[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Steroidal Sapogenins. XVIII. Partial Hydrolysis of Steroidal Saponins of *Yucca schidigera*[2,3]

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Partial hydrolysis of the oligosaccharide moiety of water-soluble steroidal saponins produces water-insoluble intermediates. After collecting the intermediates by centrifuging or filtering, their hydrolysis to the aglycone, or sapogenin, is completed by refluxing with 2 *N* HCl. The partial hydrolysis of yucca saponin can be produced by the action of native enzymes or of dilute hydrochloric or sulfuric acids. Products of enzymic cleavage were obtained in crystalline form and consisted primarily of sarsasapogenin linked to glucose–galactose oligosaccharides.

Hydrolysis of several 5α-22a-spirostane glycosides by enzymes contained in the parent plant or by enzymes produced by microörganisms, cleaving these saponins to the steroidal sapogenins and component sugars, has been reported in recent papers.[4,5] During those experiments it has been observed that sarsasaponin, a 22b-spirostane glycoside, from certain species of yucca was only partially hydrolyzed, yielding glycosides intermediate in number of sugars between the natural saponin and the aglycone. This paper reports some studies of the extraction and hydrolysis of the 22b-spirostane glycosides found in *Y. schidigera*. In addition to sarsasapogenin, this species also yields markogenin (from 5 to 15% of the total aglycone[6]).

Although in the standard sapogenin assay procedure[7] saponins are extracted from plant tissue with hot 70–90% alcohol, most saponins present in agaves and yuccas are water soluble. When leaves have a high moisture content, as *A. fourcroydes* with *ca.* 75%, a majority of the saponin may be obtained simply by expression of the natural plant juices. As fresh *Y. schidigera* leaves used in these experiments had a moisture content of only 54–56%, none, or very little natural juice could be expressed. In a procedure developed for enzyme studies, two extractions with cold water (see Experimental section) removed 85% of the saponin extractable with hot ethanol.

When water extracts of *Y. schidigera* were allowed to stand so that enzyme action might occur, a crude sludge was deposited in much the same manner as in plant extracts containing saponases.[4] However, benzene–10% ethanol extracts of this sludge contained no free sapogenin. If this sludge was first refluxed with 2 *N* HCl for 4 hours and then extracted with benzene–10% ethanol, sapogenins were obtained. It became apparent that although the sludge, as deposited, did not contain the aglycone, water-soluble saponin of the plant extract had been changed to water-insoluble intermediates.

An experiment was conducted to prove that enzyme action was responsible for the formation of most of the water-insoluble intermediate. A water extract of fresh green leaves, a similar heat-inactivated control, and a water extract of brown, withered leaves of the same plant sample were layered with toluene and allowed to stand at room temperature (22–26°). The partially hydrolyzed saponins recovered from these extracts after time intervals of 2 to 15 days were hydrolyzed to sapogenins. The resulting data are shown by curves in Fig. 1. Although not centrifuged until the fourth day, the solids of the control did not appear to increase after the first day. Compared after an elapsed time of 4 days, the sludges of the extract of green leaves and the control contained 53 and 4%, respectively, of the total sapogenins recovered from each aliquot. The sludge from the leaves which had begun to dry and decompose yielded only 26% of the sapogenin recovered from this sample. These differences are interpreted as showing that the determining factor in the amount of partial hydrolysis in these extracts was enzyme action. In the cardiac glycosides, partial hydrolysis by enzymes is the common, rather than the exceptional type of cleavage.[8]

Studies now in progress indicate that the enzymes of *Y. schidigera* leave two or more simple sugars attached to the steroid nucleus. Some of the partial hydrolysis products have been recrystallized from 70–80% ethanol to yield a mixture of glycosides. One fraction, further purified by recrystallization from ethanol–benzene (1–1), melted 265–268°. Products of 2 *N* HCl hydrolysis of this glycoside were sapogenins, about 45% by weight, and a mixture of simple sugars. The sapogenins were identified as sarsasapogenin and markogenin.[6] Paper chromatography indicated that the sugar mixture (Fig. 2, unknown 2) consisted mostly of galactose and glucose, with a minor fraction of xylose.

Another glycoside fraction, m.p. 293–297°, contained about 30% sapogenin and the sugar components, as indicated by paper chromatography (Fig. 2, unknown 1), had the same composition as unknown 2. Neither of the glycoside fractions from *Y. schidigera* resembled the sarsasaponin which van der Haar[9] obtained from his "Radix sarsaparillae," or that described by Power and Salway[10] as the saponin of *Smilax ornata*. Further research on sapo-

(1) A laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture. Article not copyrighted.

(2) Paper XVII, M. E. Wall, S. Serota and C. R. Eddy, THIS JOURNAL, **76**, 1230 (1954).

(3) Presented in part at the 125th National Meeting of the American Chemical Society, Kansas City, Mo., March, 1954.

(4) M. M. Krider and M. E. Wall, THIS JOURNAL, **76**, 2938 (1954).

(5) M. M. Krider, T. C. Cordon and M. E. Wall, *ibid.*, **76**, 3515 (1954).

(6) M. E. Wall, C. R. Eddy, S. Serota and R. F. Mininger, *ibid.*, **75**, 4437 (1953).

(7) M. E. Wall, M. M. Krider, E. S. Rothman and C. R. Eddy, *J. Biol. Chem.*, **198**, 533 (1952).

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(10) F. B. Power and A. H. Salway, *J. Chem. Soc.*, **105**, [1] 201 (1914).

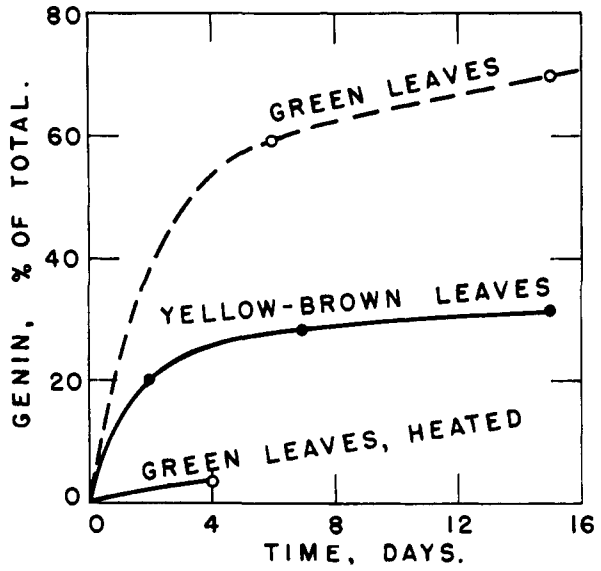

Fig. 1.—Effect of heat and condition of leaves upon enzyme activity in water extracts of *Y. schidigera*.

hins is being conducted in an attempt to correlate ease of hydrolysis with the chemical structure of the carbohydrate moieties.

The saponins of *Y. schidigera* were also partially hydrolyzed by the action of dilute hydrochloric and sulfuric acids. Comparison of these acids at two *p*H levels is presented in Table I. The results show that at *p*H 1.5 only the hydrochloric acid gives an optimal yield of the intermediate after 8 hours reflux. At *p*H 1.2 optimal yields of the intermediate are achieved with both acids: hydrochloric in 4 to 6 hours and sulfuric in 8 hours.

TABLE I

RATE OF HYDROLYSIS OF SAPONIN TO INTERMEDIATES BY HCl AND H_2SO_4 AT TWO *P*H VALUES, IN % OF CONTROL

	*p*H 1.2		*p*H 1.5	
Hr.	HCl	H_2SO_4	HCl	H_2SO_4
2	73	41	56	20
3	83	51	72	38
4	95	63	80	42
6	105	85	86	56
8	102	102	100	70

In order to evaluate the partial hydrolysis to be expected from enzymes naturally present in *Y. schidigera*, leaves collected in the same general geographic area were received April 30, and at weekly intervals from August 20 to September 30, 1953. Most of these shipments were also analyzed by the acid partial hydrolysis procedure. Following partial hydrolysis by either enzymes or dilute acids, the intermediates were collected by centrifugation or filtration, and completely hydrolyzed by refluxing with 2 *N* HCl.[11] The regular assay procedure, consisting primarily of hot ethanol extraction, concentration of extract, transfer of saponin to butanol, removal of butanol, 2 *N* HCl hydrolysis of saponin, benzene extraction, hot methanolic KOH treatment and chromatography, served as a control.[7]

From inspection of data in Table II it is apparent

(11) E. S. Rothman, M. E. Wall and H. A. Walens, THIS JOURNAL, **74**, 5791 (1952).

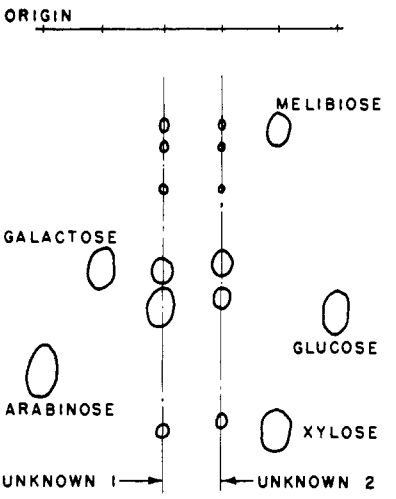

Fig. 2.—Facsimile of a paper chromatogram showing carbohydrate products of hydrolysis of intermediate steroidal glycosides by 2 *N* HCl.

that the activity of plant enzymes is not reliable. Three out of seven yields (fourth day solids) were 7% or less, the maximum 47%, of the control. The mild acid procedure gave yields equal to those obtained by the regular procedure. Thus, water extractions, followed by mild acid reflux, steps by which a saponin concentrate was obtained without the use of organic solvents, formed the basis of a new process for recovering sarsasapogenin from yucca.

TABLE II

COMPARATIVE HYDROLYSIS BY ENZYME AND BY DILUTE ACID OF SAPONINS FROM SEVEN LOTS OF LEAVES, IN % OF CONTROL

Receipt of leaves	Enzyme	Acid
April 30	47	..
Aug. 26	44	..
Sept. 4	7	80
Sept. 10	16	135
Sept. 16	7	104
Sept. 23	0	88
Sept. 30	23	107

Experimental

Yucca schidigera leaves collected in Arizona and kept in cold storage were used in these studies. The grinding and extraction procedures were identical with those described previously.[4] In each experiment a 50-g. sample of the freshly ground leaves was assayed for moisture. For comparative purposes all data incorporated into a table or graph were based upon combined weights of sarsasapogenin and markogenin recovered from any one sample.

Extraction of Saponins with Cold Water.—A 1-kg. sample of ground leaves was immediately extracted with 4 l. of ice-water. The extract was rapidly heated to boiling to destroy the enzymes, cooled and the saponins extracted with butanol. Following established procedures,[7] 5.8 g. of sapogenins was obtained from the extracted saponin. The press cake was twice extracted with boiling ethanol to recover residual saponin. Sapogenins obtained from this source weighed 1.8 g. Thus the water extracted saponins represented 76% of the total recoverable saponins.

Similarly, 1 kg. of leaves extracted first with 3 l., then with 1 l., of ice-water yielded 76 and 10% of the contained sapogenins. Again, a succession of 2, 1 and 1 l. yielded 75, 11 and 1%. A succession of four 1 l. extractions gave 84% in the first two and nothing in the last two.

Demonstration of Partial Hydrolysis by Enzyme Action.—Freshly ground green leaves, 3 kg. wet or 1.56 kg. dry weight, were extracted with 6 and 3 l. of ice-water, respectively. Half the clarified extract, or 4.4 l., with *p*H 4.95, was poured into a small carboy, layered with toluene, and allowed to stand at room temperature (22–26°). The other half was handled similarly, with the exception that it was first heated to boiling to inactivate the enzymes. This heated control was centrifuged after 4 days. The solids, consisting of a soft brown sludge, were slurried with water and transferred to a small flask, made 2 *N* with HCl, and refluxed 4 hours. The granular solids of the hydrolysate were collected on a filter pad, washed thoroughly with water, and dried in an oven at 100°. Sapogenins were extracted from the dried solids with hot benzene and hot benzene containing 20% chloroform, and chromatographed without preliminary KOH in methanol treatment.[7] After chromatography the yield of sapogenin was determined from infrared spectral data of the sapogenin acetates. Weight of pure sapogenins recovered was 0.5 g. The supernatant of the heated extract was butanol extracted and following established procedures,[7] 12.3 g. of sapogenins was obtained. According to these values, each half of the original water extract contained saponins equivalent to 12.8 g. of sapogenins, of which amount the 0.5 g. obtained from the sludge represented 4%.

Solids were still settling out of the unheated portion on the fourth day, so it was allowed to stand six days before centrifuging. No free sapogenins could be detected in the water-insoluble solids, but upon hydrolysis with strong acid and subsequent purification procedures, 7.6 g. of sapogenins was obtained. The supernatant from this unheated portion of the extract was returned to its carboy and allowed to stand nine more days. New solids which formed during this time contained 1.4 g. of sapogenin. Hence, the total sapogenins (9.0 g.) obtained in 15 days by this enzyme procedure represent 70% of the 12.8 g. which this extract was calculated to contain.

In an accompanying experiment, leaves of the same plants, but which varied in color from yellow to black, were used as the source of saponin and enzymes. Ground leaves, 1.73 kg. dry weight, were extracted. The water extract, 10.3 l. with *p*H 4.75, was dark brown instead of the usual greenish-brown color. After standing two days at room temperature, a layer of sludge had accumulated. Upon assay of these solids, 4.8 g. of sapogenins was obtained. The clarified extract was returned to the carboy. Solids collected after seven and 15 days, contained, respectively, 1.9 and 0.9 g. of sapogenins. Added together, a total of 7.6 g. of sapogenin was obtained from solids formed in 15 days in this extract. The supernatant was extracted with butanol and, using the regular procedure, 16.2 g. of sapogenins was obtained. From these values the original extract is calculated to have contained saponin equivalent to 23.8 g. of sapogenins, of which weight the total sapogenins recovered following partial enzymic hydrolysis represent 32%.

Glycosides Obtained by Enzymic Partial Hydrolysis.—A water extract of leaves, 1.7 kg., was layered with toluene, allowed to stand six days at 22–26°, and centrifuged. The soft sludge was slurried with a total of 1.5 l. of 70% ethanol and transferred to a 2-l. flask. Upon heating on a steam-bath, most of the sludge dissolved. Reddish-brown solids were removed by filtration. The filtrate was concentrated, then cooled. A large crop of light colored crystals was obtained. Upon concentration of the mother liquor several smaller crops of crystalline solids were recovered. All fractions were recrystallized several times from 70% ethanol. A typical fraction melted 280–287°; range 269–293° for all crops. The crystals were light gray in color. All glycoside fractions (totaling 31 g.) were combined. They contained 33% of sapogenin.

A portion of the glycoside mixture, 10 g., was refluxed in 200 ml. of an equal volume ratio of absolute ethanol and benzene, and filtered while hot. The cake, 4.3 g., was dissolved in 90% ethanol, filtered through a pad of Florisil to remove yellow gums, and, after concentration, recrystallized several times from 70% ethanol. The major fraction recovered, 2.3 g., m.p. 293–297°, $[\alpha]^{25}D$ −46.8 (*c* 1% in pyridine), was designated unknown 1. A sample of unknown 1, 148 mg., was hydrolyzed with 2 *N* HCl in 20% aqueous ethanol for four hours, the solids in the hydrolysate collected on a tared, sintered-glass micro funnel, washed with water, dried and weighed. The solids, 60 mg. dry weight, contained 70% (or 42 mg.) sarsasapogenin and traces of markogenin. By difference, this unknown glycoside could theoretically contain 5 or 6 hexoses per molecule. Calcd. sarsasapogenin pentasaccharide, $C_{57}H_{94}O_{28}$: C, 55.75; H, 7.72. *Anal.* C, 55.98; H, 7.71.

The mother liquor from unknown 1 was evaporated to dryness. The solids, 5.7 g., were not soluble in benzene or ether. After treatment similar to that described for purifying glycoside unknown 1, a crop of white crystals, 2.1 g., m.p. 265–268, $[\alpha]^{25}D$ −48.3 (*c* 1% in pyridine), was obtained. On hydrolysis, it yielded 45% of aglycone, mostly sarsasapogenin. This would correspond to a glycoside with 3 hexose molecules. Calcd. sarsasapogenin trihexoside, $C_{45}H_{74}O_{18}$: C, 59.83; H, 8.26. *Anal.* C, 60.34; H, 8.34.

The acid filtrates from the 2 *N* HCl hydrolysis of the unknown fractions were evaporated, *in vacuo*, at or below room temperature, in order to remove all HCl. Portions of the resulting sugar mixtures were dissolved in 0.1 ml. of water, spotted on paper chromatographs, and, in the presence of appropriate known sugars, resolved with 1-butanol–pyridine–water (3:1:1.5).[12] As indicated by the results (Fig. 2), the mixtures are mostly glucose and galactose, with a minor quantity of xylose. Samples of unhydrolyzed glycosides were also chromatographed, using butanol–ethanol–water (10:1:2) as the developer. These chromatographs were sprayed with benzidine–citric acid. No spots appeared.

Partial Hydrolysis by Action of Dilute Hydrochloric Acid.—Freshly ground leaves, 6.8 kg. wet or 3.26 kg. dry weight, were extracted with cold water. The clarified extract, 20.4 l., *p*H 4.84, was immediately adjusted to *p*H 2.55 by adding 178 ml. of concd. HCl, then apportioned into 7 equal aliquots. The sapogenin content of one aliquot, considered the experimental control sample, was determined by the regular procedure; yield 5.9 g. or 1.3% (moisture-free basis). An aliquot at *p*H 2.55 was refluxed 4 hours. The water-insoluble intermediates produced were treated in the same way as solids collected after enzymic partial hydrolysis. The sapogenin recovered, 1.2 g., was 19% of that obtained for the control. Similarly, sapogenin content of solids which formed in aliquots adjusted to *p*H values of 2.0, 1.8, 1.6, 1.4 and 1.0 and refluxed for 4 hours were determined. Weights of sapogenin obtained from the other aliquots represented, respectively, 71, 80, 86, 86 and 90% of that recovered from the control aliquot.

Comparison of Partial Hydrolysis by Dilute Hydrochloric and Sulfuric Acids. A.—Freshly ground leaves, 6 kg. wet or 3.12 kg. dry weight, of the September 30, 1953, shipment were extracted with 12 and 6 l., respectively, of cold water. Half the clarified extract, or 8.9 l., was acidified to *p*H 1.2 with 174 ml. of concd. HCl. The other half was acidified to the same *p*H by adding 78 ml. of concd. H_2SO_4. Volumes were equalized by adding 96 ml. of water to the latter. The extracts were heated under reflux, with continuous stirring, in 12-l. flasks. When boiling began (designated as zero time) a 1.5-l. sample was withdrawn, cooled rapidly and centrifuged. Sapogenins in the recovered sludge were determined as previously described. Other samples were taken at 2, 3, 4, 6 and 8 hours. The supernatant of the zero time sample was butanol extracted and sapogenin assay made by the regular procedure. Results are presented in Table I.

B.—A comparison of partial hydrolysis by the same acids at *p*H 1.5 was conducted in the same manner as in A. The ground leaves, 7.42 kg. wet or 3.12 kg. dry weight, were from a shipment received October 6, 1953. Clarified extract, 9 l., was acidified to *p*H 1.48 by adding 139 ml. of concd. HCl. The other 9 l. was acidified to *p*H 1.47 by adding 58 ml. of concd. H_2SO_4 and 81 ml. of water. Heating and sampling were performed as in A, and results are included in Table I.

Comparison of Sapogenin Yields Obtained by Partial Hydrolysis and Regular Procedures.—In a typical experiment 4 kg. of freshly ground leaves were well mixed. A 1-kg. sample was extracted with hot alcohol and assayed for sapogenin by the regular procedure; yield 5.9 g. sapogenin, or 1.3% (m.f.b.).

The remaining 3 kg. of leaves was extracted with 6 and 3 l. of cold water, respectively. Two-thirds of the clarified extract, with *p*H 4.95, was layered with toluene and held four

(12) A. Jeanes, C. S. Wise and R. J. Dimler, *Anal. Chem.*, **23**, 415 (1951).

days at 30°. The intermediate produced yielded 2.3 g. of purified sapogenin. This was an over-all yield of 23% of the sapogenin which should have been obtained from this sample by using the regular procedure.

The remainder of the extract, containing saponins extracted from 1 kg. leaves, was adjusted with concd. HCl to *p*H 1.5 and refluxed 4 hours. From the resulting partial hydrolysis solids 6.2 g. of sapogenin was recovered, or 107% of the yield by the regular procedure. The data are given in Table II.

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Enzymatic Hydrolysis of Xylan[1]

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An enzyme preparation from the medium of *Aspergillus foetidus* hydrolyzed corn cob xylan primarily to xylobiose with no production of D-xylose. An extract of the mold mycelium was separated on a cation-exchange resin into two fractions; one which hydrolyzed xylan and xyloöligosaccharides to D-xylose without producing significant amounts of transient oligosaccharides and one which hydrolyzed xylan to a mixture of D-xylose and xyloöligosaccharides. Fractionation of the mycelium extract on either celite or carboxymethylcellulose produced a fraction which did not hydrolyze xylan but did hydrolyze hemicellulose-B.

Xylan is hydrolyzed by animals,[2] plants,[3] and by a large number of microörganisms.[4] Hydrolysis of xylan by microörganisms was probably first examined by Hoppe-Seyler.[5] Sorenson[6] recently gave evidence that certain bacteria act on xylan to produce xyloöligosaccharides and D-xylose.

Since the use of specific xylan splitting enzymes would be of great assistance in examining the structure of the hemicelluloses, this Laboratory has undertaken the separation of the enzymes of *Aspergillus foetidus* into preparations with specific hydrolytic actions. Herein is described the initial separations achieved.

An extract of *A. foetidus* mycelium hydrolyzes xylan to D-xylose and xyloöligosaccharides as indicated by chromatographic comparison of the products with authentic samples.[7] This enzyme preparation can be separated on a cation exchange resin into two fractions. A fraction (I) splits xylan to D-xylose, cellulose to D-glucose and hydrolyzes hemicellulose-B. Fraction II splits xylan to D-xylose and xyloöligosaccharides, cellulose to D-glucose and cellobiose but does not hydrolyze hemicellulose-B. A further fractionation of fraction I on a column of carboxymethylcellulose yields a portion (IA) that like fraction I hydrolyzes xylan, cellulose and hemicellulose-B and a portion (IB) that does not hydrolyze xylan and cellulose but does hydrolyze hemicellulose-B.

The mold mycelium extract can also be separated on columns of either celite or carboxymethylcellulose into a fraction of unchanged activity which hydrolyzes xylan, cellulose and hemicellulose-B, and another fraction which hydrolyzes hemicellulose-B but does not hydrolyze xylan or cellulose.

Fractional precipitation of the medium with ammonium sulfate yields a preparation which hydrolyzes xylan to xylobiose and higher xyloöligosaccharides but produces no noticeable D-xylose. This "xylo-dextrinase" has no effect on cellulose.

It is recognized that chromatographic comparisons do not constitute absolute identifications of D-xylose and xyloöligosaccharides. However such rapid and simple chromatographic comparisons with authentic specimens provide strong indications of the components present. This is particularly true in examining the enzymatic hydrolysis products of xylan because the structure of the polysaccharide is fairly well established and because its acid hydrolytic products have been well characterized as crystalline substances.[7]

Experimental

Materials.—Xylan was prepared from corn cob as previously described.[8] Uronic acid: 2.9%, $[\alpha]^{25}D$ −116.3° (*c* 1.0 in 1 *N* NaOH); ash, 0.45%; molecular weight by light scattering, 34,500; D.P., 261. Hemicellulose-B was also prepared as previously described. Swollen or "reactive" cellulose was prepared according to the phosphoric acid procedures outlined by Walseth.[9,10] The mold, *A. foetidus*[11] was

(1) Journal Paper No. 804 of the Purdue Agricultural Experiment Station.

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