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FERULIC ACID AS A COMPONENT OF A COMPLEX CARBOHYDRATE POLYMER OF BROMELAIN

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Abstract—Ferulic acid was identified bound to a complex carbohydrate polymer of bromelain via ester bonds. Other components of the polymer are glucose, galactose, mannose, ribose, xylose, glucuronic acid and galacturonic acid.

INTRODUCTION

STANDARD bromelain, a protease preparation made by precipitating the colloids in pineapple stem juice with acetone[1] contains in addition to proteases smaller amounts of other enzymes,[2] neutral maltose polymers[3] inorganic salts, and an acidic carbohydrate polymer. The u.v. spectrum of this enzyme mixture shows, in addition to the typical protein absorption, a moderate shoulder at 325–330 nm, a very slight shoulder at 290 nm and a partial filling in of the protein trough at 255 nm. In weak alkali the 325 shoulder shifts to a definite peak at 375 nm; in strong base the peak slowly disappears and gives rise to a new peak at 345 nm.

The material responsible for some of the anomalies in the normal protein u.v. spectrum can be separated by various techniques. By passing low ionic strength enzyme mixture over either CMC Sephadex or carboxylic anion resin columns at pH 6·0+0·4, the basic proteins can be adsorbed, allowing the acidic and neutral carbohydrate polymers to pass through. The polymer may also be partitioned between either a water–phenol system[4] or an ammonium sulfate acetone system,[5] the proteins staying in the aqueous phase and the acidic carbohydrate polymers going into the organic phase. Sideris[6] prepared a material which was undoubtedly similar to the preparation described in this paper, by precipitating the acidic carbohydrate polymer as a calcium salt. The simplest method of preparing large amounts of the crude carbohydrate complex is to precipitate the protein with trichloroacetic acid and to recover the carbohydrate polymer from the supernatant.

This report is part of a study of some of the non-protein components of standard bromelain. The specific aim of this study was to identify the non-protein material responsible for the 325–330 nm absorption shoulder.

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[2] R. M. HEINICKE and W. A. GORTNER, *Econ. Botany* **11**, 225 (1957).
[3] J. TU and R. M. HEINICKE, unpublished data (1956).
[4] A. PUSZTAI, *Biochem. J.* **99**, 93 (1966).
[5] S. CHRISTIAN and R. M. HEINICKE, unpublished data (1958).
[6] C. SIDERIS, *Plant Physiol.* **3**, 309 (1928).

RESULTS

By trichloroacetic acid precipitation of the proteins followed by dialysis and freeze drying, we obtained about a 5 per cent yield from standard bromelain of a complex carbohydrate polymer mixture. This material was white and fluffy, contained about 52 per cent sugars calculated as glucose by the anthrone test,[7] 0·1–0·3 per cent phenolic acids[8] and about 1 mole of carboxyl groups per 3000 g of material. Figure 1 illustrates the absorption spectrum of the material in neutral solution, in moderately basic solution, and in strong alkali held for 1 hr.

Saponification of this polymer yielded easily crystallizable amounts of a material suspected to be ferulic acid. Table 1 illustrates the close similarity in properties between authentic

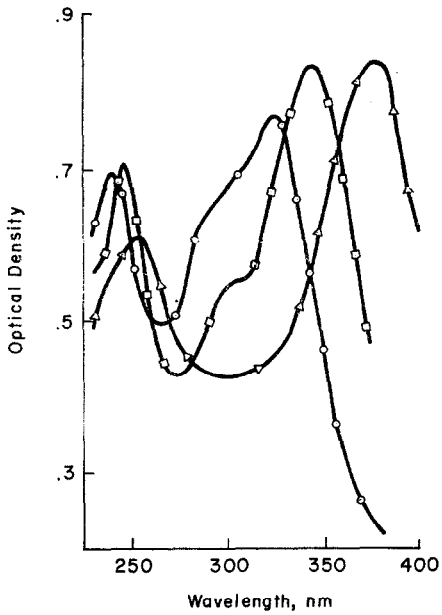

FIG. 1. U.V. SPECTRA OF 0·1% COMPLEX CARBOHYDRATE POLYMER OF BROMELAIN.
○, in water.
△, in 0·005 N NaOH.
□, in 1 N NaOH held for 1 hr.

ferulic acid and the crystals isolated from the pineapple stem. The isolated crystals also had the same R_f value as authentic ferulic acid in a number of thin-layer chromatographic solvent systems. Acid hydrolysis of the complex gave ferulic acid plus other fluorescent compounds. One of those was a degradation product of ferulic acid. Heating ferulic acid with the same strength of acid gave similar degradation products.

The neutral sugars, identified in the acid hydrolysate, were glucose, galactose, mannose, arabinose and xylose. The uronic sugars were glucuronic and galacturonic acids. Although we suspected that glucosamine or acetylglucosamine might be present[9] we were unable

[7] T. A. SCOTT and E. H. MELVIN, *Anal. Chem.* **25**, 1656 (1953).
[8] H. G. BRAY, B. G. HUMPHRIS, W. V. THORPE, K. WHITE and P. B. WOOD, *Biochem. J.* **52**, 416 (1952).
[9] R. M. HEINICKE and R. SUGAI. These materials are present in the unfractionated enzyme mixture, unpublished data (1958).

to find any trace of these sugars in the complex mixture.[10] Either they are not present or our isolation procedures destroyed them.

Although we suspected that at least trace amounts of *p*-coumaric and caffeic acid might also be present, we failed to find any traces of these acids even when we used sodium borohydride to prevent oxidation of the caffeic acid.[11]

TABLE 1. SPECTRAL PROPERTIES OF FERULIC ACID

	λ_{max} (nm) in				
	EtOH	EtOH+ 5% $AlCl_3$	EtOH+ NaOEt	EtOH Sat. with NaOAc	EtOH+Sat.'d. with NaOAc+H_3BO_3
Ferulic acid (authentic)	240	241	241	233	237
	295*	297*	308*	286	289
	321	325	344	310	313
Ferulic acid (isolated)	240	241	241	235	236
	295*	297*	308*	287	289
	321	325	346	312	313

* Shoulder.

DISCUSSION

Although the occurrence of ferulic acid carbohydrate complexes is undoubtedly widespread in plants, so far only one other plant, wheat, has been reported in the literature to contain such a complex. The glycoprotein reported in wheat has been shown to contain ferulic acid linked in an ester linkage to the carbohydrate moiety.[12–15] It has been suggested that this ferulic ester of pentosan protein is deposited in the cell-wall lignin fraction as a method of disposing of a toxic material.

The material from the pineapple plant resembles the wheat pentosan but also differs from it in several respects. The pentoses, typically cell-wall sugars, form a smaller part of the total sugars in the pineapple polymer than in the wheat. Also in the pineapple polymer, peptides, while present, are relatively minor constituents. As in the wheat material the ferulic acid is attached as an ester.

We still have no clear picture of the function of these materials. Some preliminary observations on the increases in the amounts of these materials when stems are stored for long periods at 38° suggest that these polymers may play a role in water retention.

EXPERIMENTAL

Materials

The standard bromelain was obtained from our pilot plant. The ion retardation resin, AG 11A8, was bought from the Bio-Rad Laboratories. All solvents were distilled before use and ether was freed from peroxides with acidic ferrous sulfate[16] prior to distillation.

[10] Stoffyn and Jeanloz method. R. W. WHEAT, in *Methods in Enzymology* (edited by E. F. NEUFELD and V. GINSBERG), Vol. 8, p. 60, Academic Press, New York (1966).
[11] H. A. SCHROEDER, *Phytochem.* **6**, 1589 (1967).
[12] W. KÜNDIG, H. NEUKOM and H. DEUEL, *Helv. Chim. Acta* **44**, 823 (1961).
[13] H. FAUSCH, W. KÜNDIG and H. NEUKOM, *Nature* **199**, 287 (1963).
[14] W. KÜNDIG and N. NEUKOM, *Helv. Chim. Acta* **46**, 1423 (1963).
[15] H. NEUKOM, *Getreide Mehl* **14**, 13 (1964); *Chem. Abstr.* **61**, 2392 (1964).
[16] L. F. FIESER, *Experiments in Organic Chemistry*, p. 287, D. C. Heath and Company, Boston (1957).

Separation of Complex Carbohydrate Polymer of Bromelain

Standard Bromelain (100 g) was suspended in 200 ml water and centrifuged. Solid trichloroacetic acid was added to the clear supernatant to make it 10 per cent solution and then centrifuged. Acetone was added to the milky supernatant in ratio of 2 to 1 and then allowed to stand overnight at 0°. The main portion of supernatant was decanted and the residue was centrifuged, washed with acetone, centrifuged and then dissolved partly in 200 ml water. After removal of insolubles, the supernatant was dialyzed against cold running water for 5 days, and then freeze-dried. The white fluffy solid weighed 5 g. In neutral aqueous solution, the u.v. spectrum showed a 325 nm with a shoulder at 290 nm and the minimum at 268 nm. In dilute alkali, the maximum was at 375 nm and the minimum at 318 nm.

Saponification and Identification of Ferulic Acid

3 g dialyzed carbohydrate polymer of bromelain in 150 ml 2 N NaOH was allowed to stand at room temperature for 4 hr, after which time the solution was cooled, acidified with conc. HCl, saturated with NaCl and extracted with ethyl acetate. After drying over Na_2SO_4, the ethyl acetate was removed under reduced pressure and then ether was added to the residue. After drying over Na_2SO_4, the ether was evaporated to about 3 ml for the thin-layer chromatography. The residue was then streaked on a silica gel G plate and developed in benzene–dioxane–acetic acid (90:25:4 v/v).[17] The most prominent fluorescent band was scraped off, eluted with alcohol and rechromatographed. By comparison of thin-layer plates and u.v. spectrum with an authentic sample, the fluorescent band was identified as ferulic acid.

Acid Hydrolysis and Identification of Sugars

4 g dialyzed carbohydrate polymer of bromelain were refluxed in 200 ml 0·1 N HCl for 24 hr. The solution was filtered and evaporated to dryness under reduced pressure. To the residue was added water and evaporated to dryness to remove the excess of HCl. This was repeated two more times. The residue dissolved in water was passed through an Amberlite IR-120 resin in H^+ form and then Duolite A-14 resin in OH^- form. The effluent was evaporated under reduced pressure at 65° to about 10 ml and chromatographed on Whatman No. 1 paper using first *n*-propyl alcohol–benzyl alcohol–85 per cent formic acid–water (50:72:20:20 v/v)[18] and then *n*-butanol–pyridine–water (10:3:3 v/v).[19] The dried chromatogram was developed with *p*-aminobenzoic acid spray[20] and the five spots were identified as glucose, galactose, mannose, arabinose and xylose.

The acidic fraction from Duolite A-14 was recovered by elution with 1 N NaOH. The effluent was neutralized with conc. HCl, evaporated to about 15 ml under reduced pressure, and then passed through an ion retardation resin, AG 11A8, to remove inorganic salts. The salt-free sample was chromatographed on Whatman No. 1 paper using *n*-butanol–acetic acid–water (50:15:35 v/v).[21] After spraying the dried chromatogram with ammoniacal silver nitrate[22] two brown spots appeared on heating which were identified as glucuronic and galacturonic acid. In the basic fraction from the Amberlite IR-120 resin no amino sugars could be detected.

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[21] E. D. DAVIDSON, In *Methods in Enzymology* (edited by F. NEUFELD and V. GINSBURG), p. 56, Academic Press, New York (1966).
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