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3,4-DIHYDROXYBENZALDEHYDE, A FUNGISTATIC SUBSTANCE FROM GREEN CAVENDISH BANANAS

DAWN MULVENA, EDWIN C. WEBB and BURT ZERNER

Department of Biochemistry, University of Queensland, St. Lucia, Queensland, Australia

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Abstract—A fungistatic substance has been isolated from the outer skin of green Cavendish bananas and identified as 3,4-dihydroxybenzaldehyde. The compound has been shown to inhibit the growth of *Gloeosporium musarum*, a fungus which causes ripe fruit rot in the banana.

INTRODUCTION

WHILE investigating the factors controlling latent infections by *Colletotrichum* in tropical fruits, Simmonds[1] found that hot water extracts of green banana skins were toxic to *Gloeosporium musarum*, one of the fungi parasitic on the banana. *G. musarum* is one of the species of *Colletotrichum* and it causes ripe fruit rot in the banana. In 1929, Link, Angell and Walker[2] isolated from the outer scales of onions resistant to infection by *Colletotrichum circinans* (the fungus which causes the disease known as onion smudge), a toxin which they identified as 3,4-dihydroxybenzoic acid (protocatechuic acid). The preformed toxin[3] was found only in resistant varieties and none could be isolated from onions susceptible to this disease. The conditions of isolation were, however, rather severe. Link and Walker[4] later demonstrated that catechol was an approximately equal contributor to the toxicity of a resistant onion-scale extract.

It was therefore of interest to determine the chemical nature of the fungistat isolated from green Cavendish bananas and to examine its possible relationship to protocatechuic acid.

RESULTS

Variation of Fungistatic Activity

The fungistatic activity extracted from banana skins falls to low levels as the fruit ripens, and this is reflected in the development of the fungus on the banana. The infection is latent in the green banana, but as the banana ripens the fungus develops and causes rotting of the fruit. Simmonds[1] reported that other factors (nutritional and enzymic) in addition to the fungistatic activity of the skin affected the development of the fungus. He concluded that the mechanism of latent infection is a complex interrelationship of these factors.

The fungistatic activity also showed a seasonal variation within the green tissue. The activity gradually increased to a maximum in the winter months (July–September) and then decreased to a minimum in the summer months (December–January), when the anthracnose infections are most troublesome.

[1] J. H. SIMMONDS, *Queensland J. Agr. Sci.* **20**, 373 (1963).
[2] K. P. LINK, H. R. ANGELL and J. C. WALKER, *J. Biol. Chem.* **81**, 369 (1929).
[3] P. J. ALLEN, in *Plant Pathology* (edited by J. G. HORSFALL and A. E. DIMOND), Vol. 1, p. 435, Academic Press, New York (1959).
[4] K. P. LINK and J. C. WALKER, *J. Biol. Chem.* **100**, 379 (1933).

Association of Fungistatic Activity with Compounds Isolated from Banana Skins

Chromatography on paper in propan-1-ol–water (2:98) of exhaustive ether extracts of charcoal to which the fungistatic activity had been adsorbed, showed that a number of compounds had been extracted. In the first year of this investigation, the fungistatic activity was associated with two spots (R_fs 0·57 and 0·67; purple–black in u.v. light) designated B1 and B2. In the following season, the amount of B2 decreased markedly and appeared only in trace amounts at the time of maximum fungistatic content (August–September). Therefore the material isolated and identified in this study is B1.

Identification of B1

KOH fusion of B1 gave protocatechuic acid as the major decomposition product. B1 was purified (see Experimental) and identified as 3,4-dihydroxybenzaldehyde by m.p., spectral and chromatographic comparison with authentic material.

Fungistatic Activity of 3,4-Dihydroxybenzaldehyde (B1)

Both natural and synthetic compounds were found to inhibit the germ tube growth of *Gloeosporium musarum* to the same extent and in a similar manner. A concentration of 250 μg/ml of B1 inhibited the growth of the germ tube 35 per cent in comparison to the germ tube length in a control. A concentration of 500 μg/ml completely inhibited germination. However, it was noted during these experiments that there was some variation in these figures. Walker[5] reported similar results in his studies and showed that variation in the results was due to a difference in the age of the cultures used.

DISCUSSION

With the identification of B1 as 3,4-dihydroxybenzaldehyde, the possibility is raised that the toxin of onions resistant to *Colletotrichum circinans* may not be protocatechuic acid but the more labile aldehyde, which could have been oxidized during the isolation procedure reported for protocatechuic acid.[2,6] It further suggests that 3,4-dihydroxybenzaldehyde may exhibit a spectrum of fungistatic activity against *Colletotrichum*, and to this end we are checking its activity against several species of *Colletotrichum*.

The nature of the other compound B2, however, is less clear. From the limited amount of work we were able to do on this material, it would appear to be a phenolic derivative other than protocatechuic acid or catechol. Its fungistatic activity appears to be of the same order as that of B1.

Field tests of the efficiency of 3,4-dihydroxybenzaldehyde in the control of latent anthracnose are currently being made. The properties of 3,4-dihydroxybenzaldehyde (fungistatic activity, solubility in water (~0·3 M) and its relatively low cost) would appear to make it a suitable additive to wash water in the presentation of bananas for marketing.

EXPERIMENTAL

Bioassay of Fungistatic Activity

The fungistatic activity was assayed with spores of *Gloeosporium musarum*, type 14902-E3. The germ tube growth of the spores is inhibited by the fungistat, that is, the greater its concentration, the smaller the germ tube length. The relationship between germ tube length under test conditions expressed as a percentage of a control and fungistatic activity is negatively sigmoidal. The spores were developed in drops containing nutrient for 15–20 hr at 22°. The germ tube lengths were measured by means of a graticule fitted into a microscope

[5] J. C. WALKER, C. C. LINDEGREN and F. M. BACHMANN, *J. Agr. Res.* **30**, 175 (1925).
[6] K. P. LINK, A. D. DICKSON and J. C. WALKER, *J. Biol. Chem.* **84**, 719 (1929).

eyepiece. An arbitrary standard activity curve was set up in which a given percentage growth was related to a given number of units/ml. Therefore, a semiquantitative estimation of fungistatic activity was available. This method was used to follow fungistatic activity throughout the various experiments.

Extraction of Toxin from the Skins

The outer skin of the banana, which contains all the fungistatic activity,[1] was cut into thin slices and homogenized with 95% ethanol in a Waring blendor (1 g/3 ml). The ethanol was evaporated to give a crude aqueous extract of the toxin (solution A). For the green tissue, the pH of this extract was 4·3–4·5, while that obtained from ripe tissue had a slightly higher pH (4·5–4·7). Initially, the extract was red, but it darkened to brown on standing. Since it was found that heating of the crude extract markedly decreased the fungistatic activity, and also that standing overnight at room temperature caused a 25 per cent loss of activity, all solutions were stored at 4° where the crude extract could be kept for over a month before there was any measurable decrease in activity. This extraction procedure was used to follow the variation of fungistatic activity as the banana ripens and also the seasonal variation in the green skin.

Purification of B1

Charcoal (B.D.H. activated) was added to solution A until all the fungistatic activity had been adsorbed. The charcoal was extracted with diethyl ether until all the activity was recovered. The ether extract (solution B) was evaporated and the solid material obtained was dissolved in propan-1-ol–water (2:98) and chromatographed on a cellulose column with the same solvent. A yellow band was collected which contained the fungistatic activity. This solution was extracted three times with equal volumes of ethyl acetate. The ethyl acetate solution was streaked on Whatman 3MM paper and chromatographed in propan-1-ol–water (2:98). The fungistatic activity band was eluted with ethanol or ethyl acetate. This purification on paper was repeated several times, as quickly as possible to avoid loss of activity due to oxidation. The purified solution was evaporated to a small volume, water was added and, on standing, light yellow crystals formed. These were shown by chromatography and by mass spectral analysis to have a slight impurity of protocatechuic acid (< 1 per cent). Protocatechuic acid was shown in a separate experiment to have only one-quarter the activity of B1 towards *G. musarum*.

Correlation of Compounds in Solution B and Fungistatic Activity

Solution B was chromatographed in the propan-1-ol–water (2:98) and the spots visualized in u.v. light. The spots were cut out and extracted for 6 hr at 4° with 1 or 2 ml of citrate–phosphate buffer, pH 4·5. The various solutions were assayed using the bioassay already described.

Identification of B1

Crystalline B1 (1 mg) was fused with KOH and the major product of the fusion was identified as protocatechuic acid, by comparison with an authentic sample. B1 and 3,4-dihydroxybenzaldehyde had identical R_f values on Whatman No. 1 paper in butan-1-ol–acetic acid–water (6:2:1) (0·78), 6% HOAc (0·61) and chloroform–propan-1-ol (2:98) saturated with water (0·28). The i.r. spectra of B1 and 3,4-dihydroxybenzaldehyde determined (KBr disc) on a Perkin Elmer-457 i.r. spectrometer were identical. Both B1 and recrystallized 3,4-dihydroxybenzaldehyde melted at 153–154° (lit.,[7] m.p. 153°). The mass spectrum of B1 was determined on an MS9 mass spectrometer using the direct insertion lock. The ion source temperature was 83°. Peaks occurred at *m/e* 138, 137, 109, 81, 55. Accurate mass measurement showed the parent peak at *m/e* 138 to be due to $C_7H_6O_3$. The spectrum is consistent with the following fragmentation:

$$\underset{m/e\ 138}{[C_6H_3(OH)_2\text{—}CHO]^{+\cdot}} \xrightarrow{-H\cdot} \underset{m/e\ 137}{C_6H_3(OH)_2\text{—}C\equiv\overset{+}{O}} \xrightarrow{-CO} \underset{m/e\ 109}{C_6H_3(OH)_2^{+}} \xrightarrow{-CO} m/e\ 81$$

In addition, the spectrum showed a very small peak at *m/e* 154, almost certainly due to protocatechuic acid. Both B1 and 3,4-dihydroxybenzaldehyde had identical u.v. spectrum in 95% ethanol.

Fungistatic Activity

Solutions (1 mg/ml) were made up of B1 and 3,4-dihydroxybenzaldehyde and appropriate dilutions made. Germ tube growths in these solutions were measured after development as described in the bioassay procedure.

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[7] *Chemistry of Carbon Compounds* (edited by E. H. Rodd), Vol. III^B, p. 746, Elsevier, Amsterdam (1963).