J. Pharm. Pharmacol., 1965, **17**, 274–280 Received November 30, 1964

Effects of enzymes on *Yucca glauca* Nutt. and other steroid-yielding monocotyledons

G. BLUNDEN,* R. HARDMAN* AND W. R. WENSLEY

A routine assay procedure for steroidal sapogenins in plant tissue is described in which the endogenous enzymes are allowed to function before the tissue is treated with acid. Inhibition of this enzyme action either by disintegration of the tissue in acid or by autoclaving gave a low yield from the leaf, corm, rhizome, tuber or root of the genera so tested (*Agave*, *Aletris*, *Asparagus*, *Smilax*, *Trillium* and *Yucca*). Oven-dried material, in powder, needed to be incubated with water for one day for the full sapogenin-affording enzyme activity to occur. When autoclaved leaf of *Yucca glauca* Nutt. was incubated with commercial cellulase it gave about 80% of the yield of sapogenin afforded by the unheated leaf. The yield is also given when emulsin or pectinase is used instead of the endogenous enzymes.

MONOCOTYLEDONOUS plants, such as species of *Dioscorea*, *Agave* and *Yucca*, are the main source of starting material for the steroid industry. The yield of steroidal sapogenin from the tubers of *Dioscorea belizensis* Lundell, family Dioscoreaceae, is controlled by an endogenous enzyme system (Blunden & Hardman, 1963). The general applicability of such control is now demonstrated in two more families, Amaryllidaceae and Liliaceae, with leaves, roots, rhizomes and corms from six genera: *Agave*, *Aletris*, *Asparagus*, *Smilax*, *Trillium*, and *Yucca*. The extent and nature of the enzyme control is indicated by experiments with *Yucca glauca* Nutt., a member of the Liliaceae found growing in dry soil in the U.S.A. from Iowa and South Dakota to Montana, south to Missouri, Texas and Arizona (Britton & Brown, 1913). Botkin, Shires & Smith (1943) found the plant sufficiently abundant in New Mexico and its leaf yielded sufficient fibre, equal in strength to hemp, for it to serve as a possible source of hard fibre. Marker, Wagner, Ulshafer, Wittbecker, Goldsmith & Ruof (1943) showed that the leaf contained saponins which on hydrolysis yielded sarsasapogenin 0·5% of the dry weight of leaf. The highest yield of this genin isolated from leaf in the experiments described below was 1·94% of the dry weight.

Experimental

The aerial parts of *Yucca glauca* were collected from wild plants in October 1963 from southern Montana and western North Dakota, and in May 1964 from central South Dakota and eastern Wyoming. The outer dead leaves of the plants were removed and discarded. The remaining leaves were stored in a dry room at 21° for up to 6 months, and yellowed during this time. The moisture content of the leaves at the time of assay varied from 3·7–9·5%. Unless otherwise stated this was the leaf used; that it yielded sarsasapogenin was confirmed by the isolation of this steroid. The powdered leaf, after extraction with benzene, was extracted

From the School of Pharmacy, University of Manitoba, Winnipeg, Canada.

* Present address: GB: School of Pharmacy, Portsmouth College of Technology, Portsmouth. RH: Department of Pharmacy, The University, Nottingham.

with 95% ethanol. The solvent was removed from the ethanolic extract and the residue treated with boiling 2N hydrochloric acid for 30 min. The acid-insoluble material was collected, washed with water, sodium carbonate solution and water until neutral, and after drying was extracted with light-petroleum (b.p. 40–60°). Yellow crystals separated from the petroleum extract; these recrystallised from acetone as colourless prismatic needles, m.p. 198–199°, giving the appropriate infrared spectrum for sarsasapogenin and a mixed m.p. of 198° with that compound.

ASSAY

The yucca leaves from one plant were cut into pieces approximately 5 mm in length and these were well mixed. From this bulk supply about 10 g was taken for each sapogenin determination and about 1 g for each of two moisture determinations. The latter was obtained by drying at 100–105° for 16 hr and the mean value used. One of the following three procedures was used for the sapogenin determination.

Procedure A. The pieces of leaf (10 g) were disintegrated for 5 min in a Waring Blendor in the presence of 70 ml water. The mixture, with water rinsings, was incubated at 37° for 24 hr in a plugged flask. After concentrated hydrochloric acid had been added to make the acid concentration 2N, the mixture was boiled for 2 hr, and cooled. The acid-insoluble material collected at the pump was neutralised by washing with water, 20% sodium carbonate solution and again with water. The residue was dried at 80° overnight, powdered and the sapogenins extracted with light petroleum (b.p. 40–60°) in a Soxhlet apparatus for 20 hr. The petroleum-soluble material (0·1–0·4 g) was acetylated with 2 ml acetic anhydride using the procedure of Wall, Eddy, McClennan & Klumpp (1952) and the benzene-soluble material was assayed in carbon disulphide at a concentration of 2·5–8·5% in a 0·422 mm cell of a Beckman I.R.8 spectrophotometer. The estimation was based on the band at 982 cm^{-1} at which pure sarsasapogenin acetate had the molecular extinction coefficient (ϵ) 5·291.

Procedure B. Procedure A was followed with the exception that 2N hydrochloric acid replaced water when the leaf was disintegrated and subsequently incubated.

Procedure C. The leaf was autoclaved at 121° for 30 min before procedure A was followed.

These assays were also applied to plant material other than *Yucca glauca*. In all instances the suitability of the actual plant specimen for these experiments was easily checked, for saponins by the haemolysis procedure (Blunden & Hardman, 1963) and for sapogenins by thin-layer chromatography (Blunden & Hardman, 1964).

Results

The yields are calculated as the genin acetate from the infrared spectra and expressed as % of the moisture-free plant material: the molecular

extinction coefficients at 982 cm^{-1} for diosgenin acetate and hecogenin acetate were 3·547 and 4·594, respectively.

Using yucca leaf there was no significant difference in the yield of sarsasapogenin when the period of boiling with 2N hydrochloric acid was changed to 1, 3 or 4 hr in assay procedure A.

From the mixed bulk supply of 5 mm pieces of yucca leaf of plant no. 1, each of seven samples were treated by one of the assay procedures in Table 1. The experiments were repeated with leaves from other plants collected

TABLE 1. YUCCA LEAF: SAPOGENIN YIELDS* AFTER VARYING ASSAY PROCEDURES

Plant No.	Month collected	Assay procedure A (Incubation 24 hr)	Assay procedure A with varied incubation periods					Assay procedure B	Increase % Assay A relative to Assay B
			None	1 hr	2 days	3 days	4 days		
1	October	1·70	1·66	1·65	1·64	1·66	1·67	0·81	110
2	October	1·91	1·94	1·94	1·94	1·94	1·92	1·14	68
3	May	0·50	0·32	0·47	0·50	0·49	0·51	0·23	117
4	May	0·90	0·78	0·88	0·89	0·90	0·91	0·62	29
5	May	1·13	1·11	1·12	1·12	1·11	1·13	0·75	51

* As % of moisture-free leaf calculated as sarsasapogenin acetate from the infrared spectra.

from the same and from different areas in October and in the following May. The results are shown in Table 1. The yields obtained by disintegrating the leaf in water were always greatly in excess of those when acid was used. While a high yield of sarsasapogenin was made available after 5 min disintegration in water at 20°, the May-collected leaves, with their lower sapogenin content, needed incubation for up to 1 day at 37° for the maximum yield of genin to be subsequently obtained. One day was chosen as the standard incubation period at 37° in assay procedure A, and adopted in procedures B and C for comparison of the results.

Besides yucca leaf, other steroidal saponin-containing plant material which had been stored at 21° was assayed by procedures A, B and C (Table 2). In every instance procedure A gave a result in excess of that afforded by procedures B and C.

TABLE 2. VARIOUS MONOCOTYLEDONS: COMPARISON OF SAPOGENIN YIELDS* BY ASSAY PROCEDURES A, B AND C

Species	Morphological part	Genin	Assay procedure			Increase % A relative to	
			A	B	C	B	C
Yucca glauca, plant no. 6	Leaf	Sarsasapogenin	1·16	0·62	0·63	87	84
Yucca glauca, plant no. 7	Leaf	Sarsasapogenin	1·31	0·83	0·82	58	60
Asparagus officinalis ..	Root	Sarsasapogenin	2·21	1·64	1·10	35	101
Smilax aristolochiaefolia	Root	Sarsasapogenin	1·65	1·26	1·40	31	18
Smilax aristolochiaefolia	Root	Sarsasapogenin	1·45	1·15	1·23	26	18
Aletris farinosa	Root and rhizome	Diosgenin	0·17	0·06	0·04	183	325
Aletris farinosa	Root and rhizome	Diosgenin	0·29	0·16	0·09	81	222
Trillium erectum	Corm	Diosgenin	0·22	0·16	0·14	37	57
Agave americana	Leaf	Hecogenin	1·94	1·00	0·92	94	111
Agave americana	Leaf	Hecogenin	2·08	1·30	1·18	60	76

* As % of moisture free plant material calculated as the acetate of the genin indicated from the infrared spectra.

For further comparison with earlier results obtained with the tubers of *Dioscorea belizensis* Lundell (Blunden & Hardman, 1963), the leaves of one yucca plant were cut up and appropriately sub-sampled as before and one sample dried for 16 hr at 80°, then powdered so that all the material passed through a No. 60 sieve, and assayed. The results are given in Table 3.

TABLE 3. YUCCA LEAF: SAPOGENIN YIELD* AFTER DRYING AT 80°

Plant No.	Leaf stored at 21°		Leaf dried at 80°, then powdered		Increase (%) when enzymes allowed to function	
	Assay procedure		Assay procedure		Column 2 relative to column 3	Column 4 relative to column 5
	A	B	A	B		
8	0·89	0·50	0·90	0·69	78	30
9	1·94	1·14	1·96	1·44	70	36

* As % of moisture free leaf calculated as sarsasapogenin acetate from the infrared spectra.

Autoclaved leaf mixed with untreated leaf from the same sample was assayed by procedure A; this procedure was also applied to a mixture of untreated leaf and the acid-insoluble material obtained by procedure C from a sub-sample of the same leaf. The results are shown in Table 4.

TABLE 4. YUCCA LEAF: SAPOGENIN YIELD* FROM AUTOCLAVED MATERIAL IN THE PRESENCE OF UNTREATED LEAF.

Plant No.	Assay procedure		Mixture used as starting material in assay procedure A						
			Autoclaved leaf mixed with untreated leaf			Acid-insoluble material by assay procedure C mixed with untreated leaf			
			Weight of leaf g		Yield from mixture	Weight of leaf g		Yield from mixture	Calculated yield if no sapogenin released from acid insoluble material by enzymes
	A	C	Autoclaved	Untreated		Autoclaved	Untreated		
6	1·16	0·63	3·972	4·694	1·16	3·207	4·264	0·94	0·93
7	1·31	0·82	3·980	3·889	1·29	3·553	3·427	1·02	1·05

* As % of moisture free leaf calculated as sarsasapogenin acetate from the infrared spectra.

Samples (10 g) of leaf were assayed by procedure A, by procedure C, and were also autoclaved at 121° for 30 min, then disintegrated in the Waring Blendor for 5 min in the presence of an enzyme (or enzyme mixture) (Table 5), followed by the addition of an equal weight of the same enzyme (or enzyme mixture) after the Blendor had been stopped, and before incubation at 37° for 3 days. All the enzymes were purchased from the Nutritional Biochemicals Corporation, Cleveland 28, Ohio, U.S.A. and were labelled as in their "Technical Specification, 1963." The total weight used for 10 g leaf (moisture content 3·7–9·5%) in each instance is given in brackets: "Cellulase (Tech) 4,000 units per g" (1·0 g); "Emulsin" (0·2 g); "Pectinase" (1·0 g); "Maltase (Standardised 600 *p*-nitrophenyl glucoside units)" (0·2 g); mixture of equal weights of "Cellulase (Tech)" and "Pectinase" (2·0 g). All the added enzymes, except the maltase, caused an increase in the yield compared with that from autoclaved leaf

TABLE 5. YUCCA LEAF: SAPOGENIN YIELD* FROM AUTOCLAVED LEAF IN THE PRESENCE OF THE COMMERCIAL ENZYME NAMED

Plant No.	Assay procedure		Assay procedure C with the named enzyme added after the leaf had been autoclaved				
	A	C	"Cellulase (Tech)"	"Emulsin"	"Pectinase"	"Maltase"	"Cellulase (Tech)" plus "Pectinase"
10	0·97	0·60	0·88 (76)†	0·77 (46)	0·71 (30)	0·63 (8)	—
11	0·50	0·23	0·41 (67)	0·42 (70)	0·32 (33)	0·22 (0)	0·43 (74)
12	0·59	0·26	0·49 (70)	—	0·40 (42)	—	0·50 (73)
13	1·32	0·75	1·11 (63)	—	1·02 (47)	—	1·15 (70)
14	1·30	0·71	—	0·90 (32)	—	0·73 (3)	—
15	1·68	1·16	—	1·41 (48)	—	1·10 (0)	—

* As % of moisture-free leaf calculated as sarsasapogenin acetate from the infrared spectra.
† Figures in brackets are the increases caused by the enzyme named (e.g. column 4 minus column 3), expressed as % of increases caused by endogenous enzyme (column 2 minus column 3).

(Table 5). The increase never brought the yield up to that given by the endogenous enzymes; "Cellulase (Tech)" was about 70% as effective as the endogenous enzymes. The mixture of "Cellulase (Tech)" and "Pectinase" gave about the same result as did "Cellulase (Tech)" alone, as also did "Cellulase (N.B.C.)", a more expensive grade, when used alone.

Discussion

The stem of *Yucca glauca* showed a higher sapogenin content than the leaf (stem 0·92 and leaf 0·41%; stem 1·74 and leaf 1·32%) in the two plants examined. This variation between stems and leaves is in agreement with the findings of Panouse & Mamlok (1963) for *Y. guatemalensis*.

Our present preliminary results indicate a seasonal variation in the sarsasapogenin content of the leaf of *Yucca glauca*. The average sarsasapogenin content of the moisture-free leaf from 10 plants collected in October was 1·1% (range 0·39–1·94%) and for 9 plants collected in the following May 0·75% (range 0·41 to 1·32%).

The leaf of *Yucca glauca* was used to assess the effects of enzyme action. The yield of sapogenin from harvested plant material when the endogenous enzymes were allowed to function (Assay Procedure A) was compared with the yield when the enzymes were prevented from functioning by acid (Assay Procedure B) or heat (Assay Procedure C) (Table 1). These assay procedures were then applied to other plants (Table 2). In each instance the genin named in Table 2 was known to occur in the plant tissue chosen (Jacobs & Simpson, 1935; Marker, Turner, Shabica, Jones, Krueger & Surmatis, 1940; Marker, Turner & Ulshafer, 1940). The results obtained with this range of plant specimens showed the general applicability of endogenous enzyme control on the yield of sapogenin from harvested material. In all instances the plant material afforded a higher yield of steroid when the enzymes were allowed to function (Table 2). The increase for *Yucca glauca* leaf in nineteen plants varied from 29 to 127 % relative to the same material in which the enzymes had been inactivated at the start of the assay.

The yield of endogenous enzyme-afforded sapogenin was not limited by the amount of enzyme activity available: a given weight of yucca leaf which

had been stored at 21° after harvesting, when added to an equal weight of the same leaf which had been autoclaved, afforded sapogenin in yield equal to that from two parts by weight of leaf which had not been autoclaved (Table 4). As with the enzymes of the tubers of *Dioscorea belizensis* (Blunden & Hardman, 1963) those of yucca leaf responsible for a part of the sapogenin yield were destroyed by autoclaving but were not permanently inactivated by being heated at 80° for 16 hr. They showed normal activity when the powder was incubated with water. Plant enzymes are often heat resistant when in the cell-wall-adsorbed state. Neither drying at 80° for 16 hr nor autoclaving at 121° for 30 min affected the sapogenin-affording "substrate"; the normal increase in sapogenin yield occurred when the dried powdered leaf was incubated with water (Table 3) or when untreated leaf was supplied as the enzyme source for autoclaved leaf (Table 4). Acid-insoluble material from the assay of autoclaved leaf (Assay Procedure C) failed to yield additional sapogenin when incubated with a sample of untreated leaf (Table 4). The sapogenin-affording enzymic process was a rapid one. It is likely that most freshly harvested plant material would require only disintegration in water for 5 min for much of the sapogenin resulting from enzyme action to be made available. Incubation with water for a day or so would be necessary when the plant tissue had been oven-dried before being powdered. During this drying process some sapogenin-affording enzyme activity may occur before the conditions become unfavourable; the yields by Assay Procedure B were lower than those obtained when the leaf was first dried at 80° before Assay Procedure B was applied (Table 3).

As observed, variations in the ratio of the sapogenin yield by Assay Procedure A to that by Assay Procedure B will be caused by varying the conditions experienced by the plant tissue from the time of harvesting to the time of commencement of the assay. The ratio of yields may also vary with the metabolic state of the plant at the time of harvesting: a sapogenin may occur in a form releasable by Assay Procedure B and also in a form only releasable by method A and the proportion of the two forms may vary with the season and with the age of the tissue as well as with the morphological part of the plant involved.

There are numerous examples of the isolation of steroidal sapogenin in the form of oligosaccharide glycosides from plant tissue and of the hydrolysis of such free glycosides to the aglycone by 2N hydrochloric acid (for example, Marker & Lopez, 1947; Krider, Branaman & Wall, 1955). Boiling 2N hydrochloric acid will also yield free sapogenin from plant tissue, as in assay procedure B above, and on several occasions it has been demonstrated that more intensive acid treatment does not increase the yield of sapogenin; the yield may be reduced (for example, Rothrock, Hammes & McAleer, 1957; Morris, Roark & Cancel, 1958; Blunden, 1962).

Harvested plant tissue affords more sapogenin if the endogenous enzymes are allowed to function before the material is treated with acid (assay procedure A, above). In these circumstances substances of greater complexity than the oligosaccharide type of saponin may be contributory

to the overall yield: for instance, where the steroid is linked to such substances as cellulose, hemicellulose and pectic substances, and also to other cell substances including the cell contents. Sapogenin may also be afforded by enzyme disruption of surfaces to which saponin or genin was formerly held by physical sorption. Some synthesis of steroid may occur in the harvested plant tissue when the numerous enzymes are caused to function.

In the sapogenin-yielding system used (Yucca leaf) the β-glycosidases, cellulase and emulsin, and the α-glycosidase, pectinase, partially replaced the endogenous enzymes in affording sapogenin (Table 5). In this, cellulase was more effective than pectinase, while maltase had no effect. Such commercial enzymes will disrupt surfaces, hydrolyse polysaccharides, release aglycones by hydrolysis, and will also yield substances such as glucose, which may contribute to synthesis of steroid. More investigation is needed to establish the contribution of these various processes to the endogenous enzyme-afforded sapogenin and to gain more information about the nature of the occurrence of steroidal compounds in plant cells.

Acknowledgements. We wish to thank the Canadian Foundation for the Advancement of Pharmacy for their financial support and Mrs. D. Mitchell for her technical assistance. We are grateful to Dr. J.-Nap. Chabot, Botaniste Economiste, Ministère de l'Agriculture et de la Colonisation, Quebec for the *Trillium erectum*, Professor Shebeski of University of Manitoba for the *Agave americana* and Dean van Horne of Montana State University for the *Yucca glauca* collected in that State.

References

Blunden, G. (1962). M. Pharm. Thesis, University of Nottingham.
Blunden, G. & Hardman, R. (1963). *J. Pharm. Pharmacol.*, **15**, 273–280.
Blunden, G. & Hardman, R. (1964). *J. Chromatog.*, **15**, 273–276.
Botkin, C. W., Shires, L. & Smith, E. C. (1943). *New Mexico Agr. Expt. Sta. Tech. Bull.*, 300.
Britton, N. L. & Brown, A. (1913). *Illustrated Flora of the Northern United States and Canada*, 2nd ed., Vol. 1, p. 512, New York: Charles Scribner's Sons.
Jacobs, W. A. & Simpson, J. C. E. (1935). *J. biol. Chem.*, **109**, 573–584.
Krider, M. M., Branaman, J. R. & Wall, M. E. (1955). *J. Amer. chem. Soc.*, 77, 1238–1241.
Marker, R. E. & Lopez, J. (1947). *Ibid.*, **69**, 2389–2392.
Marker, R. E., Turner, D. L., Shabica, A. C., Jones, E. M., Krueger, J. & Surmatis, J. D. (1940). *Ibid.*, **62**, 2620–2621.
Marker, R. E., Turner, D. L. & Ulshafer, P. R. (1940). *Ibid.*, **62**, 2542–2543.
Marker, R. E., Wagner, R. B., Ulshafer, P. R., Wittbecker, E. L., Goldsmith, D. P. J. & Ruof, C. H. (1943). *Ibid.*, **65**, 1199–1209.
Morris, M. P., Roark, B. & Cancel, B. (1958). *J. Agr. Food Chem.*, **6**, 856–858.
Panouse, J. & Mamlok, L. (1963). *Ann. pharm. franç.*, **21**, 735–741.
Rothrock, J. W., Hammes, P. & McAleer, W. J. (1957). *Industr. Engng Chem.*, **49**, 186–188.
Wall, M. E., Eddy, R. C., McClennan, M. L. & Klumpp, M. E. (1952). *Analyt. Chem.*, **24**, 1337–1341.