allowed to stand at 0° for 24 hours. The orange needles, m.p. 126–130°, that separated were again chromatographed through an alumina layer in benzene and a free hydrocarbon was obtained from the effluent. This hydrocarbon failed to crystallize but gave a picrate of orange needles, m.p. 133.5–134°, from alcohol (picrate of XIIa).

Anal. Calcd. for $C_{19}H_{18}{\cdot}C_6H_3O_7N_3$: C, 63.15; H, 4.4; N, 8.8. Found: C, 63.52; H, 4.21; N, 8.66.

The oily hydrocarbon (XIIa), obtained by passing the benzene solution of the picrate of m.p. 133.5–134° through an alumina layer, was dried at a reduced pressure; λ_{max}^{EtOH} 260 mμ (Fig. 1).

Anal. Calcd. for $C_{19}H_{18}$: C, 92.65; H, 7.35. Found: C, 92.42; H, 7.62.

The crude XIIb was dissolved in 20 cc. of petroleum ether, passed through a column containing 30 g. of alumina, and the column was eluted with 120 cc. of petroleum ether. The effluent was fractionated into 20-cc. portions, and the oily substance obtained from fractions 2–4 was converted into a picrate. This picrate was also chromatographed through alumina as a benzene solution, as in the foregoing, and the free hydrocarbon obtained from the effluent was again converted to the picrate which recrystallized from alcohol to yellowish-brown microcrystalline powder, m.p. 149–150° (picrate of XIIb).

Anal. Calcd. for $C_{19}H_{18}{:}C_6H_3O_7N_3$: C, 63.15; H, 4.4; N, 8.8. Found: C, 62.79; H, 3.88; N, 8.78.

The oily hydrocarbon (XIIb), obtained by passing the benzene solution of this picrate through an alumina layer, was dried at a reduced pressure; λ_{max}^{EtOH} 266 mμ (Fig. 1).

Anal. Calcd. for $C_{19}H_{18}$: C, 92.65; H, 7.35. Found: C, 92.87; H, 7.00.

The crude XIIc was dissolved in 20 cc. of benzene, passed through a column containing 30 g. of alumina, and the column was washed with 80 cc. of benzene. The effluent was fractionated into 20-cc. portions, and the crystals obtained from fractions 1–3 were recrystallized from alcohol to colorless needles (XIIc), m.p. 210–212°.

Anal. Calcd. for $C_{19}H_{18}O$: C, 87.0; H, 6.8. Found: C, 86.57; H, 6.92.

HAKATA, FUKUOKA, JAPAN

[CONTRIBUTION FROM THE EASTERN UTILIZATION RESEARCH BRANCH, AGRICULTURAL RESEARCH SERVICE, U. S. DEPARTMENT OF AGRICULTURE]

Steroidal Sapogenins. XIV. Hydrolysis of 5α,22a-Spirostane Glycosides by Plant Enzymes[1-3]

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Enzymes present in leaves of Agaves and Yuccas containing saponins with 5α,22a-spirostane aglycones will cleave many saponins to their aglycones and component sugars or polysaccharides. These enzyme systems are called saponases. Saponases are active in the present of 10% ethanol. Higher concentrations of ethanol or 10% butanol inhibit saponase activity. Tigogenin, hecogenin, gitogenin and manogenin are typical aglycones recovered from the hydrolysates. The aglycones or sapogenins produced by the action of saponases are identical with sapogenins obtained from the same source by hydrolysis with 2–4 *N* HCl.

A preliminary communication[4] from this Laboratory reported the hydrolysis of steroidal saponins by enzymes that occur in the same plants as the saponins. In presenting a more detailed account of the occurrence and properties of these enzymes, the researches reported will be restricted to a discussion of the hydrolysis of steroidal saponins by enzymes present in plants containing saponins with 5α,22a-spirostane aglycones; *e.g.*, tigogenin, hecogenin, gitogenin and manogenin. The hydrolysis of saponins having aglycones of different configuration will be discussed in other papers.

Partial enzymatic hydrolysis of cardiac glycosides to monoglycosides has been known for 20 years.[5] More recently, plant and mold enzymes have been discovered which can split the carbohydrate–steroid linkage[6-8] in the cardiac series. Except for a rather inconclusive note by Canham and Warren,[9] however, neither type of enzymatic hydrolysis has been demonstrated previously on steroidal saponins.

We have found that plant enzymes can cleave 5α,22a-spirostane glycosides to the steroidal sapogenin and component sugars. We will call such enzyme systems saponases, reserving the term hemisaponases for enzymes that leave one or more sugars attached to the steroid nucleus.

Saponases have been found in the leaf tissues of a number of Agave species and also in *Yucca gloriosa*. As shown in Table I, the saponin substrates prepared from these plants all yielded 5α,22a-spirostanes. Since extensive sapogenin surveys[1,10] have failed to show the presence of free sapogenins in freshly harvested plants, apparently *in vivo* the enzymes and substrate are kept rigidly separate.

That saponases do exist is easily demonstrable. By grinding fresh leaf tissue of the species given in Table I, and extracting the ground material with cold water, a solution is obtained that becomes turbid on standing. Part of the turbidity is due to the water-insoluble sapogenins formed by hydrolysis of the water-soluble saponins. Although research now in progress may modify the present statements, the best experimental conditions for saponase activity seem to be a temperature of 30–37° for 48–96 hr. with the *p*H about 5.3. The crude sludge thus obtained contains 25–40% sapogenins. As described previously,[11] the sapogenins

(1) Paper XIII, M. E. Wall, *et al.*, AIC-363, February, 1954.

(2) Presented in part at the Philadelphia Meeting-in-miniature of the American Chemical Society, January 29, 1953. Not copyrighted.

(3) Previous work at this Laboratory has shown that steroidal saponins can be regarded as spirostane-3 glycosides. E. S. Rothman, M. E. Wall and C. R. Eddy, THIS JOURNAL, **74**, 4013 (1952).

(4) M. M. Krider and M. E. Wall, *ibid.*, **74**, 3201 (1952).

(5) A. Stoll, *et al.*, *Helv. Chim. Acta*, **34**, 1933 (1951), and W. A. Jacobs, *Physiol. Rev.*, **13**, 222 (1933).

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(7) A. Stoll, J. Renz and A. Brack, *ibid.*, **34**, 397 (1951).

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(9) P. A. S. Canham and F. L. Warren, *J. S. African Chem., Inst.*, [N. S.], **3**, 9 (1950).

(10) R. E. Marker, *et al.*, THIS JOURNAL, **69**, 2167 (1947).

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

TABLE I

COMPARISON OF ACID AND ENZYME HYDROLYSIS OF 5α,22a-SPIROSTANE GLYCOSIDES

Species	Total sapogenins, % (moisture-free basis) Acid	Enzyme[b]	Sapogenins found[a]
Agave fourcroydes	0.2	0.2	Hecogenin 70%, tigogenin 30%
Agave schottii	.8	0.1	Chlorogenin 70%, tigogenin 30%
Agave toumeyana	.4	1.1	Hecogenin 50%, manogenin 50%
Agave nelsonii	.7	0.6	Hecogenin, manogenin Δ^9-hecogenin, Δ^9-manogenin
Agave cerulata	.1	0.1	Hecogenin, manogenin, Δ^9-hecogenin, Δ^9-manogenin
Yucca gloriosa	.2	0.4	Mangonenin 65%, hecogenin 25%, tigogenin 10%

[a] The nature and proportions of sapogenins produced by the two types of hydrolysis were quite similar. In some cases, presence of sapogenins such as 9(11)-dehydrohecogenin and manogenin made calculation of proportions more difficult, and in these cases percentages are omitted. [b] In each case, the enzyme and substrate were from the same plant.

can be easily purified by trituration with appropriate solvents or by chromatography.

Table II gives data on the effects of ethanol and butanol on saponase activity. Plant saponases were not affected by ethyl alcohol in concentrations up to 10% by volume. Higher concentrations of ethanol markedly reduced the activity, as shown by reduced yields of sapogenin. Butanol added in quantities sufficient to saturate aqueous enzyme solutions (approximately 10% by volume) inhibited enzyme activity, but extraction of the butanol from aqueous enzyme solutions with benzene resulted in complete restoration of saponase activity (*cf.*, experiment 3).

TABLE II

EFFECT OF ETHANOL AND BUTANOL ON SAPONASE ACTIVITY IN AQUEOUS SYSTEMS[a]

Ethanol, v./v. %	Butanol v./v., %	Sapogenins found, g.
0	..	1.2
5	..	1.2
10	..	1.2
25	..	0.8
50	..	0.3
..	0	1.1
..	10 (satd.)	0.5
..	0 (after extn. of BuOH with Bz)	1.1

[a] Experiments conducted on aqueous extracts of *Agave toumeyana*.

The fact that plant saponases can tolerate some ethanol is useful because certain saponin preparations that are insoluble in water are solubilized by the addition of ethanol. One can thereby obtain a homogeneous solution or fine dispersion of the saponin in the extract containing the enzymes. Restoration of saponase activity by the removal of butanol with benzene permitted accurate observations of enzyme action in cross-enzyme experiments (enzymes from one species, substrate from another). Saponins that accompany saponases in crude aqueous extracts can be extracted with butanol, and the saponase activity can be restored by removing the butanol with benzene. Then a saponin substrate from a different source can be mixed with the enzyme solution with full assurance that sapogenins produced by enzyme action came only from the added substrate.

Saponase activity has been demonstrated in various ways: (1) in systems in which the enzyme was extracted with ice-cold water (4°) and combined with an aqueous saponin concentrate prepared by extracting the residual leaf tissue with ethanol and subsequently evaporating the ethanol, (2) on crude aqueous extracts containing both saponase and substrate, (3) in systems in which saponin was removed from the enzyme solution by butanol extraction, and the saponin-free enzyme solution was then combined with saponin substrates prepared from the same species of plant by methods described previously,[11,12] (4) in systems in which the enzymes came from one Agave species and the saponins from another, and (5) in systems in which the enzymes came from an Agave and the substrate from a Dioscorea. Except for (5), the products of hydrolysis were 5α,22a-spirostanes; in (5), diosgenin (Δ^5-spirosten-3β-ol) was isolated. In most examples of the enzymatic hydrolysis of saponins by plant saponases, the nature and proportions of sapogenins isolated were quite similar to those found in duplicate samples in which the sapogenins were obtained by the use of our conventional acid hydrolysis procedure[12] (Table I). These results are in contrast to the results reported by Marker and Lopez,[13] who proposed that sapogenins obtained by acid hydrolysis are artifacts. Our findings conclusively show that the sapogenin structure has not been changed, confirming previous infrared data from our laboratory,[4] and indicating that saponins and sapogenins have similar structures.

The results of experiments presented in this paper show that saponase activity is not specific for any individual plant or saponin; the enzymes of one Agave species hydrolyzed the saponins of another. In fact, this action is not necessarily genus specific, since Agave saponase hydrolyzed saponins from Dioscorea.

In these experiments, the saponase replaced strong (2–4 *N*) acid as the agent for cleaving the carbohydrate–steroid linkage. Many fiber plants discard huge quantities of waste material, which contain a low concentration of saponin. Acid hydrolysis of these saponins is impractical, but in many instances utilization of native plant saponases for this hydrolysis should make possible the recovery of sapogenins as a by-product of the fiber industry.

Experimental

(1) **Quantitative Determination of Enzymatic Hydrolysis of Saponins.**—In a typical experiment *Yucca gloriosa* leaves, collected in North Carolina and kept in cold storage, were ground to pass through a 1-inch screen in a Ball and Jewel rotary cutter. Any coarse pieces of leaf were recycled. The finely shredded leaves were rapidly mixed and sampled: 50 g. was taken for moisture determination and a 1000-g. portion for sapogenin analysis by the regular procedure.[12] The remaining 2230 g. was immediately stirred

(12) E. S. Rothman, M. E. Wall and C. R. Eddy, THIS JOURNAL, **74**, 4013 (1952).

(13) R. E. Marker and J. Lopez, *ibid.*, **69**, 2389 (1947).

with 4.5 l. of ice-water to extract the enzymes. In 10 to 15 minutes, the mixture, at temperature 10°, was squeezed in a cider press. The volume of press liquor was about 4 l. The cake was re-extracted with 2.5 l. of ice-water. The extracts were run through a small Sharples centrifuge at 25,000 r.p.m. and combined to give an enzyme solution of 7.1 l. The temperature was 18°, and the *p*H 5.58. Solids from the centrifuge and the press cakes were twice extracted with boiling 95% ethanol to recover the saponins in these materials. The alcohol was partially removed from the combined extracts by heating, and, after cooling, fat-soluble materials were removed by extracting with benzene. The aqueous solution was heated on a steam-bath, adding water as needed, until all the benzene and ethanol had evaporated. In this way, 780 ml. of water-soluble concentrate containing residual saponins was obtained. The *p*H was 4.9. The aqueous enzyme and saponin solutions were combined, layered with toluene, and held at 37° for 90 hr. The *p*H was 5.5. Bacterial growth, as determined by microscopic examination, was negligible during this time interval. The resultant mixture was centrifuged. The clear supernatant solution was extracted once with benzene containing 10% ethanol to recover sapogenins dissolved in the toluene. The sludge was extracted four times with the same solvent mixture, more ethanol being added to break emulsions when necessary. The combined benzene extracts were concentrated, partially purified by treatment with 10% KOH in methanol, and evaporated to dryness. The mixture of sapogenins was resolved by chromatography on alumina. The fractions recovered, further purified by recrystallization from methanol, were as follows: tigogenin (5α,22a-spirostan-3β-ol), 0.5 g., m.p. 195–196°; hecogenin (5α,22a-spirostan-3β-ol-12-one), 1.4 g., m.p. 250–252°; and manogenin (5α,22a-spirostan-2α,3β-diol-12-one), 3.6 g., m.p. 246–248°. Identity of the sapogenins was firmly established by infrared spectra of the respective sapogenin acetates.[14,15] The total of 5.5 g. of sapogenin represents a yield of 0.4% of the dry weight of the sample. Identical sapogenins were isolated from the acid-hydrolyzed control sample.

(2) **Action of Saponases upon Saponins Extracted Simultaneously with the Enzymes.**—The aqueous extract of leaves contained not only saponase but also most of the 5α,22a-spirostane glycosides present in the plant sample.[16] In a typical experiment, 2 kg. of *Agave fourcroydes* (71% moisture) was ground, and then extracted with 8 l. and 4 l. of ice-water, respectively. The combined centrifuged extracts, 12.3 l. with a *p*H of 5.7, were held at 34° for 102 hr. The extracted and purified sapogenin was identified as hecogenin. The yield was 1.1 g. or 0.2% of the dry weight of the sample. The hecogenin obtained from a 2-kg. sample by the regular 2 *N* acid hydrolysis procedure weighed 1.4 g.

(3) **Hydrolysis of Saponin Preparation by Saponin-free Enzyme Solution from the Same Plant Sample.**—Freshly ground *Agave toumeyana* leaves, 1.36 kg., were extracted with ice-cold water as described in 1. The final volume was 6 l.; the *p*H was 5.35. Sapogenins (I) were determined by the regular acid hydrolysis procedure in an aliquot (1.5 l.) representing one-fourth of the final volume. An equal aliquot (II) was held for the hydrolysis of saponins by saponases. The remaining half (III) of the final volume was extracted with butanol to remove saponins. One-half of III, equivalent to one-fourth of the original extract, was extracted with benzene to remove butanol (IV). To both the benzene-extracted aliquot (IV) and the half of (III) still saturated with butanol (V) was added 250 ml. of a saponin concentrate prepared from leaves of the same sample of *A. toumeyana*. Volumes were equalized at 1.75 l. and buffered to *p*H 5.3 with Na_2HPO_4. The saponins added were equivalent to 1.5 g. of sapogenins, the amount estimated to be present in I and II. Fractions II, IV and V were layered with toluene and held at 37° for 94 hr. Sapogenins from each fraction were isolated and identified by the procedures described above. The yields were: (I) 1.5 g., (II) 1.1 g., (IV) 1.1 g., and (V) 0.5 g.

(4) **Hydrolysis of Saponin Preparations by a Saponin-free Enzyme Solution from a Different Species.** A.—Freshly ground *Agave nelsonii* leaves, 4.5 kg., were extracted with ice-water. The centrifuged extract, 15.5 l. with a *p*H of 5.1, was divided into three equal parts. Sapogenins in one aliquot (VI) were determined by the regular acid hydrolysis procedure.[12] Another aliquot (VII) was adjusted to *p*H 5.3 and stored at 30° for 96 hr. The third aliquot (VIII) was extracted with butanol to remove *A. nelsonii* saponins and with benzene to remove the butanol. A water-soluble saponin concentrate containing the equivalent of 3 g. of sapogenin (as determined by acid hydrolysis) was prepared from leaves of *A. toumeyana* and was added to VIII. This system was then treated as was VII. The enzymatically hydrolyzed sapogenins of VII and VIII were recovered by the procedure described in part 1. The yields were: VI, 2.2 g.; VII, 1.8 g.; VIII, 2.2 g.

B.—Freshly ground *A. toumeyana* leaves, 1.18 kg., were extracted with ice-water. The aqueous extract was centrifuged and extracted as in A to remove saponins and butanol. To the final volume of extract, 5.2 l., *p*H 5.35, was added 250 ml. of absolute ethanol. Then 14 g. of dioscin, which by acid hydrolysis would yield 40% or 5.6 g. of diosgenin, was dissolved in 250 ml. of abs. ethanol and added slowly, with continuous stirring, to the enzyme solution. There was no immediate precipitation of the saponin. The mixture was held at 37° for 120 hr. with the stirrer operating at low speed. The sapogenin IX was recovered, purified and identified as in part 1. The yield of IX was 4.1 g. diosgenin (Δ⁵-spirosten-3β-ol), m.p. 194–195°. After elution of the diosgenin with benzene–$CHCl_3$ mixtures, the alumina column was eluted with benzene–ethanol (4–1) and ethanol–water (1–1). From the latter eluate was obtained 4.0 g. of unreacted dioscin.

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