et al. 1984b). In this way 230 mg of light-cream coloured powder of saponin cholesterol precipitable fraction (PP) was obtained.

ACID HYDROLYSIS

CS and PP saponins were hydrolysed during 4 and 20 h, respectively by boiling under reflux with 2N HCl in 50% methanol, using 1 cm^3 of acid solution per 10 mg of saponins. In both cases aglycones were filtered, washed with water and dried. Small volumes of filtrates were evaporated until dry, dissolved in water and evaporated repeatedly under reduced pressure at a water bath temperature of 40°C. The dry residue was dissolved in 1 cm^3 of 10% isopropanol and sugar constituents were analyzed by TLC.

ISOLATION AND IDENTIFICATION OF AGLYCONES

Aglycones (180 mg) of CS saponins were chromatographed in a Kieselgel 60 column using 0.5–1% methanol in benzene. This furnished 12 mg of soyasapogenol C, C+D — 22 mg, D — 11 mg, B — 45 mg, B+F — 38 mg and F — 12 mg. They were compared by TLC to standard soyasapogenols B, C, D and F. Soyasapogenol B was crystallized from chloroform-methanol mixture yielding needles mp. 256–259°C, MS m/z (rel. int) 458 (2), 440 (2), 425 (1), 422 (1), 234 (100), 224 (10), 219 (44), 216 (13), 175 (30); soyasapogenol C crystallized from chloroform-methanol, furnishing needles mp. 239–240°C, MS m/z 440 (8), 425 (5), 255 (5), 224 (12), 216 (100), 206 (15), 201 (30), 187 (22), 175 (40), 133 (34); soyasapogenol D crystallized from chloroform-methanol giving prisms mp. 295–297°C MS m/z 472 (65), 457 (8), 454 (9), 440 (20), 425 (6), 248 (62), 235 (42), 224 (34), 223 (16), 216 (30), 206 (28), 203 (100), 175 (32), 99 (30), soyasapogenol F crystallized from aqueous dioxan affording plates mp. 314–317°C, MS m/z 458 (31), 443 (6),

440 (8), 425 (5), 234 (78), 224 (24), 221 (40), 220 (33), 206 (30), 205 (53), 203 (100), 175 (87).

Aglycones of PP saponins (80 mg) were dissolved in ethyl acetate and chromatographed on a Kieselgel 60, 10 cm × 6 cm column using ethyl acetate as solvent. The solvent volume was reduced and aglycone crystallized readily, then it was filtered and crystallized again from dioxan-water, yielding needles (15 mg) mp. 347–350°C, MS m/z 502 (1), 456 (6), 248 (100), 233 (12), 221 (4), 203 (85), 189 (18), 173 (15), 133 (20).

THIN LAYER CHROMATOGRAPHY

The glycosides and sugars were analysed by TLC according to previously described methods (Górski et al. 1984b). The haemolytic activity of individual glycosides was tested by covering two-dimensionally developed TLC plates with gelatine blood suspension.

RESULTS AND DISCUSSION

Exhaustive extraction of fresh black medic trefoil flowers with boiling ethanol yielded a fraction containing triterpene glycosides. Evaporation of alcohol followed by suspension of dry residue in water and overnight storage at 6°C afforded a crystalline compound. After repeated crystallizations this compound was easily soluble in alcohols and little or not soluble in water, and melted at 238°C with previous browning. Thin layer chromatography revealed that it is a mixture of three different glycosides possessing very close R_f values in the two developing systems used. They gave cherry-coloured spots when developed with Liebermann-Burchard reagent. Two of the three glycosides appeared as dominant, whereas only a trace of the third was found. Acid hydrolysis of this mixture furnished four aglycones the structure of which was established by TLC and spectroscopic means as soyasapogenols B, C, D and F. However, it was previously proven (Jurzysta 1982, 1984) that soyasapogenols C, D and F are not naturally occurring compounds but artifacts arising during acid hydrolysis. Thus, crystalline saponins of black medic trefoil flowers are a mixture of three different glycosides of soyasapogenol B. Xylose, rhamnose, glucuronic acid, galactose and glucose were found to be their sugar chain components.

The water fraction left after separation of crystalline saponins gave copious lather when shaken and haemolyzed red blood cells, indicating the presence of a large amount of saponins. Their haemolytic activity suggested that it would be possible to separate them from the solution

by cholesterol precipitation. Thus, using this method a cholesterol-precipitable fraction of saponins was obtained. Two-dimensional TLC revealed that it consisted of the glycosides (Table 1). Four of them were blue and three

Table 1

TLC characteristics of cholesterol-precipitable black medic trefoil blossom saponins

Spot number	R_f value in solvent		Colour after visualization with Liebermann-Burchard reagent		Haemolysis [1]
	S_1	S_2	in natural light	UV	
1	0.09	0.15	pale-green	green	+
2	0.08	0.21	blue	green	+
3	0.12	0.23	pale-green	green	+
4	0.11	0.28	blue	green	+
5	0.16	0.32	blue	green	+
6	0.18	0.34	pale-green	green	+
7	0.22	0.38	blue	green	+
8	0.23	0.30	cherry	cherry	−
9	0.24	0.34	cherry	cherry	−
10	0.26	0.36	cherry	cherry	−

S_1: ethyl acetate-acetic acid-water (7:2:2), S_2: n-butanol-acetic acid-water (4:1:1)
[1] (−) no haemolysis, (+) haemolysis

were pale-green in natural light and green in UV light when visualized with Liebermann-Burchard reagent. All of them haemolyzed blood cells when TLC plates were developed with gelatine-blood suspension. Three of the ten glycosides occurring in trace amounts were cherry-coloured when visualized and their R_f values were the same as those for crystalline saponins. From hydrolysis products of this fraction a single aglycone was separated in crystalline form. Its melting point and R_f values and spectroscopic data were consistent with those for medicagenic acid. Besides this compound, traces of soyasapogenol B, C, D and F were also detected when hydrolysis products were chromatographed by TLC. The glucose, xylose, rhamnose and traces of glucuronic acid were found as sugar components of the precipitable fraction. These findings clearly prove that the dominant components of cholesterol precipitable fractions are medicagenic acid glycosides.

The seven compounds that were green under UV light and which haemolyzed red blood cells were glycosides possessing medicagenic acid as an aglycone molecule. They differed only in sugar chain structures. These three cherry coloured saponozides traces of which were found in the precipitable fraction were evidently soyasapogenol B glycosides identified in the crystalline saponin fraction.

There has hitherto been no report on saponin occurrence in *M. lupulina* flowers, but some research was done on alfalfa blossom saponins (Morris and Hussey 1965). An individual triterpene saponin which was a medicagenic acid glycoside with glucose, rhamnose and glucuronic acid in the sugar chain was isolated from alfalfa flowers. No soyasapogenol presence was reported.

Considering the aglycone structures, saponins of black medic trefoil flowers seem to be very similar to those found in seeds (Jurzysta 1973) or tops (Górski et al. 1984a) of this species. Both, flowers and seeds or leaves contain medicagenic acid and soyasapogenol B glycosides. They differ strongly in saponosides composition, as determined by sugar chain combinations. However, the physiological activity depends mostly on the aglycone structure. Our present findings as well as those previously reported (Checke 1971, Jurzysta 1982, Górski et al. 1984b) clearly show that, independently of the source or origin of medicagenic acid glycosides, they are haemolytically and fungistatically active, whereas soyasapogenol B glycosides lacked such activity.

Some difference between *M. media* and *M. lupulina* aerial part saponins can be pointed out. None of the aerial parts eg. tops, seeds or flowers of *M. lupulina* contain hederagenine glycosides, whereas their presence in *M. media* tops was reported (Jurzysta 1982).

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Badania nad saponinami Medicago lupulina.
5. Wyodrębnianie i charakterystyka chemiczna saponin z kwiatów

Streszczenie

Z kwiatów *Medicago lupulina* L. wyizolowano dwie frakcje saponinowe: krystaliczną i wytrącalną cholesterolem. Frakcja krystaliczna była mieszaniną trzech glikozydów o zbliżonych wartościach R_f. Z jej hydrolizatów wyizolowano w postaci krystalicznej sojasapogenol B i jego artefakty soyasapogenole C, D i F oraz stwierdzono chromatograficznie obecność ksylozy, ramnozy, kwasu glukuronowego, galaktozy i glukozy. Frakcja wytrącalna cholesterolem składała się z dziesięciu glikozydów, z których siedem barwiących się na niebiesko lub szaro-zielono po wywołaniu odczynnikiem Liebermana-Burcharda hemolizowało czerwone ciałka krwi. Zdolności hemolityczne jak i barwa wskazywały, że były to glikozydy kwasu medikagenowego. Potwierdzeniem tego przypuszczenia było wyizolowanie z hydrolizatu tej frakcji krystalicznego kwasu medikagenowego. Składniki cukrowe frakcji wytrącalnej cholesterolem stanowiły glukoza, ksyloza, ramnoza i kwas glukuronowy występujący w ilościach śladowych.