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Isolation, chemical characterization and biological activity of alfalfa (*Medicago media* Pers.) root saponins

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Abstract

Saponins from alfalfa (*Medicago media* Pers.) roots were isolated and their acid hydrolysis revealed several aglycones that were obtained in crystalline form and characterized. Medicagenic acid, hederagenin and soyasapogenols A, B, C, D, E and F were found. Crude saponins were separated into cholesterol-precipitable and non-precipitable fractions. The precipitable fraction consisted of medicagenic acid glycosides; glucose, arabinose, xylose and rhamnose were found as their sugar chain components. The nonprecipitable fraction was a mixture of hederagenine and soyasapogenol glycosides, and glucose, arabinose, xylose, galactose and glucuronic acid were found in the sugar components. The medicagenic acid glycosides made up 6% of root dry matter and showed high biological activity. They cuased red blood cells lysis (haemolytic index 3000), completely inhibited *Trichoderma viride* growth at the concentration of 2.5 mg·100 cm^{-3} of growth medium and retarded wheat seedling growth at concentrations as low as 100 ppm. The cholesterol-nonprecipitable fraction caused no blood cell lysis and fungus growth inhibition, although it did inhibit seedling growth, but to a much lesser extent than medicagenic acid glycosides. The detrimental effect of alfalfa root saponins on winter wheat crop after alfalfa is discussed.

Key words: Medicago media roots, saponins, isolation, biological activity

INTRODUCTION

Saponins are naturally occurring chemical constituents of many plant species. Their detrimental effect on some animals (Cheeke et al. 1977), microorganisms (Zimmer et al. 1967, Gestetner et al. 1971) and plant seedling growth (Mishustin and Naumova 1955, Nord and Van Atta 1960, Jurzysta 1970, Marchaim et al. 1975) is quite well recognized.

They can be found in alfalfa seeds, blossoms, leaves and stems (Morris and Hussey 1965, Pedersen 1975, Jurzysta 1982). Some attention has also been paid to saponins from alfalfa roots (Morris et al. 1961, Gestetner et al. 1970, 1971, Shany et al. 1970), but the available data is not complete. It was found by thin layer chromatography that alfalfa root saponins are a mixture of medicagenic acid, soyasapogenols and hederagenine glycosides (Shany et al. 1972). These saponins display a very high inhibitory effect on *Tribolium castaneum* larvae growth, and a high ability to cause red blood cell lysis.

Saponins were found in root periderm and phloem tissue. Their abundance in the surface of the root may play an important physiological role (Pedersen 1975). They may serve as protectors against root parasites and diseases or they may escape into the soil and affect soil microorganisms or the growth of other plants. This allelopahtic effect has been observed. It is known that an alfalfa stand is much poorer for cotton grown as a subsequent crop than other legume crop stands. It is believed, but not completely proven, that this detrimental effect is caused by alfalfa root saponins introduced into the soil by leaching or by release during root decomposition. For better understanding of all of these effects, saponins from alfalfa roots were isolated and their composition and biological activity was fully documented.

MATERIAL AND METHODS

ISOLATION AND FRACTIONATION OF SAPONINS

Roots of alfalfa (*Medicago media* L. cv. Kleszczewska) were sampled from field grown plants, washed under running water, air-dried and ground. Three kg of powdered roots were defatted with methylene chloride in a Soxhlet apparatus. Saponins were then extracted under standard conditions (Wall et al. 1952). Crude saponins (100 g) were purified and separated into cholesterol-precipitable (7.5 g) and nonprecipitable (2.5 g) fractions according to commonly used methods (Jurzysta 1982). Both fractions were chromatographed on silica gel, and two dimensional TLC revealed eleven spots in the cholesterol-precipitable and nine spots in the nonprecipitable fraction.

ACID HYDROLYSIS

Crude saponins (170 g) were hydrolyzed in 3000 cm^3 of 2 N H_2SO_4 in 50% methanol for 4 hours. Two volumes of water (6000 cm^3) were

added and the suspension filtered. The filtrate was discarded and the precipitate (53 g) was extracted with chloroform for 25 h. The chloroform fraction was extracted with 5% NaOH. By the evaporation of the chloroform, 4 g of dry residue containing a soyasapogenol mixture were obtained. The precipitate remaining after chloroform extraction was dissolved in 1200 cm^3 of 2 N H_2SO_4 in 50% methanol and hydrolyzed again for a period of 86 h. After the addition of 2400 cm^3 of distilled water, the solution was filtered. The filtrate was discarded and the precipitate dissolved in 500 cm^3 5% NaOH and extracted with ethyl acetate (four times, 300 cm^3 each). The solvent was then removed and the residue (8 g) containing hederagenine was obtained. The alkaline solution was acidified with HCl to pH 4 and extracted with ethyl acetate. The solvent was removed and 20 g of residue containing medicagenic acid was obtained.

Acid hydrolysis of the cholesterol-precipitable (20 mg) and nonprecipitable (20 mg) fraction with 5 cm^3 2N HCl in 50% methanol for 16 h, under reflux, followed by water precipitation gave 9 mg and 10 mg aglycones respectively. The water-acidic solutions containing sugars were evaporated several times under reduced pressure at 40°C until HCl was completely removed. The dry residue was dissolved in 0.2 cm^3 10% isopropanol. Aglycones and sugars were chromatographed by TLC together with the appropriate standards. The aglycone fraction of cholesterol-precipitable saponins contained medicagenic acid as the sole aglycone and the sugar fraction was a mixture of glucose, arabinose, xylose and rhamnose. The nonprecipitable saponins had hederagenine and soyasapogenols A, B, C, D, E and F as aglycones and glucose, arabinose, xylose, galactose and glucuronic acid in the sugar fraction.

ISOLATION AND IDENTIFICATION OF AGLYCONES

The crude hederagenine (8 g) and medicagenic acid (20 g) were dissolved in a small volume of ethyl acetate and chromatographed on Kieselgel 60, 10 cm × 6 cm column using ethyl acetate to obtain fractions of pure aglycones. After removal of the solvent, medicagenic acid was dissolved in dioxan-water and crystallized yielding 13 g of white, crystalline compound, mp. 352-353°C (lit. (Boar and Allen 1973) 349-350°C), MS m/z (rel. int.) 502 (1), 487 (1), 457 (1), 456 (4), 249 (100), 235 (4), 233 (4), 203 (41), 189 (7), 133 (1). Hederagenine was crystalized in ethanol giving 850 mg of white compound mp. 330-332°C (lit. (Boar and Allen 1973, Jurzysta 1982) 330-332°C), MS m/z 472 (1), 458 (1), 248 (100), 224 (2), 235 (2), 233 (5), 206 (9), 203 (41), 195 (5), 175 (6), 133 (16).

The crude soyasapogenol fraction was chromatographed on a Kieselgel 60, 40 cm × 3 cm column using 0.5-1.5% methanol in benzene to yield:

soyasapogenol A (45 mg), white, crystalline, mp. 309-310°C (lit. (Boar and Allen 1973) 310-313°C), MS m/z 471 (1), 250 (100), 235 (33), 224 (8), 206 (10), 175 (20), 133 (11), 119 (14); soyasapogenol B (197 mg), crystalline, mp. 255-258°C (lit. (Boar and Allen 1973, Jurzysta 1982) 257-259°C), MS m/z 458 (2), 443 (1), 440 (2), 234 (100), 224 (10), 206 (10), 219 (35), 216 (10), 175 (26), 133 (11), 119 (15); soyasapogenol E (27 mg), white, mp. 249-250°C (lit. (Woitke et al. 1970), 250°C), MS m/z 456 (1), 441 (1), 438 (1), 232 (100), 224 (14), 217 (21), 206 (19), 175 (39), 133 (19), 119 (30); soyasapogenol F (13 mg), crystalline, mp. 312-314°C (lit. (Jurzysta 1982) 316-318°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). Soyasapogenols C and D were obtained in trace amounts. Melting points were measured with a Büchi apparatus and MS with a LKB 9000 spectrometer.

THIN LAYER CHROMATOGRAPHY

TLC of glycosides was carried out on silica gel (DC-Fertigeplatten Kieselgel 60 Merck, 0.25 mm) developed with S_1: ethyl acetate-acetic acid-water (7:2:2) or/and S_2: n-butanol-acetic acid-water (4:1:1). Sapogenines were chromatographed on silica gel using S_3: petroleum ether-chloroform-acetic acid (7:2:1) or S_4: benzene-methanol (92:8) as a developing solvents. Spots were detected after spraying with the Liebermann-Burchard reagent and heating in 120°C.

TLC of sugars was carried out on cellulose (DC-Fertigeplatten Cellulose, Merck) developed with S_5: benzene-n-butanol-pyridine-water (1:5:3:3, upper layer), and visualised by spraying with ammonified silver nitrate solution.

BIOASSAYS

The Haemolytic Index was determined according to methods described by Borkowski (1959). The haemolytic activity of individual saponins was checked by covering two-dimensional TLC with a gelatine-blood suspension.

The antifungal activity was measured using the fungus *Trichoderma viride* by the method of Zimmer et al. (1967) modified by Jurzysta (1979). Both haemolytic (Majko 1978) and antifungal activity (Jurzysta 1979) methods were applied for quantification of the cholesterol-precipitable fraction in alfalfa roots. Wheat seedling growth tests were performed in Petri dishes. Twenty seeds were put on Whatman filter paper and 5 cm^3 of 100, 500, 1000 ppm solutions were added per plate. Distilled water was used as a control. The lengths of seedling roots and tops were measured after 7 days. Each treatment was assayed in 5 replicates.

RESULTS AND DISCUSSION

Preliminary purified ethanol extracts of alfalfa (*Medicago media* Pers.) roots yielded crude saponin that was a mixture of several glycosides. Acid hydrolysis of these glycosides afforded a few aglycones that were separated into individual components and identified by chromatographic and spectroscopic methods. This way, medicagenic acid, hederagenin and soyasapogenols A, B, C, D, E and F were found. This result confirms the data obtained by Shany et al. (1970), who by the TLC method found the same aglycones, excluding soyasapogenol F. But this soyasapogenol is an artifact formed from soyasapogenol B during acid hydrolysis, as previously documented by Jurzysta (1982, 1984). Oleanolic acid found earlier in alfalfa roots by West (1979) was not identified in this study. Based on the isolation efficiency, it can be said that medicagenic acid makes up the highest percentage (92%) of the total sapogenin content. The hederagenin and soyasapogenols yielded efficiencies of 6 and 2% respectively.

Crude root saponins were separated into cholesterol precipitable and nonprecipitable fractions. On the basis of two-dimensional TLC patterns, it was established that the cholesterol-precipitable fraction consisted of eleven components (Table 1) and the nonprecipitable fraction included nine (Table 2) saponosides strongly differing in their relative quantities. Acid hydrolysis of the

Table 1

Two-dimensional TLC characteristics of cholesterol-precipitable alfalfa root saponins

Spot number	R value in solvent		Relative quantity[1]	Colour after visualization with Liebermann-Burchard reagent		Haemolysis[2]
	S_1	S_2		in natural light	UV	
1	0.72	0.65	+ +	violet	green	−
2	0.61	0.41	+ +	blue	green	−
3	0.38	0.26	+ + +	green	green	+
4	0.30	0.21	+	light-green	green	+
5	0.26	0.19	+	light-green	green	+
6	0.23	0.18	+ + + +	green	green	+
7	0.20	0.15	+ + +	gray-green	green	+
8	0.17	0.14	+	gray-green	green	+
9	0.17	0.12	+	gray-green	green	+
10	0.15	0.13	+	gray-green	green	+
11	0.15	0.11	+	gray-green	green	+

[1] Relative quantities were determined visually from the spot colour intensities, the highest quantites are represented by + + + + and the lowest by +.

[2] (−) no haemolysis, (+) haemolysis.

S_1: ethyl acetate-acetic acid-water (7:2:2), S_2: n-butanol-acetic acid-water (4:1:1).

Table 2

Two-dimensional TLC characteristics of cholesterol-nonprecipitable alfalfa root saponins

Spot number	R_f value in solvent		Relative quantity[1]	Colour after visualization with Liebermann-Burchard reagent		Haemolysis[2]
	S_1	S_2		in natural light	UV	
1	0.49	0.37	+	violet-pink	pink	–
2	0.49	0.32	+ +	violet-pink	pink	–
3	0.44	0.25	+ +	gray	gray	–
4	0.39	0.28	+ +	brown	pink-bricky	–
5	0.42	0.22	+ + + +	violet-pink	pink	–
6	0.37	0.20	+ +	violet	bricky	–
7	0.36	0.17	+ +	gray-blue	gray	–
8	0.33	0.17	+ +	brown	bricky	–
9	0.32	0.13	+	gray	gray	–

[1] [2]. S_1 and S_2 as in Table 1.

precipitable fraction revealed medicagenic acid as the sole aglycone and glucose, arabinose, xylose and rhamnose as the sugar components, whereas the nonprecipitable fraction revealed hederagenin and soyasapogenols, and glucose, arabinose, xylose, galactose and glucuronic acid. It may be concluded then, that from among all of the saponins present in the alfalfa root, only medicagenic acid glycosides are able to form insoluble in water, cholesterol complexes. This result does not agree with data reported by Gestetner et al. (1970) who found medicagenic acid and hederagenin in precipitable fractions and only soyasapogenols in nonprecipitable ones. They also have not found any difference in the sugar composition between these fractions. This disagreement may arise because of the different analytical procedures that were used. It is well known that the saponin-cholesterol complex is difficult to filter and thus washing with water when it is treated as a filter cake is very inconvenient and may not always be brought to completion. To avoid these difficulties, several washings followed by centrifugation were done.

The two-dimensional TLC chromatograms of both fractions, developed with blood-gelatine suspension, revealed that nine of the eleven components of the precipitable fraction caused red blood cell lysis. Two glycosides that had the highest R_f values showed no such activity. These two components were medicagenic acid glycosides and the reason for their lack of haemolytic activity remains unclear. A reasonable explanation of the inactivity may be the low water solubility of these saponins. This data does not support results reported by Gestetner et al. (1971) who found that the haemolytic index of alfalfa root saponins depended strongly on their sapogenin to

sugar ratio. They found that the haemolytic index of saponins with high R_f values was very high and fell as the R_f value dropped, and stated that the extent of haemolytic activity is related to the amount of medicagenic acid in the saponin moiety. Our findings as well as previous literature data (Hostettmann et al. 1982) show that the biological activity of saponins depends not only on the aglycone or aglycone to sugar ratios, but is also determined by the sugar chain composition or by some other factors, such as water solubility. Further proof for such an interpretation is the haemolytic index of whole cholesterol-precipitable fractions. The obtained value (3000) is very close to that (3500) obtained by Jurzysta (1982) for alfalfa tops. The nonprecipitable alfalfa root saponin fraction showed no haemolytic activity.

The antifungal activity of alfalfa root saponins was tested with the fungus, *Trichoderma viride*. Very strong inhibition of fungus growth was observed if cholesterol-precipitable saponins were present in the growth medium whereas the effect of the nonprecipitable fraction was very low or none (Fig. 1). A linear correlation between growth medium saponin concentrations (0-2 mg·100 cm^{-3}) and growth inhibition was found for the precipitable

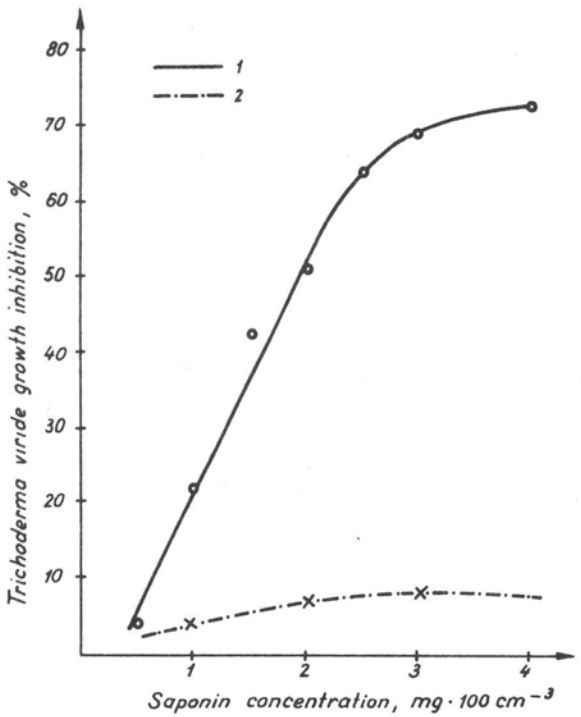

Fig. 1. Effect of alfalfa root saponins on *Trichoderma viride* growth. 1 — cholesterol-precipitable; 2 — cholesterol-nonprecipitable fraction

fraction, and this part of the curve was successfully made use of for the quantification of medicagenic acid glycosides in alfalfa roots. For concentrations higher than 2 mg·100 cm^{-3} of growth medium, the curve broke and slowly evened to a plateau. This level of *T. viride* growth inhibition was four times higher than the activity of analogical fractions from *Medicago media* (Jurzysta 1982) or *Medicago lupulina* (Górski et al. 1984) tops. This also supports the data of Gestetner et al. (1971) obtained for *Sclerotium rolfsii* Sacc. and of Shany et al. (1970) for *Tribolium castaneum* larvae growth, pointing out the much higher activity of medicagenic acid glycosides from alfalfa roots than from alfalfa tops. In respect to the allelopathic or phytotoxic activities of alfalfa root saponins towards the growth of crop plants routine Petri dish tests with winter wheat seeds were carried out. It was found (Table 3), that the cholesterol-precipitable fraction at concentrations as low as 100 ppm inhibited wheat seedling root growth by 50%. The 500 and 1000 ppm concentrations caused at first the browning of

Table 3

Effect of cholesterol precipitable and nonprecipitable alfalfa root saponins on wheat seedling growth

Saponin concentration, ppm	Length of wheat seedlings, mm			
	cholesterol-precipitable fraction		cholesterol-nonprecipitable fraction	
	tops	roots	tops	roots
100	72.0	49.3	77.7	68.3
500	52.0	21.3	62.0	41.3
1000	31.3	9.3	60.3	31.0
Control	87.7	88.3		
Tukay's s.d.d. p = 95%	7.5	6.0	7.5	6.0

root tips and then the complete decay of the root system. At 1000 ppm, wheat seed germination dropped by 30%. The inhibition of wheat seedling top growth was also observed, but to a lesser extent than that for root system growth. These findings are in good agreement with data obtained by Jurzysta (1970), who found the phytotoxic effect of *Medicago lupulina* seed saponins on wheat seedling growth, and with results published by Mishustin and Naumova (1955) concerning the effect of alfalfa root saponins on cotton seed germination. But the phytotoxic activity of alfalfa root medicagenic acid glycosides to wheat seedling growth is about ten times higher than the activity of the saponins isolated by Musayelan and Grigorian (1977) from *Saponaria viscosa* plants. The cholesterol-

-nonprecipitable fractions, which showed no haemolytic and antifungal activity, retarded wheat seedling growth, although about five times weaker than medicagenic acid glycosides. It may be assumed, but not fully proved, that hederagenine glycosides are mainly responsible for this activity.

Comparing the biological activity of precipitable and nonprecipitable fractions as well as their part in the total saponin content, it can be stated that medicagenic acid glycosides are the fundamental factors responsible for the detrimental effects of alfalfa root saponins on the tested organisms. It is therefore important to determine their quantity in alfalfa roots. Based on haemolytic and *T. viride* tests, it was found that medicagenic acid glycosides made up about 6% of root dry matter. This is quite a large amount, much larger than that 1.21-2.19% obtained by Shany et al. (1970) or 0.96% found by Tencer et al. (1972). Both quoted quantities seem to be underestimated. Shany's result was obtained from the evaluation of the extraction efficiency and represents the total alfalfa root saponin content, and Tencer's result is based on the titrimetric method. This method gave the result of 0.11% for alfalfa tops, a value characteristic only of selected low saponin alfalfa varieties. For nonselected alfalfa plants, this is an unlikely low percentage. Assuming the 6% alfalfa root saponin content and taking under consideration that alfalfa root dry matter ranges from 6000 to 10000 kg per hectar, it can be easily calculated that plowing under three year old alfalfa stands introduces approximately 500 $kg \cdot ha^{-1}$ of toxic medicagenic acid glycosides into the top 20 cm of the soil. Their high biological activity may be responsible for poor winter wheat crop after alfalfa stands. This phenomenon has been a topic of our research and will be published soon.

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Wyodrębnianie, charakterystyka chemiczna i aktywność biologiczna saponin korzeni lucerny (Medicago media Pers.)

Streszczenie

Z korzeni lucerny mieszańcowej wyodrębniono saponiny, z których po przeprowadzeniu hydrolizy kwasowej otrzymano szereg aglikonów. Aglikony te po oczyszczeniu, rozdzieleniu i krystalizacji zidentyfikowano metodami chemicznymi i spektroskopowymi jako kwas medi-

kagenowy, hederageninę oraz sojasapogenole A, B, C, D, E i F. Surowe saponiny rozdzielono na frakcję wytrącalną i nie wytrącalną cholesterolem. Frakcja wytrącalna składała się z glikozydów kwasu medikagenowego, a w jej składnikach cukrowych stwierdzono obecność glukozy, arabinozy, ksylozy i ramnozy. Frakcja niewytrącalna była mieszaniną glikozydów hederageniny i sojasapogenoli, a w skład jej części cukrowej wchodziły glukoza, arabinoza, ksyloza, galaktoza i kwas glukuronowy. Glikozydy kwasu medikagenowego stanowiły 6% suchej masy korzeni lucerny i wykazywały dużą aktywność biologiczną. Powodowały one hemolizę krwi (indeks hemolityczny 3000), całkowicie hamowały wzrost grzyba *Trichoderma viride* w stężeniu 2,5 mg·100 cm^{-3} pożywki i hamowały wzrost siewek pszenicy już w stężeniu 100 ppm. Glikozydy nie wytrącalne cholesterolem nie wywoływały hemolizy i nie wykazywały wpływu na wzrost grzyba, jednakże hamowały wzrost siewek pszenicy lecz w stopniu znacznie mniejszym niż glikozydy kwasu medikagenowego. Wskazano na szkodliwy wpływ jaki mogą wywierać saponiny zawarte w korzeniach lucerny na pszenicę ozimą uprawianą na stanowisku po lucernie.