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DEGRADATION OF TANNINS FROM CAROB PODS (*CERATONIA SILIQUA*) BY THIOGLYCOLIC ACID*

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Abstract—Tannins isolated from ripe carob pods were subjected to thioglycolic acid degradation and mild and strong acid hydrolysis. Mild acid hydrolysis did not degrade the tannins, strong acid hydrolysis resulted in the production of delphinidin, cyanidin and pelargonidin. The ethyl acetate-soluble thioglycolic acid degradation products were identified by paper chromatography, UV and IR spectral analysis as (−) epigallocatechin, (−) epigallocatechin gallate and (−) epicatechin gallate. The minimum molecular weight of the polymers, 3200, was estimated on Sephadex columns. It is concluded that the polymeric condensed tannins from ripe carobs consist of subunits of flavan-3-ols and their gallate esters.

INTRODUCTION

THE MAIN constituents of carob polyphenols were found to be condensed tannins, containing the flavan nucleus and insoluble in the usual organic solvents.[1] The authors suggested that the catechins and leucoanthocyanidins present in green carob pods may be regarded as precursors of these tannins. Joslyn *et al.*[2] stated that the 'major leucoanthocyanins in carob pods are highly polymerized leuco-delphinidins'. The dimeric proanthocyanidins isolated from wild strawberry leaves[3] and avocado seeds[4] support the suggestion that condensed tannins are composed of flavan-3-ol and flavan-3,4-diol subunits.

The structure of condensed tannins has until recently not been fully investigated, due to the lack of suitable degradation procedures. Mineral acid hydrolysis of condensed tannins results in the formation of flavan-3,4-diols. Complex insoluble polymers (phlobaphenes) are however the main product of mineral acid treatment, as a result of acid-catalysed polymerization.[5]

Betts *et al.*[6] successfully employed thioglycolic acid to degrade the tannins from common heather (*Calluna vulgaris*). This tannin was found to consist of cyanidin subunits linked C-4-O-C-7. The authors suggested that the procedure is suitable for the degradation of condensed tannins in general. It has subsequently been employed to degrade the polyflavanoid bark fraction of *Tsuga heterophylla*.[7]

In a study on the composition of condensed carob tannins, they were subjected to thioglycolic acid degradation and the result compared with that obtained by mineral acid

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[1] E. NACHTOMI and E. ALUMOT, *J. Sci. Food Agric.* **11**, 153 (1963).
[2] M. A. JOSLYN, H. NISHIRA and S. ITO, *J. Sci. Fd Agric.* **19**, 543 (1968).
[3] L. L. CREASY and T. SWAIN, *Nature* **208**, 151 (1965).
[4] T. A. GEISSMAN and H. F. K. DITTMAR, *Phytochem.* **4**, 359 (1965).
[5] E. C. BATE-SMITH, *Methods in Polyphenol Chemistry, Proc. Plant Phenolic Group Symp.* (edited by J. B. PRIDHAM), p. 74, Pergamon Press, Oxford (1963).
[6] M. J. BETTS, B. R. BROWN, P. E. BROWN and W. T. PIKE, *Chem. Commun.* 1110 (1967).
[7] K. D. SEARS and R. L. CASEBIER, *Phytochem.* **9**, 1589 (1970).

hydrolysis. The minimum molecular weight of the polymer was estimated on Sephadex columns.

RESULTS

Thioglycolic acid degradation.[6] The degradation of carob tannins by thioglycolic acid (TGA) resulted in the formation of several ethyl acetate-extractable compounds. No S-containing compounds precipitated in the water solution after neutralization. The same ethyl acetate-soluble compounds were obtained on repeated TGA hydrolysis of the residues remaining after each degradation. The ethyl acetate extract was studied by two dimensional paper chromatography. Six platinic iodine[8] reacting spots (S-containing compounds) were detected. These spots gave no colour reactions with reagents used to detect the catechol nucleus[9] or the flavan-3,4-diols[10] and were not further investigated. Six spots were found reacting with diazotized *p*-nitroaniline.[9] The ethyl acetate extract after TGA degradation was compared with the ethyl acetate extract of green carobs, which may contain the precursors of the condensed tannins (Fig. 1). Spots Nos. 1–6 gave R_f values and color reactions identical to those obtained after TGA degradation. Spots No. 3 and 6 were identified as phloroglucinol and gallic acid, respectively, by comparing their R_f values and colour reactions with authentic samples. Co-chromatography with the authentic samples confirmed their identity. The results obtained for spots No. 1, 2 and 4 led to the tentative consideration of three flavan-3-ol derivatives:[11,12] (−) epigallocatechin gallate, (−) epicatechin gallate and (−) epigallocatechin.

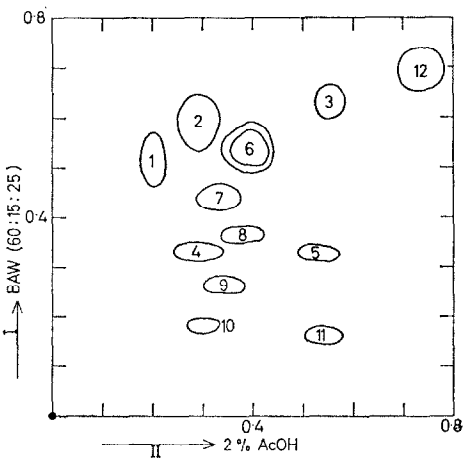

FIG. 1. TWO-DIMENSIONAL PAPER CHROMATOGRAM OF ETHYL ACETATE-SOLUBLE COMPOUNDS FROM GREEN CAROB PODS.

1, Epigallocatechin gallate; 2, epicatechin gallate; 3, phloroglucinol; 4, epigallocatechin; 6, gallic acid and (+)-catechin.

[8] R. J. BLOCK, E. L. DURRUM and G. ZWEIG, *Paper Chromatography and Paper Electropheresis*, Chap. 5, p. 94, Academic Press, New York (1955).
[9] D. E. HATHWAY, *Chromatographic and Electrophoretic Techniques* (edited by I. SMITH), Chap. 17, p. 322, Heinemann, London (1963).
[10] D. G. ROUX and A. E. MAIHS, *J. Chromatog.* **4**, 65 (1960).
[11] E. A. H. ROBERTS and D. M. WILLIAMS, *J. Sci. Food Agric.* **9**, 217 (1958).
[12] L. VUATAZ, H. BRANDENBERGER and R. H. EGLI, *J. Chromatog.* **2**, 173 (1959).

The compounds were obtained chromatographically pure by elution from two-dimensional paper chromatograms. The eluates were freeze-dried and the dry powders used for further identification procedures.

The UV absorption maxima recorded for spots No. 1, 2 and 4 (276, 278 and 274 nm, respectively) corresponded to those reported in References 11 and 12 for (—) epigallocatechin gallate, (—) epicatechin gallate and (—) epigallocatechin. The $\Delta\lambda$ max (nm) recorded after the addition of $AlCl_3$[13] were 10, 32 and 0, respectively.

Three absorption maxima in the OH stretching, phenyl ring and CH bending regions of the IR are characteristic of the flavan-3-ol structures.[14] A strong band at 1680 and less pronounced bands at 1080 and 1315 cm^{-1} are indicative of the existence of ester-linked gallates.[15] Absorption maxima at these frequencies are present in the IR spectra of (—) epicatechin gallate and spot No. 2, but are absent from the spectrum of (—) epigallocatechin. A strong similarity exists between the spectrum of (—) epicatechin gallate and spot No. 2. The spectrum of spot No. 1 corresponded to that of (—) epigallocatechin gallate and that of spot No. 4 to (—) epigallocatechin.

The spectrum of the isolated carob tannins corresponded closely to that of the flavan-3-ol derivatives. This led to the conclusion that the tannin is probably a polymer of these compounds.

The products of alkaline fusion[16] of spot No. 2 were identified chromatographically as gallic acid, phloroglucinol and catechol. This is an additional confirmation of the proposed identity of this spot as (—) epicatechin gallate.

2 N HCl *hydrolysis*.[17] One major and two secondary compounds were detected on paper chromatograms developed with 'Forestal' solvent. The R_f values and absorption maxima of the chromatographically pure compounds, and data reported in Reference 18 are given in Table 1.

Spots No. 1 and 2 gave the anticipated bathochromic shifts on the addition of $AlCl_3$,[13] which were larger than previously reported.[18] The three compounds obtained by 2 N HCl hydrolysis were identified as delphinidin, cyanidin and pelargonidin, in decreasing order of concentration.

TABLE 1. 2 N HCl HYDROLYSIS—R_f VALUES IN 'FORESTAL' SOLVENT, λ_{max} (nm) AND ALUMINUM CHLORIDE SHIFTS

Spot No.	R_f values	λ_{max} (nm) Me-OH-HCl	$\Delta\lambda_{max}$ (nm)
1	0·29	550	36
2	0·48	540	20
3	0·68	—	—
delphinidin*	0·30	546	23
cyanidin*	0·50	535	18
pelargonidin*	0·68	520	0

* Data from J. B. Harborne.[18]

[13] L. JURD, *Phytochem.* **8**, 445 (1969).
[14] H. L. HERGERT and E. F. KURTH, *J. Org. Chem.* **18**, 521 (1953).
[15] C. TSAI SU and V. L. SINGLETON, *Phytochem.* **8**, 1553 (1963).
[16] D. G. ROUX, *J. Am. Leather Chem. Ass.* **53**, 384 (1958).
[17] S. ITO and Y. OSHIMA, *Agric. Biol. Chem.* **26**, 156 (1962).
[18] J. B. HARBORNE, *Biochem. J.* **70**, 22 (1958).

0·1 N HCl *hydrolysis*.[19] The mild acid hydrolysis did not degrade the condensed carob pod tannins—the ethyl acetate extracts obtained after refluxing carob tannins in 0·1 N HCl or water (blanks) gave identical paper chromatograms. The main product in the ethyl acetate extracts was identified as gallic acid. No flavan-3-ol derivatives were present.

Molecular Weight Estimation of Isolated Carob Pod Tannins

The resolution obtained on Sephadex G-25 was unsatisfactory, as most of the carob constituents were eluted from the column with the void volume. This indicates that their molecular weight exceeds 3200.[20]

The resolution on Sephadex G-50 (Fig. 2) was more satisfactory. Three distinct peaks were obtained. The first peak was eluted from the column in Vt 0·28–0·55 and contained 12 per cent of the sample applied. The second peak was eluted in Vt 0·75–2·25 and constituted 75 per cent of the sample. The shape of the peak suggested that it was composed of more than one fraction. By subsequent fractionation on Sephadex G-75 it was found to consist of approximately equal amounts of two fractions. The third peak was eluted from the G-50 column in Vt 5·0–5·8. This minor fraction accounted for 6 per cent of the sample. The eluates comprising each fraction were combined, concentrated and freeze-dried. The total recovery was 90 per cent.

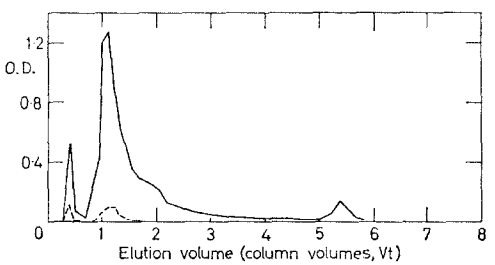

FIG. 2. RESOLUTION OF CAROB TANNINS ON SEPHADEX G-50 (FINE GRADE) COLUMN IN aq. 50% (v/v) METHANOL.
Column length 17·5 cm, volume 2·12 ml/cm, total volume (Vt) 36·0 ml and void volume (Vo) 14·5 ml. Flow rate approx. 30·0 ml/hr. — diluted 1:10; read at 280 nm: ---- no dilution; read at 540 nm.

The calculated minimum molecular weight for complete exclusion from G-50 column is 3600.[20] The majority of the carob tannin constituents were excluded from the G-25 column (M.W. exceeding 3200), but were fractionated on the G-50 column (M.W. less than 3600). The estimated molecular weight of the condensed carob pod tannins soluble in 50% aqueous methanol ranges between 3200 and 3600.

The tannin fraction insoluble in 50% aqueous methanol, and the three fractions separated on the G-50 column, were subjected to acid hydrolysis and TGA degradation as described above. The results obtained with each of the fractions were identical with those of the condensed carob tannin *per se*. These results suggest that the carob tannin is made up of polymers of varying molecular weights (minimum M.W. 3200) of the same repeating subunits (flavan-3-ol derivatives).

[19] E. HASLEM, *J. Chem. Soc.* (C), 1824 (1969).
[20] G. I. FORREST and D. S. BENDALL, *Biochem. J.* **113**, 757 (1969).

DISCUSSION

Joslyn *et al.*[2] identified delphinidin as the main product of strong acid hydrolysis of carob polyphenols and suggested that the major polymeric proanthocyanidin from carob consists of leuco-delphinidin.

Strong mineral acid hydrolysis of carob tannins produced, in addition to the highly polymerized insoluble fraction, three anthocyanidins: delphinidin, cyanidin and pelargonidin. These compounds may be derived from the polymeric catechin, the copolymer of catechin and leucoanthoyanidin, or the polymeric leucocyanidin.[21]

Flavan-3,4-diol derivatives were absent from the degradation products of TGA and the extracts from green carob pods. This strengthens the assumption that they are not the true precursors of carob tannins. The three major products of TGA degradation were identified as (−) epicatechin gallate, (−) epigallocatechin gallate and (−) epigallocatechin.

A comparison of the paper chromatograms obtained with the ethyl acetate extracts of green carob pods and the TGA degradation products (Fig. 1), reveals a strong similarity, particularly with regard to the major components, the catechins and gallic acid. The presence of gallic acid is attributed to hydrolysis of the gallate esters. Some free gallic acid may also be present.[2] Phloroglucinol in TGA extracts is accounted for by further degradation of the monomeric subunits.

The carob fraction insoluble in aq. 50% (v/v) methanol, and the fraction soluble in this solvent and resolved on the Sephadex G-50 column, gave degradation products identical to those obtained with the carob tannins *per se*. In addition, the similarity between the IR spectra obtained for the flavan-3-ol derivatives and the condensed tannin *per se*, led us to conclude that the condensed tannins from carob pods consist of polymers of different molecular weights (minimum M.W. 3200), built from the same structural subunits (flavan-3-ol derivatives).

The relationship between flavonoids and condensed tannins has long been known.[22,23] The mode of linkage between the subunits of the polymers has received much attention. An ether linkage between C-4 and C-6 or C-8 has been considered the most likely mode of linkage.[6,24,25] The existence of a carbon–carbon bond between C-4 and C-6 or C-8 has been confirmed in dimeric proanthocyanidins.[3,4] Originally, Betts *et al.*[6] reported the cleavage of ether linkages by thioglycolic acid. They subsequently demonstrated the cleavage of C-C linkages in a synthetic proanthocyanidin by TGA.[26] Sears and Casebier[7] degraded the condensed tannin in Western hemlock (*Tsuga heterophylla*), believed to have structural units linked C-C, with TGA. Haslam[19] found that a polymeric proanthocyanidin from the roots of *Bergenia* species consists entirely of (+) catechin-3-gallate. A scheme of polymerization which results in C-C bonds was prepared. This scheme may be applicable to the tannins of ripe carobs, with the exception that in contrast to the uniformly built *Bergenia* root tannin, carob tannin is probably formed from various catechin units.

It is probable that many tannins proposed to be composed of leucoanthocyanidin subunits, on the basis of acid hydrolysis, may be shown to consist of catechin subunits, on the basis of TGA degradation.

[21] E. C. Bate-Smith and T. Swain, *Chem. & Ind.* **1953**, 377 (1953).
[22] K. Freudenberg, *Ber. Chem.* **53**, 1416 (1920).
[23] D. G. Roux, *Chem. & Ind.* **161** (1948).
[24] H. I. Hergert, *The Chemistry of Flavonoid Compounds* (edited by T. H. Geissman), p. 553, Pergamon Press, Oxford (1962).
[25] D. G. Roux, *J. Am. Leather Chem. Ass.* **54**, 614 (1959).
[26] M. J. Betts, B. R. Brown and M. R. Shaw, *J. Chem. Soc.* **1178** (1969).

EXPERIMENTAL

Isolation of condensed tannins from ripe carob pods. Ripe carob pods (75 g) were refluxed twice in 1500 ml H_2O for 2 hr each time. The tannins were isolated from the water extracts as described by Vuataz *et al.*[12] A 4% yield was obtained.

Thioglycolic acid degradation. As described by Betts *et al.*[6] for tannins from common heather (*Calluna vulgaris*). The test solution consisted of 0·5 g condensed carob tannins and 36% TGA in aqueous solution. A control in which the TGA was replaced by water was also carried out.

Mineral acid hydrolysis. 2 N HCl;[17] 0·1 N HCl.[19]

Identification of the acid hydrolysis and TGA degradation products. Paper chromatography—Whatman No. 1 paper was used in most cases; 3 MM paper was used for preparative chromatography of acid hydrolysis products.

The two-dimensional solvent system used was: (i) *n*-BuOH–HOAC–H_2O (BAW, 60:15:25) and (ii) 2% AcOH. For the detection of anthocyanidins, one-dimensional chromatograms were developed with HOAc–HCl–H_2O ('Forestal', 30:3:10). Spots on two-dimensional chromatograms were detected with: UV, diazotized *p*-nitroanaline,[9] platinic iodide (for S-containing compounds),[8] vanillin-HCl (for catechol nucleus)[9] or *p*-toluene sulphonic acid (for flavan-3,4-diols).[10]

The acid hydrolysis products were eluted from the preparative one-dimensional chromatograms as described by Harborne.[27] The TGA degradation products were eluted with 70% EtOH from two-dimensional chromatograms after detection with UV. The eluates were concentrated and freeze-dried.

Spectral analyses. UV and visible spectra of the compounds were determined in EtOH or MeOH plus 0·01% HCl. Aluminium chloride shifts were determined.[13] IR spectra of the TGA degradation products were obtained using the KBr pellet technique.

Alkaline fusion. Spot No. 2 was subjected to alkaline fusion by a micro-method described by Roux.[16]

Molecular weight estimation. Gel filtration was carried out on Sephadex G-25 (fine grade), G-50 (fine grade) and G-75 (Pharmacia, Uppsala, Sweden) columns using aq. 50% (v/v) MeOH as the eluant. The minimum molecular weight for complete exclusion was calculated to be 3200, 3400 and 17,000 for G-25, G-50 and G-75, respectively.[20] Samples resolved on the G-25 and G-50 columns were prepared by dissolving 200 mg ripe carob tannins in 5 ml 50% MeOH. The solution was centrifuged for 20 min at 5000 rpm. Fifty per cent of the tannins were soluble in the solvent, and 2 ml of the supernatant was applied to the column.

Fractions of approximately 4·5 ml were collected, and their optical densities determined at 280 and 540 nm. The eluates corresponding to a particular fraction were combined and freeze-dried. The powders were subjected to chemical composition determination as described for carob tannins *per se*.

[27] J. B. HARBORNE, *J. Chromatog.* **1**, 473 (1958).