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BETA VULGARIS L.: THE CHAR ACTERIZATION OF THREE POLYPHENOLS ISOLATED FROM THE LEAVES

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Abstract—An alcoholic extract of leaves of *Beta vulgaris* L. was found to contain five phenolic compounds. Two of these compounds were characterized using chromatographic and absorption spectral techniques. One of them yielded glucose and quercetin upon acid hydrolysis; the second yielded xylose, glucose and a C-glycoside whose spectral and chromatographic behavior was similar to those of vitexin. An isolation procedure for milligram quantities of the C-glycoside was devised. An acetyl derivative was prepared. A third phenolic compound was shown to be 3-hydroxytyramine by i.r. and u.v. spectra and further confirmed by the preparation of two derivatives. Previous work has shown this phenol when oxidized to be closely associated with resistance to the causal organism of sugar beet leaf spot *Cercospora beticola* in culture.

INTRODUCTION

THE FIRST of the C-glycosides to be isolated was vitexin,[1] a C-glycoside of apigenin. Other C-glycosides have since been discovered and include orientin[2], the luteolin analog of vitexin which was later given the name of lutexin[3], barbaloin,[4] bergenin[5] and deoxyvitexin.[6] Vitexin, its rhamnosides and its glucosides have been discovered in many plant species.[7–9]

The authors would like to report the characterization of a C-glycoside isolated from the leaves of *Beta vulgaris* L. (sugar beet) which upon spectral and chromatographic analysis is identical with vitexin. In addition to this glycoside the leaf extract was also found to contain a glucoside of quercetin.

Harrison[10] reported the characterization of a phenolic compound present in extracts of sugar beet leaves that when oxidized was toxic to *Cercospora beticola* in culture. The compound was found in higher concentrations in leaves from resistant varieties than in those from susceptible varieties. The authors now report the identification of this phenolic compound isolated as 3-hydroxytyramine which has been reported in several plant species.[11]

RESULTS

A two-dimensional chromatogram of an alcoholic leaf extract run in 1-butanol/ethanoic acid/water (4:1:5) and 5% acetic acid gave five major spots reacting with diazotized sulfanilic acid and diazotized-4-benzoylamino-2,5-dimethoxyaniline.[12]

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The hydrolysis of Spot *4* in 2 N hydrochloric acid yielded glucose and a phenolic component *4-A*. The phenolic component gave the same u.v. spectrum (Table 1), the same R_f values in several solvent systems, and the same colors with chromogenic reagents (Table 2) as were obtained with an authentic sample of quercetin. Under the same conditions of hydrolysis, Spot *3* produced glucose, xylose, and a phenolic component *3-A*. Severe hydrolysis of the phenolic component *3-A* in 6 N hydrochloric acid produced arabinose.

TABLE 1. ULTRA VIOLET ABSORPTION SPECTRA MAXIMA IN NM OF 4, 3, 4-A, 3-A, QUERCETIN AND VITEXIN

Compound	95% Ethanol	95% Ethanol + NaOH	95% Ethanol + $AlCl_3$
4	254, 264†	—	—
3	271, 333	—	—
4-A	256, 373	—	—
3-A	271, 334	282, 333, 401	280, 304, 343, 385
Quercetin	256, 373	—	—
Vitexin*	270, 334	280, 331, 400	279, 303, 341, 382

* Values given are literature values.[10]
† This maximum appears as a distinct shoulder of the first maximum.

TABLE 2. R_f VALUES OF 4, 3, 4-A, 3-A, QUERCETIN AND VITEXIN IN VARIOUS SOLVENT SYSTEMS AND THE COLORS PRODUCED WITH ULTRA-VIOLET LIGHT (U.V.), DIAZOTIZED SULFANILIC ACID (DSA) AND DIAZOTIZED 4-BENZOYLAMINO-2, 5-DIMETHOXYANILINE (BADA)

Solvents*	1	2	3	4	5	6	U.V.	DSA	BADA
Compound 4	0·52	0·36	0·72	0·53	0·38	—	d-Bl	Br	Bl-G
Compound 3	0·63	0·57	0·76	0·71	0·57	—	d-Br	Br	R
Compound 4-A	0·84	0·00	0·49	0·11	0·82	—	b-Y	Br	Bl-G
Compound 3-A	0·61	0·15	0·67	0·37	—	0·51	R-Br	Br	R
Quercetin	0·85	0·00	0·49	0·12	0·83	—	b-Y	Br	Bl-G
Vitexin†	0·63	0·14	—	—	—	0·49			

Bl = blue, Br = brown, G = grey, R = red, Y = yellow, b = bright, d = dark.

* Solvents
1. 1-Butanol/ethanoic acid/water (4:1:2)
2. Ethanoic acid/water (5:95)
3. Ethanoic acid/water (60:40)
4. 2-Propanol/water (22:78)
5. 1-Propanol/ethyl ethanoate/water (140:20:40)
6. Ethanoic acid/water (30:70)

† Values given are literature values.[7]

Milligram quantities of the compound *3-A* were obtained using a 1-pentanol extraction procedure. An acetyl derivative of the compound *3-A* was prepared and the i.r. spectrum of *3-A* and its acetyl derivative indicated the presence of at least one free hydroxyl group. Alkaline fusion of the *3-A* produced apigenin, *p*-hydroxybenzoic acid, and phloroglucinol. These were identified by chromatographic comparison with known samples of the compounds. A mild NaOH degradation of *3-A* yielded the same components as alkali fusion, except the relative concentration of phloroglucinol to that of apigenin and *p*-hydroxybenzoic acid was much greater than in the alkali fusion. The maximum in the u.v. spectrum

of the compound *3-A* (Table 1) in 95% ethanol, 95% ethanol plus $AlCl_3$, and 95% ethanol plus NaOH closely resembles that of vitexin.[13]

The R_f values (Table 2) of the compound *3-A* in 5% ethanoic acid, 1-butanol/ethanoic acid/water (4:1:2) and 30% ethanoic acid compare favorably with those reported for vitexin. The i.r. spectrum of the compound *3-A* is similar to the i.r. spectrum of vitexin.[14]

The phenolic compound in spot *1* was isolated in milligram quantities from a 1-pentanolic leaf extract by use of multiple cellulose column chromatography, employing 2-butanone–propanone–methanoic acid–water (40:2:1:6) as the solvent.

The phenolic compound appeared to be a catecholamine on the basis of chromogenic tests and paper chromatographic behavior in certain solvent systems. The purified plant phenol and 3-hydroxytyramine had identical R_f values in eleven solvent systems.

The colors produced by 3-hydroxytyramine and the purified plant phenol *1* upon spraying the chromatograms with detection reagents are shown in Table 3. In all cases the colors produced by 3-hydroxytyramine and the phenolic compound were identical in shade, intensity and time of development.

TABLE 3. COLOR TESTS GIVEN BY UNKNOWN PHENOLIC COMPOUND (*1*) AND 3-HYDROXYTYRAMINE

Reagents	Unknown compound (1)	3-hydroxytyramine
Diazotized sulfanilic acid	Reddish-purple	Reddish-purple
2,6-Dibromoquinone-4-chloroimide	Blue-black (grey-brown)*	Blue-black (grey-brown)*
0·44% potassium ferricyanide	Grey	Grey
Diazotized 4-benzoylamino-2,5-dimethoxyaniline	Red (blue-grey)*	Red (blue-grey)*
Ninhydrin	—(reddish-brown)*	—(reddish-brown)*

* Color observed 3–4 hr after spraying.

When a mixture of 3-hydroxytyramine and purified *1* was two-dimensionally chromatographed and dyed with suitable detection reagents, only one spot was observed. The u.v. spectra of 3-hydroxytyramine hydrochloride and the purified plant phenol *1* were determined to be the same by using dilute hydrochloric acid (pH 2·0) as a solvent.

Infra-red spectra of the purified plant phenol and authentic 3-hydroxytyramine hydrochloride were also observed to be similar.

The picrate derivatives prepared from the purified *1* and 3-hydroxytyramine were both fine yellow crystals identical in appearance. The melting range of both was 188–189°. The melting undepressed on mixing.

The styphnate derivatives of *1*- and 3-hydroxytyramine were red–orange, needle-shaped crystals identical in appearance with a melting range 266–267°. Showing no depression on mixing.

Intra-red spectra of the picrates and styphnates from both sources were determined and found to be similar. Ultra-violet spectra for picrates and styphnates were determined using 95% ethanol as a solvent. Both spectra for picrates show identical maxima 285 and 359 nm and minima at 272 and 299 nm. The styphnates yield an intense maximum at 400 nm.

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DISCUSSION

The similarity of *3-A* to vitexin is apparent. The R_f values in various solvent systems, the u.v. spectrum in 95% ethanol, as well as in this solvent with $AlCl_3$ and NaOH additives, compare with those reported in the literature for vitexin. The identical nature of *3-A* to vitexin is further substantiated by the finding of *p*-hydroxybenzoic acid, phloroglucinol and apigenin as degradation products in the NaOH degradation and alkali fusion. Also the i.r. spectrum of *3-A* closely resembles that given in the literature for vitexin[14] and orientioside[12] (which was later reported to be vitexin[15]). Additional similarity in these two materials is indicated by the production of arabinose with 6 N HCl acid hydrolysis.[14]

The acid hydrolysis of the compound *4* yielded glucose and a phenolic compound *4-A*. The compound *4-A* gave the same u.v. spectrum and R_f values in several solvent systems and the same colors with chromogenic reagents as quercetin. This information characterized the compound *4* as a phenolic compound containing glucose and quercetin.

The low concentration of the penolic compound *1* in the original extract, the ease of oxidation of the compound, and the fact that its physical properties were so closely related to sugars and some amino acids, made separation of the plant phenol difficult. The similar chromatographic behavior and the similar colors produced upon dyeing with chromogenic reagents indicate that the compound *1* is the substance that Harrison obtained, oxidized and found toxic to *Cercospora beticola* in culture.

The plant phenol *1* was isolated in milligram quantities by use of multiple cellulose column chromatography. The results, of chromogenic tests, R_f behavior in different solvents, melting points, u.v. absorption and i.r. absorption spectra and the preparation of derivatives, show that phenol *1* and 3-hydroxytyramine are the same.

EXPERIMENTAL

Preparation of Plant Extract (Ethanol Extraction)

Leaves of *Beta vulgaris* L. were cut at the junction of the petiole and the blade, frozen immediately and stored at minus 10° until analysis. At the time of analysis 15 g of frozen leaves were placed in a Waring Blendor and ground in 75 ml of 95% ethanol. The mixture was filtered using an additional 25 ml of 95% ethanol to rinse the blender. The filtrate was allowed to stand at room temperature for an hour and then refiltered. The filtrate was taken to dryness under vacuum and the residue dissolved in 10 ml of water. This aqueous solution was adjusted to pH 2·5 using HCl. This acidic solution was extracted with ether (3 × 10 ml). The aqueous layer was concentrated by vacuum to a volume of a few milliliters and used for chromatographic analysis.

A second method of extraction (1-pentanol-extraction) was used for phenolic compound *1*. Fifteen g of frozen leaves were ground in 75 ml of 1-pentanol containing 0·5% conc. HCl. The leaves were ground in a Waring Blendor for a period of 3 min, filtered, washed, and the filtrate extracted with a 15 ml portion of distilled water. The aqueous phase was adjusted to a pH of 1·5 using HCl, placed in small vials, and stored in a freezer.

Chromatographic Isolation of Compounds 3, 4 and 1

The isolation of the compounds corresponding to Spots *3* and *4* was accomplished by chromatography on Whatman No. 3 paper using 5% acetic acid.

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Several two-dimensional ascending chromatograms of the extract obtained by the pentanol procedure were developed first in butanol–ethanoic acid–water (4:1:2) and then in 5% acetic acid for the isolation of compound *1*. The air-dried chromatograms were dipped with ninhydrin, diazotized 4-benzoylamino-2, 5-dimethoxyaniline and aniline diphenylamine, and sprayed with Arnow's reagent.

Hydrolysis of Compounds 3 and 4

The concentrated extracts of compounds *3* and *4* were hydrolyzed with an equal volume of 2 N HCl for 30 min. The solution was filtered, concentrated, and examined chromatographically.

Isolation of Compound 3-A

One hundred g of leaves were hydrolyzed in 2 N HCl at 100° for 20 min. The cooled filtrate was extracted with 1-pentanol (3 × 50 ml), and the extract concentrated and allowed to stand for 24 hr. The yellow amorphous precipitate which formed was dissolved in boiling 95% ethanol. The solution was filtered and taken to dryness. The recrystallized (80% dioxane) yellow residue obtained was identical in chromatographic behavior to the hydrolysis product of compound *3* (m.p. 262–263°) (Found: C, 57·09, 57·18; H, 4·69, 4·80. $C_{21}H_{20}O_{10}$ required: C, 58·33; H, 4·66%). The acetate (pyridine/acetic anhydride had m.p. 263–266°) (Found: C, 57·59, 57·41; H, 4·77, 4·78; acetyl, 43·26, 43·71%, M.W., 784, 792. $C_{35}H_{34}O_{18}$ required: C, 56·60; H, 4·61; acetyl, 40·76%, M.W., 742).

6 *N HCl Hydrolysis of Compound 3-A* was carried out for 14 hr at 95°. The products were examined chromatographically.

KOH Fusion of Compound 3-A

Fifty mg of the *3-A* and 0·5 g KOH in 1 ml H_2O was heated until a syrup formed. The syrup was cooled, acidified, and extracted with ether. The ether was evaporated and the residue subjected to two-dimensional chromatography using 1-butanol/ethanoic acid/water (4:1:2) and 5% acetic acid. The compounds were located using diazotized sulfanilic acid and diazotized 4-benzolylamino-2,5-dimethoxyaniline. They were identified in the usual manner using authentic markers. Confirmation was made in four solvent systems.

Degradation of Compound 3-A with 5 N Sodium Hydroxide

Fifty mg of the *3-A* in 45 ml of 5 N NaOH was refluxed under N_2 for 30 min. The mixture was acidified with HCl and extracted with ether (3 × 25 ml). The ether extract was examined chromatographically as previously described.

Cellulose Column Chromatography

To 150 ml of a solution composed of 2-butanone, formic acid, propanone and water (40:1:2:6) were added with vigorous stirring 15 g of cellulose (Whatman column chromedia CF11).

Preparation of Extracts for Cellulose Columns (Phenolic Compound (1))

A 100 ml portion of sugar beet leaf extract was extracted with two 15 ml portions of diethyl ether to remove traces of 1-pentanol. The aqueous phase was lyophilyzed and the residue taken up in 0·25% conc. HCl in methanol, dried and taken up in 2 ml of 0·25% conc. HCl in methanol. The solution was applied to the cellulose column and separated

with 2-butanone–propanone–methanoic acid–water (40:2:1:6). Fractions were collected and those giving a positive Arnow's test were combined (45 ml) and extracted with ether (25 ml). The organic phase was extracted seven times with 1·2 ml portions of 0·2 N HCl and the combined aqueous phase adjusted to a pH of 2·0.

Absorption Spectra

The u.v. spectra were determined using a Bausch and Lomb Spectronic 505 spectrophotometer. The i.r. spectra were obtained using KBr pellets on a Beckman Model IR-5.

Preparation of Derivatives

The picrate and styphnate of the purified plant phenol *1* and of 3-hydroxytyramine were prepared as described in Shriner[16] and Buelow and Gisvold.[17]

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