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SPECIFIC AND RAPID DETERMINATION OF D-APIOSE

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Abstract—A sensitive and specific determination of D-apiose based on the reaction with cysteine after acid degradation in the presence of fructose is described. The influence of complexing agents on the chromatographic and electrophoretic properties of D-apiose is demonstrated.

INTRODUCTION

THE BRANCHED-chain pentose D-apiose (3-*C*-hydroxymethyl-D-*erythro*-furanose) occurs in higher plants as part of glycosides or cell-wall polysaccharides. Extensive paper chromatographic studies by Duff[1,2] and Beusekom[3] have yielded interesting results on the distribution of apiose. It is hoped that the specific test described in the present communication may be useful in future work on the chemotaxonomy of D-apiose.

RESULTS AND DISCUSSION

The Colorimetric Determination of Apiose

Duff[1,2] and Beusekom[3] estimated the amount of isolated apiose visually from the intensity of the colour produced on paper with the benzidine–trichloroacetic acid reagent. During work aimed at the isolation of UDP-apiose,[4] apiose was found to be highly reactive in the cysteine–H_2SO_4 test, described by Dische and Dische[5] for the determination of tetroses, in which it gives an absorption maximum near 463 nm (Fig. 1). Maximum colour is produced about 8 hr after addition of cysteine and remains essentially stable for a further 24 hr. Apiosides react similarly without prior hydrolysis. The readily water-soluble furcatin [(D-apiosyl)-1 → 6-(β-D-glucosyl)]-*p*-vinylphenol[6] or the more easily accessible apiin, which is a mixture of the 7-*O*-[(β-D-apiosyl)-1 → 2-(β-D-glucosyl)]-derivatives of apigenin and chrysoeriol,[7] may be used as standards since they are stable crystalline substances.

The following sugars were tested in concentrations of 200 μg/ml: glucose, galactose, mannose, fructose, sorbose, ribose, xylose, arabinose, lyxose, glucuronic acid, glucuronolactone and galacturonic acid. A small and unspecific rise in background absorption was observed with these sugars in procedure C (see Experimental). A correction can be applied by dichromatic reading. The difference in extinction at wavelengths 463 nm and 500 nm

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[1] R. B. DUFF and A. H. KNIGHT, *Biochem. J.* **88**, 33P (1963).
[2] R. B. DUFF, *Biochem. J.* **94**, 768 (1965).
[3] C. F. VAN BEUSEKOM, *Phytochim.* **6**, 573 (1967).
[4] H. SANDERMANN and H. GRISEBACH, *Biochim. Biophys. Acta* **156**, 435 (1968); *European J. Biochem.* **6**, 404 (1968).
[5] Z. DISCHE and M. R. DISCHE, *Biochim. Biophys. Acta* **27**, 184 (1958).
[6] S. HATTORI and H. IMASEKI, *J. Am. Chem. Soc.* **81**, 4424 (1959).
[7] H. GRISEBACH and W. BILHUBER, *Z. Naturforsch.* **22b**, 746 (1967).

was found to be proportional to the concentration of apiose in the range of 5 to 500 mμmol. Differences in molar extinction coefficients ($\epsilon_{463}-\epsilon_{500}$) are $24{\cdot}0\times10^3$ for free apiose and $28{\cdot}9\times10^3$ for glycosidically bound apiose. The latter value was obtained with apiin as well as with furcatin. Rhamnose also has maximal absorption near 463 nm with a difference in extinction coefficients ($\epsilon_{463}-\epsilon_{500}$) of $4{\cdot}8\times10^3$. Rhamnose, but not apiose, yields a second absorption maximum near 388 nm ($\epsilon_{388}=12{\cdot}0\times10^3$).

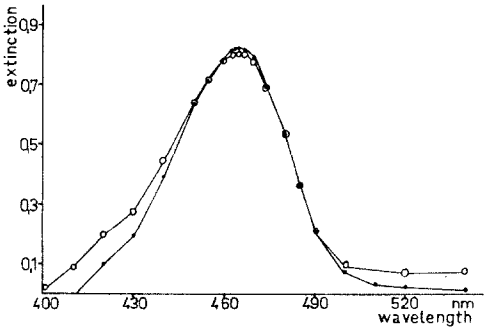

FIG. 1. ABSORPTION SPECTRA PRODUCED BY FREE OR GLYCOSIDICALLY BOUND APIOSE IN THE COLOUR TEST DESCRIBED AS PROCEDURE C BELOW.

87 mμmol of free apiose (●—●—●),
68 mμmol of furcatin (o—o—o).

Tetroses are strongly reactive in the test described below[5] but have not yet been isolated from plant cell walls. Apiose is also reactive in the secondary cysteine test with addition of mannose[8] ($\lambda_{max.}$ 467 nm). According to Dische[8] short-chain sugars do not react.

After treatment with H_2SO_4 (procedure A, see Experimental) apiose yields a sharp absorption peak at 276·5 nm ($\epsilon_{276{\cdot}5}$ $8{\cdot}0\times10^3$). In the presence of fructose (procedure B, see below) the absorption maximum is shifted to 449 nm. Subsequent addition of cysteine results in a further spectral shift to 463 nm and was found advantageous for the sharpness of the absorption peak observed and for maximum colour yield. Procedure D might be useful for further differentiation when examining sugar mixtures. For example, the absorption value ($\epsilon_{463}-\epsilon_{500}$) of apiose obtained in procedure C is lowered in procedure D by about 30 per cent whereas that of rhamnose increases in spite of the dilution with water.

TABLE 1. PROPERTIES OF COLOURED PRODUCTS FORMED IN TEST PROCEDURES A, B AND C

Procedure	Reactants	Wavelength of absorption maximum (nm)	Molar extinction value $\epsilon\times10^{-3}$ (mol^{-1} cm^2)	
A	Apiose + H_2SO_4	276·5	$\epsilon_{276{\cdot}5}$	8·0
B	Apiose + fructose + H_2SO_4	449	($\epsilon_{449}-\epsilon_{500}$)	14·1
C	Apiose + fructose + H_2SO_4 + cysteine	463	($\epsilon_{463}-\epsilon_{500}$)	24·0
C	Apioside + fructose + H_2SO_4 + cysteine	463	($\epsilon_{463}-\epsilon_{500}$)	28·9

[8] Z. DISCHE, in *Methods of Biochemical Analysis* (edited by D. GLICK), Vol. 2, p. 334, Interscience, New York (1955).

For taxonomic purposes a rapid procedure was developed for the qualitative and quantitative detection of apiose in crude cell-wall material. After the cells are broken up with liquid N_2 and extracted with ethanol, the dried residue is hydrolysed under mild conditions. Apiose is split off preferentially and can be determined in the supernatant after centrifugation. Figure 2 shows that parsley does not contain significant amounts of apiose in its cell wall, as has been demonstrated previously by acid hydrolysis and paper chromatography.[9] For *Lemna minor* strain M 11 (kindly donated by Professor R. Kandeler, Wuerzburg), which was cultivated under sterile conditions on mineral medium, an apiose content of 9 per cent of dry weight was calculated by using the difference in absorption at 463 and 500 nm (Fig. 2). *L. minor*, collected from a local pond, contained 12 per cent apiose (dry weight).

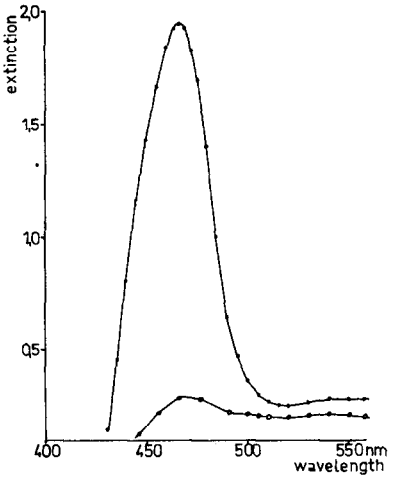

FIG. 2. REACTION OF HYDROLYSATES FROM PARSLEY (*Petroselinum crispum* Hoffm.) (o—o—o) AND *Lemna minor* L. (●—●—●) IN THE CYSTEINE–H_2SO_4 TEST FOR APIOSE, PROCEDURE C.

Each curve corresponds to 310 μg of crude cell-wall material (dry weight).

Detection of Apiose by Chromatography

Apiose has been separated from other sugars by gas chromatography of the trimethylsilyl ethers of the free sugars[10,9] or the sugar alcohols.[11] Conventional paper chromatography as applied by Duff[1,2] and Beusekom[3] is possible though restricted by the similarity in chromatographic behaviour of apiose, rhamnose, fucose and ribose.[4,11] This difficulty has recently been overcome by the use of borate-containing solvents and by use of the aniline-phthalate spray which reacts with apiose to give a specific fluorescence.[4] The phenylboronic acid-solvent of Ferrier *et al.*[12] has proven useful for separation of the pentoses.[13]

High-voltage paper electrophoresis in 0·05 m borate buffer of pH 9·2 can be useful in special cases, especially in the presence of rhamnose or mannose. In molybdate buffer of

[9] H. GRISEBACH and H. SANDERMANN, *Biochem. Z.* **346**, 322 (1966).
[10] H. GRISEBACH and U. DOEBEREINER, *Z. Naturforsch.* **21b**, 429 (1966).
[11] R. M. ROBERTS, R. H. SHAH and F. LOEWUS, *Plant Physiol.* **42**, 659 (1967).
[12] R. Y. FERRIER, W. G. OVEREND, G. A. RAFFERTY, H. M. WALL and N. R. WILLIAMS, *Proc. Chem. Soc.* 133 (1963).
[13] H. SANDERMANN, G. T. TISUE and H. GRISEBACH, *Biochim. Biophys. Acta* **165**, 550 (1968).

pH 5·0 apiose is well separated from most other sugars. According to Bourne *et al.*[15] migration of pyranoid sugars in molybdate buffer is observed only when a 1,2,3-*cis*,*cis*-triol-system can be formed. The furanose D-apiose could adopt the required stereochemistry (*a*) by using hydroxyl groups OH (1), OH (2) and OH (3) in the α-D-*erythro*-configuration, without utilizing the branch, or (*b*) by using the hydroxymethyl branch OH (3′), OH (3) and OH (2) in the α- or β-D-*erythro*-configuration. The same tridentate structures or suitable *cis*-diol groupings may be responsible for complex-formation with phenylboronic acid.

TABLE 2. INFLUENCE OF PHENYLBORONIC ACID ON R_fS OF THE PENTOSES; $R_{picr.}$-VALUES OF THE PENTOSES IN ELECTROPHORESIS WITH MOLYBDATE BUFFER

Solvent*	Arabinose	Xylose	Lyxose	Ribose	Apiose
1	0·21	0·24	0·26	0·28	0·32
2	0·20	0·26	0·34	0·73	0·84 (0·36)†
3	0·09	0·12	0·13	0·17	0·19
4	0·09	0·13	0·16	0·59	0·87 (0·26)†
5	<0·4	<0·4	0·2; 1·2‡	0·9‡	2·0 (1·6)†

* Schleicher Schuell 2043b paper was used for descending paper chromatography, Macherey Nagel MN 214 paper for high-voltage paper electrophoresis. Sugar spots were made visible with the aniline phthalate spray.

Solvents: (1) *n*-butanol/ethanol/water (4:1:5 v/v/v, upper phase); (2) solvent 1 with addition of 5% (w/w) of phenylboronic acid;[12] (3) EtOAc/HOAc/water (9:2:2 v/v/v); (4) solvent 3 with addition of 0·55% (w/v) of phenylboronic acid;[14] (5) sodium molybdate buffer of pH 5·0, prepared as described by Bourne *et al.*[15] Reference substance: picric acid.

† A second faint spot was visible after spraying with aniline phthalate, cf. Ref. 4.

‡ Some streaking was observed. The data refer to the centres of the apparent main spots.

EXPERIMENTAL

Authentic D-apiose was prepared from *Posidonia australis* via the di-*O*-isopropylidene derivative.[16] Other sugars and reagents were commercial products of analytical grade.

Parsley (*Petroselinum crispum* Hoffm.) and *Lemna minor* L. were grown in the Botanical Gardens, University of Freiburg i. Br. Test tubes of 1·5 × 16 cm were used. Molar extinction values refer to the amount of sugar or glycoside actually used in the assay. It is essential to run a parallel blank and standard sample in each assay.

Colorimetric Determination of Apiose

Procedure A. While cooling in ice water, 2·25 ml of conc. H_2SO_4/water (6:1 v/v) are carefully added to 0·25 ml of sugar solution, as described in procedure B. After heating for exactly 3 min in a vigorously boiling water bath and cooling in tap water, u.v. measurements are made. These conditions were chosen in analogy to procedures B and C. Maximal yield of u.v.-absorbing products is not achieved. The final test volume was taken to be 2·5 ml.

Procedure B. To 0·25 ml of sugar solution is added 0·25 ml of an aqueous solution of fructose (0·1% w/v). The mixture is cooled in an ice bath and 2·25 ml of conc. H_2SO_4/water (6:1 v/v) is carefully added so as to form two phases. The layers are mixed by vigorous shaking in ice water. The homogeneous mixture is held in tap water for 2 min, then transferred to a boiling water bath and heated for exactly 3 min. After cooling

[14] E. J. BOURNE, E. M. LEES and H. WEIGEL, *J. Chromatog.* **11**, 253 (1963).
[15] E. J. BOURNE, D. H. HUTSON and H. WEIGEL, *J. Chem. Soc.* 4252 (1960).
[16] D. J. BELL, in *Methods in Carbohydrate Chemistry* (edited by R. L. WHISTLER and M. L. WOLFROM), Vol. 1, p. 260, Academic Press, New York (1962).

with tap water the samples are kept at room temperature for 10 hr before absorbance is determined. The final volume was taken to be 2·75 ml.

Procedure C. 0·25 ml of sugar solution are treated as described in procedure B. After heating for 3 min and cooling with tap water 50 μl of an aqueouss olution of cysteinex HCl (3% w/v) are added with shaking and the mixture is left at room temperature. Absorbance is measured from 7–30 hr after addition of cysteine. The final test volume was taken to be 2·8 ml.

Procedure D. In presence of other sugars it might be useful to add 0·6 ml of water without cooling to a sample that has been prepared according to procedure C and of which spectral data have been taken. After 8 to 12 hr at room temperature, spectral data are redetermined.

Test of Plant Material for the Presence of Apiose

Plant tissue (1–50 mg) is placed in a centrifuge tube, frozen in liquid N_2 and thoroughly crushed with a spatula. The material is extracted several times with hot ethanol. The extract is centrifuged each time and the supernatant discarded. The residue is dried and hydrolysed with 1 ml of 0·2 N H_2SO_4 (20 min, 100°). After centrifugation an aliquot of the supernatant is tested for apiose following procedure C.

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