Microbial Transformation of Flavonoids

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The ability of a number of fungal spores, and in particular of resting vegetative mycelia, to transform naringin and naringenin was studied. In general, only hydrolytic cleavage of the sugar moieties of naringin to produce prunin and naringenin was observed. Two cultures, Penicillium charlesii and Helminthosporium sativum, also produced two unidentified flavonoid compounds but in very low yields. No transformation of aglycone was detected, although the compound was metabolized by some cultures when supplied as the glycoside prunin. A fluorodensitometric method was developed for the quantitative analysis of flavonoid compounds.

Microbial transformation of flavonoids has not been extensively studied. Udupa et al. (18, 19) incubated (±) flavanone with Gibberella fujikuroi and obtained several compounds: (−)-flavan-4α-ol, 2'-hydroxychalcone, 2',4-dihydroxydihydrochalcone, 2',4-dihydroxychalcone, (±)-4'-hydroxyflavone, and (−)-4'-hydroxyflavan-4α-ol. Degradation of flavonoids, rutin, and phlorizin in particular by various bacteria and molds has been reported (2, 4, 7, 9, 16, 20). The degradation usually involves an initial release of the sugars by intracellular glycosidases, followed by hydrolytic cleavage of the heterocyclic ring of the aglycone (16).

Recent action by the U.S. Food and Drug Administration banned the use of cyclamates as synthetic sweeteners in foods. It has been shown by Horowitz (11) and Horowitz and Gentili (12) that the chalcone and dihydrochalcones corresponding to naringin and neohesperidin are intensely sweet and appear to be free from toxicity to laboratory animals (3). However, the sensation of sweetness from the dihydrochalcones is slow in onset but of lasting duration and also imparts a menthol-like quality. These latter properties are regarded as undesirable characteristics for use in food and beverages.

During a study of the transforming potential of fungal spores, we investigated the ability of conidia to convert naringin, the bitter principle of grapefruit, to a sweetening agent lacking the undesirable properties of the dihydrochalcones. These studies were subsequently extended to include vegetative mycelia. We confined our investigation to naringin, which is available in commercial quantities, and did not investigate the use of other less readily available flavonoid compounds.

MATERIALS AND METHODS

Microorganisms. Microorganisms were maintained on Difco Y-M agar slants at 4 C. To develop inoculum, a small portion of a slant culture was inoculated into 100 ml of YES broth (yeast extract, 2%; sucrose, 15%) in 300-ml indented Erlenmeyer flasks. The inoculated flasks were incubated at 28 C for 48 hr on a rotary shaker (250 rev/min, 5-cm stroke), and the mycelia were recovered by centrifugation and washed six times with 100-ml portions of 0.1 M phosphate buffer, pH 7.0. The washed cells were resuspended in 100 ml of buffer; 10-ml portions of the suspension were added to 90 ml of buffer plus 0.75% naringin in 300-ml Erlenmeyer flasks. For larger-scale experiments, 50 ml of suspended mycelium was added to 450 ml of buffer plus 0.75% naringin in Fernbach flasks. Naringin was purchased from Sunkist Growers, Inc., Ontario, Calif. Bioconversion flasks were incubated at 28 C on a rotary shaker at 250 rev/min.

Spores. Spore suspensions were prepared as previously described (14). For bioconversions, sufficient spores were added to the flasks to give an inoculum of about 10^8 spores/ml.

Extraction of transformation products. At selected times during the incubation period, 5-ml portions were removed and centrifuged, and a small volume of supernatant fluid was spotted directly onto thin-layer chromatography (TLC) plates. For quantitative analyses, the 5-ml sample was extracted twice with 4-ml portions of butan-1-ol; the solvent extracts were combined; and the volume was adjusted to 10 ml. Further dilutions were made with butan-1-ol when required.

TLC. TLC plates, 20 by 20 cm, were coated (0.5 cm) with Silica Gel G-HR (Brinkmann Instruments, Westbury, N.Y.). After being spotted with measured volumes, plates were developed in ethylacetate-isopropanol-water (80:20:10, v/v) until the solvent front was 1 to 2 cm from the top edge. The plates were air-dried, sprayed with a freshly prepared solution of 2% sodium borohydride in methanol, and then exposed to HCl fumes for 15 min (10). Flavanones gave a bluish-red color. The plates were also examined for the yellow color often produced by chalcones, flavones, flavonols,

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isoflavones, and aurones and for the occurrence of fluorescent derivatives under ultraviolet light.

Flavonoid compounds were also detected by spraying the plates with a 2% solution of AICI₃·6H₂O in ethanol to give bright yellow-green fluorescent spots under ultraviolet light.

**Compound isolation.** Isolation of products was achieved by extraction from the supernatant fluid of two Fernbach flasks with two 1-liter portions of butanol. The extracts were combined; the solvent was removed by evaporation, and the residue was dissolved in 40 to 50 ml butanol; insoluble material was removed by centrifugation. The solvent solution was then added to a small amount of 60- to 100-mesh Florisil (Sigma Chemical Co., St. Louis, Mo.), the solvent was evaporated, and the dry solids were placed on top of a Florisil column (5 by 50 cm). The column was developed with benzene followed by 10% increment additions of methanol until the column was finally irrigated with methanol only. The eluate was collected in 15-ml portions with a fraction collector. Flavonoid elution was determined by testing every tenth tube by TLC. The first fraction (I) came off the column with 10 to 20% methanol; the second fraction (II) with 30 to 70% methanol. The solids in fraction I were dissolved in ether, the insoluble material was removed by filtration, and the filtrate was added slowly to hot water. After cooling at 4°C, white needlelike crystals were recovered by filtration, recrystallized from water, and dried in vacuo.

Fraction II was rechromatographed on a Florisil column to effect further separation of the two flavonoids present in this fraction, naringin and an unknown. The separation was still imperfect. The unknown flavonoid was separated from naringin by preparative TLC on Silica Gel G-HR with the solvent system, ethyl acetate-isopropanol-water (80:20:10, v/v/v). Zones were located by covering most of the TLC plate with a clean glass plate and spraying only a narrow vertical strip with sodium borohydride followed by concentrated HCI. The zone containing the unknown was scraped off; the compound was eluted with methanol and precipitated by slow addition of water and cooling. A second precipitation from the same solvent system was effected, but crystals were not formed. The white solids gave only a single spot on TLC in several solvent systems.

**Densitometric assay.** For quantitative densitometric analysis, the Silica Gel G-HR coating on the TLC plate was scored by a mechanical device to give 20 lanes, each 1 cm in width. For absorbance analyses, only every other lane was spotted; for fluorescence determinations, every lane except the two outer lanes was used. A Schoeffel dual-beam spectrodensitometer model SD 3000, equipped with a mechanically driven stage and attached to a Schoeffel density computer, SDC 300, and a Disc Instrument Corp. model 610 automatic printer, was used for all quantitative analyses.

Absorbance analyses were conducted with the dual-beam facility and the instrument set at a wavelength of 530 nm, the slit width on the scanner at 1.5 mm, and the slit width on the substrate at 1 mm with the gain at 1.5.

The single-beam setting was used for fluorescence analyses; excitation was set for 310 nm and emission for 304 nm; gain was at 8 and the slit of the scanner was set at 2.5.

Fluorescence maxima for the AICI₃ reaction products, obtained by adding a few drops of AICI₃ solution to the purified compounds in ethanol, were obtained with an Aminco-Bowman Spectrophotofluorometer.

Absorbance maxima of the reaction products with sodium borohydride and HCI were obtained with a Beckman model DB spectrophotometer.

**RESULTS**

**Spore studies.** Initially, 14 spore strains representing six genera and 10 species were tested for their ability to act on naringin. These organisms were: *Aspergillus candidus*, *A. niger* (three strains), *A. ochraceus* (three strains), *A. oryzae*, *Myrothecium verrucaria*, *Paecilomyces variotii*, *Penicillium charlesii*, *Rhizopus sp.*, *Trichoderma roseum*, and *T. viride*. After incubation for periods up to 1 week, some of the spores converted naringin to two new flavonones but in concentrations too small to permit ready isolation and identification. Spores of *P. charlesii* NRRL 1887 appeared to be the most active. In an attempt to increase product yield, studies were made in which the mycelium rather than the spores of this latter organism was used.

**Additional Penicillium species.** The survey was extended to include the action of washed mycelium of 11 additional *Penicillium* species upon naringin (P. atrovenerum, P. baarne, P. brev-compactum, P. chrysogenum, P. crustosum, P. cyclopium, P. duclauxii, P. expansum, P. fellutanum, P. nigricans, and P. notatum). Of these, only *P. nigricans* NRRL 915 acted on naringin, producing prunin and naringenin. The extent and rate of action were comparable to those obtained with *P. charlesii* NRRL 1887.

**Gibberella fujikuroi** (Fusarium moniliforme). *G. fujikuroi* has been reported to transform flavanone to a variety of products (18, 19). After 5 days of incubation on both naringin and naringenin, using washed mycelia of 10 strains, we were unable to detect any major by-products other than the starting compounds. However, strains NRRL 2633, 2634, 2635, and 3198 either partially or completely degraded the naringin with only slightly detectable quantities of naringenin being produced.

*A. candidus*. Two strains of *A. candidus* (ATCC 20022 and CMI 16046) produce an antifungal flavonoid antibiotic, chlorflavonin: 3'-chloro-2',5-dihydroxy-3,7,8-trimethoxyflavone (17). We incubated washed mycelia of eight strains of this organism in a medium containing phosphate buffer, 0.75% naringenin, and 0.5% KCl. The naringenin was poorly soluble and was present...
suspended mostly as fine particles. After 5 days of incubation, no transformations to a potential chlorinated derivative were observed. The cultures were extracted with butanol and the extracted substances were tested against *Mucor ramannianus* NRRL 1839 for antifungal activity; none was observed.

**P. charlesi** NRRL 1887. There was considerable variation in the rate of production of the two principal products by washed mycelia of this organism. In most experiments, there was an initial lag of 4 to 8 hr before the appearance of products; thereafter, the reaction went rapidly, peaking at about 16 hr; after this time, all flavonoid compounds were gradually degraded as determined by TLC. The two principal compounds produced were identified as: fraction I, 5,7,4′-tri hydroxyflavanone (naringenin); fraction II, naringenin-7-glucoside (prunin). Production of these compounds indicated the enzymatic action of naringinase. Compounds were identified by the following criteria.

**Naringenin.** Elemental analysis: C, 65.90%; H, 4.66%; oxygen by difference, 29.44% (theoretical, C, 66.17%; H, 4.45%; O, 29.38%). The elemental analysis and molecular formula, C15H12O7, were confirmed by high-resolution mass spectroscopy, which also gave an m/e, 272.27. There was no depression on a mixed melting point analysis with authentic naringenin, 246.7 to 247.2 C (determined with a Mettler FP-1 apparatus, 15). The compound co-chromatographed with authentic naringenin in four separate solvent systems and gave identical nuclear magnetic resonance spectra. A bathochromic shift from 289 nm in ethanol to 328, 328, and 311 nm on addition of sodium acetate, NaOH, and AlCl3·6H2O, respectively, matched literature values for naringenin (13). These shifts also confirmed the presence of OH-groups in the 7 and 5 positions. The color reaction with sodium borohydride and concentrated HCl of the experimental and authentic compounds gave a bluish-red color with a maximum at 544 nm.

**Prunin.** Elemental analysis: C, 57.71%; H, 5.27%; oxygen by difference, 37.02% (theoretical, C, 58.06%; H, 5.11%; O, 36.83%). This gave a molecular formula of C20H20O10. Mass spectral analyses were unsuccessful, the compound decomposing possibly as a result of the sugar moiety present. Hydrolysis of the substance with 0.1 N HCl revealed only the presence of naringenin and glucose as determined by TLC. The melting point, 221.2 to 222.0 C, closely matched the literature value, 225 C, uncorrected (15). A bathochromic shift from 284 nm in ethanol to 425 and 308 nm on addition of NaOH and AlCl3·6H2O, respectively, matched literature values for prunin (13) and indicated the presence of an OH-group at the 5 position. There was no shift on addition of sodium acetate, indicating the presence of a sugar moiety at the 7-hydroxyl position. Reaction of the experimental compound and an authentic compound with sodium borohydride plus concentrated HCl in ethanol gave a maximum at 534 nm.

In some experiments, naringin was acted upon by *P. charlesi* at a much slower rate (Fig. 1) so that it was possible to detect the presence of two additional substances, but at concentrations too low to permit isolation. Their lower *Rf* values, 0.40 and 0.34, as compared with naringenin, prunin, and naringin (0.91, 0.72, and 0.51, respectively) on TLC plates (Silica Gel G-HR; ethyl acetate-isopropanol-water, 80:40:10, v/v; Fig. 2), indicate that they might be the corresponding flavones oxidized at positions 2 and 3 or flavan-4-ol. These compounds reacted with sodium borohydride and HCl to give a red color and with AlCl3 to give a yellow-green fluorescence under ultraviolet light, indicating that they were not chalcones.

Growth of *P. charlesi* in a medium containing naringin resulted in rapid degradation of the flavonoid but did not eliminate the initial product lag observed when washed mycelia were placed in transformation media. Addition of 2% glucose plus the naringin to this latter medium resulted in repression of naringinase activity.

Washed cells of *P. charlesi* neither degraded naringenin nor produced derivatives after 5 days.
had the same $R_f$ values (0.40 and 0.34) as those produced by *P. charlesii* NRRL 1887. No other compounds could be detected by the sodium borohydride or AlCl$_3$ reactions. After 4 days of incubation, degradation of the by-products occurred rapidly.

**Densitometric analyses.** Two methods were developed for the quantitative densitometric analyses of the flavonoids. The first method involved the reaction of these compounds on a TLC plate after development with sodium borohydride and HCl fumes to form red to bluish-red derivatives (10). The absorption maxima of the pigmented derivatives of naringin, prunin, and naringenin are 528, 534, and 545 nm, respectively. A linear response was obtained for all three flavonoids between 0.25 and 3.5 μg when the densitometer was set at 534 nm (Fig. 3). However, the pigments formed were highly unstable, necessitating completion of the analyses within 20 min after color development.

The second analytical method involved spraying the TLC plates, after development, with a 1 to 2% ethanolic solution of AlCl$_3$. 6H$_2$O resulted in formation of a highly stable, yellow-green fluorescent derivative. Excitation and emission spectra of the AlCl$_3$ derivatives of naringin, prunin, and naringenin are shown in Fig. 4 and 5. A linear response was obtained for naringin of incubation when this aglycone was used in place of naringin.

**Steroid-transforming cultures.** Washed mycelia of 17 cultures previously described as being capable of various steroid transformations and transformation of the lactone mycotoxin, aflatoxin (5), were tested on naringin and naringenin. These organisms were: *Absidia repens*, *A. regnieri*, *Aspergillus flavus*, *Cephalothecium roseum*, *Circinella muscae*, *Cladosporium resinae*, *Cunninghamella blakesleeanana*, *Dactylium dendroides* (two strains), *Helicostylum piriforme*, *Helminthosporium sativum*, *Mucor alternans*, *M. griseo-cyanus*, *Penicillium urticae*, *Syncephalastrum racemosum*, *Thamnidium elegans*, and *Wojnowicia graminis*. Of these, only *H. sativum* NRRL 3356, *C. roseum* NRRL 1665, *A. flavus* NRRL 3357, and *W. graminis* NRRL 2472 acted on naringin, with prunin and naringenin being the principal products. Two additional compounds were produced in very small concentrations by *H. sativum*; they
naringin and naringenin to new products. In general, only two major products were observed from the action of some of these organisms on naringin. These products were prunin and naringenin, indicating the action of the glycosidase enzyme naringinase. Prunin and naringenin can also be produced readily by acid hydrolysis of naringin (1, 6). Although two additional compounds were produced by P. charlesii NRRL 1887 and H. sativum NRRL 3356, they were present in concentrations too low to be identified or to be of practical interest. Investigation of the identity of these two substances is continuing, and should they have potential practical value, it is conceivable that mutations of the organisms involved or different transforming conditions could result in higher yields.

The lack of any transformation of naringin or naringenin by organisms previously known to transform steroid and aflatoxin is somewhat surprising. The 4-keto moiety of these two compounds should be susceptible to reduction to an alcohol, as was reported to result from the action of G. fujikuroi on flavanone by Udupa et al. (18, 19). The two compounds that were produced in concentrations too low to analyze may have, in fact, had the alcohol function at position 4, since they were more polar than the parent compounds. Udupa et al. (19) had also reported the produc-

and prunin between 0.17 and 1.7 µg and for naringenin between 0.17 and 1.0 µg (Fig. 6).

This later method should be applicable to the quantitative analyses of flavones, flavonols, isoflavones, flavanones, aurones, and chalcones, since members of these compound classes also fluoresce under ultraviolet light after reaction with AlCl₃.

**DISCUSSION**

A variety of fungi have been tested for their ability to transform the flavonoid compounds

![FIG. 4. Excitation spectra of naringin, prunin, and naringenin after reaction with AlCl₃.](image)

![FIG. 5. Emission spectra of naringin, prunin, and naringenin after reaction with AlCl₃.](image)

![FIG. 6. Relation of fluorodensitometric readings in response to concentration of AlCl₃ derivatives of naringin, prunin, and naringenin.](image)
tion of chalcones from flavanone as a result of opening the heterocyclic ring between the 1 and 2 positions. Horowitz and Gentili (U.S. Patent 3,087,821, 1963) have produced chalcones from naringin, prunin, and neohesperidin by the action of 20 to 25% alkali followed by a reduction of the ethylenic double bond to yield the intensely sweet dihydrochalcones. It would appear desirable to effect opening of the heterocyclic ring by a milder process, such as microbial action, to avoid the necessity of neutralizing 20 to 25% alkali to recover the chalcone. Although none of the fungi tested acted on the heterocyclic ring, it might be worthwhile to carry out a more extensive screen. An operational difficulty, however, is the vulnerability of the sugar moieties on ring A to microbial action, as we have observed in the present investigation. Horowitz and Gentili (12) have shown that the glycosyl or neohesperidosyl radical on ring A of certain flavonoid compounds is critical for the property of sweetness.

All of the fungi examined that were capable of hydrolyzing naringin were able eventually to degrade the aglycone produced, but were unable to transform or degrade naringenin when supplied as the free aglycone. A similar observation was made by Krishnamurty et al. (16) for Butyrivibrio sp. C_2 which could anaerobically degrade quercetin when supplied as the 3-rhamnosylglucoside (rutin) or the 3-rhamnoside (quercetin) but could not degrade the free aglycone. This inability to metabolize the free aglycones may result from nonpermeability into the cells since these compounds are relatively insoluble.

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