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Bacterial Degradation of Benzyl Isothiocyanate[1]

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Bacteria that degrade benzyl isothiocyanate to benzylamine and hydrogen sulfide were isolated from papaya pulp homogenate by enrichment culture techniques. These organisms were identified as members of *Enterobacter cloacae*.

Benzyl isothiocyanate (BITC) belongs to the so-called mustard oils, a group of biologically active isothiocyanic esters formed through enzymatic hydrolysis of glucosinolates present in certain higher plants. The mustard oils and some of their derivatives account for a variety of effects that include assorted toxicological reactions in man and other animals, various germicidal, insecticidal, and anthelminthic activities, as well as the pungent flavors of several condiments (11, 13, 19, 21, 22).

BITC, a notably effective antibacterial and antifungal agent (3, 21-23), is present in several plant families and is particularly concentrated in papaya (*Carica papaya* L.) seeds (18). This paper reports the isolation from BITC-enriched papaya pulp homogenate of bacteria that can degrade BITC to yield hydrogen sulfide and benzylamine. To our knowledge, no prior reports have appeared with evidence for the microbial degradation of BITC or any other naturally occurring isothiocyanates.

MATERIALS AND METHODS

Homogenates. Homogenates were prepared from fresh papaya seeds (seeds-water, 1:15) or ripe papaya fruit (pulp-water, 1:3) by homogenization at high speed for 2 min in an Omni-mixer (Ivan Sorvall, Inc., Norwalk, Conn.). Papaya pulp homogenates were supplemented as described below with repurified (by vacuum distillation) commercial BITC (K & K Laboratories, Hollywood, Calif.). Ten grams of homogenate was placed in glass-stoppered test tubes or in screw-cap culture tubes and incubated for 18 hr at 30 C prior to chemical or microbiological analyses.

Chemical analyses of papaya pulp homogenates. H_2S was detected qualitatively with lead acetate indicator paper held over the test tubes after incubation. When necessary, H_2S formation was determined quantitatively with procedures described by Gustafsson (7), the H_2S being purged with nitrogen, trapped in lead acetate solution, and estimated by the methylene blue method. BITC in papaya homogenates was detected by gas chromatographical methods described earlier (18).

Isolation of bacteria. Papaya fruit homogenates were supplemented with several levels of BITC (200, 400, 600, and 1,200 μg/g) and placed at room temperature. After overnight incubation, samples from each homogenate were streaked on nutrient agar plates and incubated at 30 C. Four colonies, selected from plates at each level of BITC supplementation, were restreaked and inoculated on nutrient agar slants. The 16 cultures isolated were kept for classification and for subsequent studies of BITC degradation. The taxonomic properties and BITC-degrading ability of these isolates were compared to those observed with known cultures of *Enterobacter cloacae* obtained from the American Type Culture Collection (ATCC strains 13047 and 12294) and from the Center for Disease Control, Atlanta, Ga. (CDC strains 577-69, 2604-69, and 2178-70).

Identification of isolates. Unless specified, the following properties were studied by the usual methods at 35 C, the optimum growth temperature: colony appearance at 30 C on nutrient agar (Difco); flagella with Leifson flagella stain (BBL); hydrogen sulfide production in Triple Sugar Iron Agar (BBL); motility in hanging-drop preparations; indole production in indole-nitrite medium (BBL); methyl red and Voges-Proskauer reactions at 30 C in MR-VP Medium (Difco); citrate utilization in Simmons citrate agar (Difco); reaction in litmus milk (Difco); urease test in urea broth (Difco); nitrate reduction in nitrate broth (Difco); anaerobic growth at 30 C in thioglycolate medium (Difco) and Brewer Anaerobic Agar (Difco); carbohydrate fermentation reactions with phenol red broth base (Difco); catalase activity by adding 3% hydrogen peroxide to surface growth on nutrient agar plates; antibiotic sensitivity with Colab Multidisks (no. 11-160T) on nutrient agar plates; liquefaction of nutrient gelatin (Difco) in cultures incubated at room temperature; and starch

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hydrolysis as described in the *Manual of Microbiological Methods* (15).

Pectinolytic ability was studied in EMB pectate medium (20), crystal violet polypectate gel (10), and the pectate medium of Splittstoesser and Wettergreen (16). Lysine and ornithine decarboxylation in Falkow's decarboxylase medium, gluconate oxidation in gluconate broth, hippurate hydrolysis in hippurate agar, and arginine dihydrolase activity in arginine broth were conducted according to procedures described by Cowan and Steel (2). Growth in KCN medium and phenylalanine deamination in phenylalanine agar were determined as described by Edwards and Ewing (4). Oxidase (method of Kovacs) and cytochrome oxidase activities were tested as described by Gaby and Free (6).

Cultures isolated from BITC-enriched papaya pulp homogenate were identified largely from descriptions by Edwards and Ewing (4) and *Bergey's Manual*, 7th ed., supplemented by information from several other sources (1, 2, 8, 14).

BITC degradation by bacterial cells. The BITC-degrading ability of all 16 isolates was tested with aqueous cell suspensions supplemented with 100 μg of BITC per ml; H_2S production was detected qualitatively with lead acetate indicator paper. One of the isolates, strain P6A, was used for quantitative study of BITC degradation by assaying for benzylamine production. Cells were grown in flasks of nutrient broth for 15 hr at 30 C on an incubator-shaker, harvested by centrifugation, and washed twice with distilled water. A concentrated suspension (1 g wet weight of cells in 30 ml of distilled water) was supplemented with BITC (100 μg/ml, final concentration) and kept at room temperature for 2 hr. Periodically during incubation, 1-ml samples of the suspension were removed, centrifuged, and their supernatant liquids were assayed for benzylamine.

Benzylamine formation. Benzylamine was detected by gas chromatography and estimated quantitatively by comparing peak areas with those of an aqueous solution of authentic benzylamine (Aldrich Chemical Co., Inc., San Leandro, Calif.). Analyses were conducted with a Varian Aerograph 1800 gas chromatograph (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.) equipped with dual-flame ionization detectors. The column was a 6-ft by ⅛-inch (ca. 1.83 m by 0.32 cm) coil of glass tubing packed with 80 to 100 mesh Chromosorb 103 (Johns-Manville Products Corp., Los Angeles, Calif.). The operating conditions were: column temperature, 195 C; flow rate of nitrogen, 25 cc/min; hydrogen, 30 cc/min; and air, 300 cc/min.

Benzylamine also was identified by thin-layer chromatography. Ten milliliters of supernatant liquid was extracted with an equal volume of ether, and the ether phase was removed and concentrated to ca. 0.1 ml. Samples were spotted on Silica Gel G precoated sheets (MN-Polygram, Brinkmann Instruments, Inc., Westbury, N.Y.) and chromatographed in two separate solvent systems (the upper phase of a butanol-acetone-water, 40:10:50 mixture; and phenol-water, 80:30). The R_F values of the samples tested were compared with those of authentic benzylamine, which had R_F values of 0.38 and 0.47, respectively, in the two solvent systems. The chromogenic reagent was 0.2% ethanolic ninhydrin (17).

RESULTS

Homogenates of mature papaya seeds held overnight at room temperature developed an odor of H_2S and gave positive qualitative tests for sulfide. Microscopy smears of seed homogenates contained numerous gram-negative rods. Under similar conditions, essentially no sulfide was produced in papaya pulp homogenates. These observations indicated that H_2S might have resulted from microbial degradation of BITC because this compound occurs at high concentrations in papaya seeds but is extremely low in ripe papaya pulp (18).

Hydrogen sulfide formation in BITC-enriched papaya pulp homogenates. When BITC was added to ripe papaya pulp homogenates, H_2S was produced during overnight incubation under air or nitrogen atmosphere, and gram-negative rods were present. No H_2S formed if the homogenate was heated or if chloramphenicol was added before enrichment with BITC. For supplementations up to 2 mg per 10 g of pulp homogenate, H_2S production was proportional to the amount of BITC added (Fig. 1). A corresponding disappearance of BITC was observed in conjunction with the production of H_2S in BITC-enriched homogenates (Fig. 2).

Identification of isolates. All 16 bacterial cultures isolated from BITC-enriched papaya pulp homogenates were gram-negative, moderate-sized rods and formed moist, round, opaque, unpigmented colonies on nutrient agar. Based on a considerable number of characteristics examined (Table 1), 14 of the isolates were classified as members of *E. cloacae*, with properties resembling those observed with the known *E. cloacae* cultures obtained from ATCC and CDC.

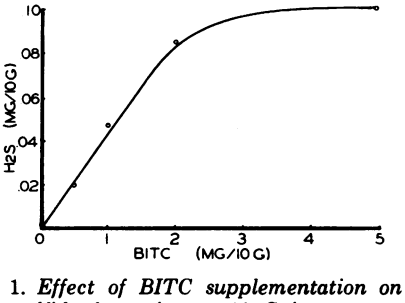

FIG. 1. *Effect of BITC supplementation on hydrogen sulfide formation at 30 C in papaya pulp homogenate.*

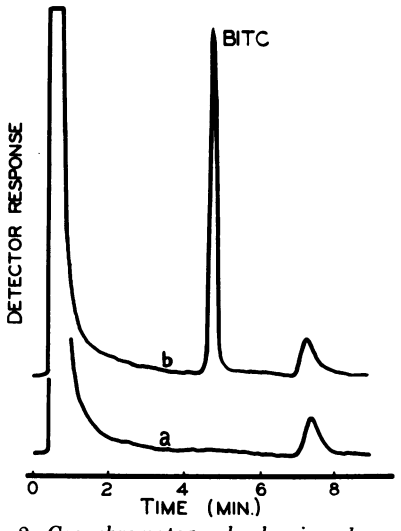

FIG. 2. *Gas chromatograph showing degradation of BITC at 30 C in BITC-enriched (2 mg/10 g) papaya pulp homogenate. (a) Unheated and (b) heated 5 min at 100 C prior to supplementation with BITC.*

The two remaining isolates also belonged to the family *Enterobacteriaceae* but were classified as members of the *Hafnia* and *Citrobacter* groups. These latter two cultures were isolated from homogenate with the lowest BITC supplementation tested, i.e., 200 μg of BITC per g of papaya pulp homogenate.

BITC degradation by cell suspensions. All 14 of the *E. cloacae* isolates obtained from BITC-enriched papaya pulp homogenates produced H_2S from BITC. On the other hand, the ATCC and CDC *E. cloacae* strains, as well as the *Hafnia* and *Citrobacter* isolates, were either negative or gave feeble qualitative tests for sulfide production from BITC.

Additional studies with one *E. cloacae* isolate (strain P6A) were conducted to determine the kinetics of BITC degradation by measuring the amount of benzylamine formed during incubation (Fig. 3). The kinetics of benzylamine formation corresponded to the production of H_2S (*data not shown*). Further identification of benzylamine in these samples was established by the similarity of their R_F values to those of authentic benzylamine on thin-layer chromatographs in two solvent systems.

DISCUSSION

The detection of *p*-hydroxybenzylamine in white mustard (12), a rich source of *p*-hydroxybenzylglucosinolate, prompted Ettlinger and Kjaer (5) to suggest that natural pathways probably exist for the biodegradation of isothiocyanates. In another study Kakimoto and Armstrong (9) also found *p*-hydroxybenzylamine in the urine of human subjects who had ingested mustard previously.

TABLE 1. *Characteristics of Enterobacter cloacae cultures isolated from BITC-enriched papaya pulp homogenates*

Property tested	Response[a]
Motility	+
Peritrichous flagella	+
Facultative anaerobe	+
Catalase	+
Hydrogen sulfide production	−
Indole production	−
Methyl red test	−
Voges-Proskauer reaction	+
Citrate utilization	+
Growth in KCN	+
Gelatin liquefaction	+
Nitrate reduction	+
Gluconate oxidation	+
Hippurate hydrolysis	−
Starch hydrolysis	+
Oxidase	−
Cytochrome oxidase	−
Urease	−
Lysine decarboxylase	−
Ornithine decarboxylase	+
Arginine dihydrolase	+
Phenylalanine deaminase	−
Pectinolytic ability	−
Litmus milk reaction	A, C, G, P
Rapid fermentation of: cellobiose, fructose, glucose, galactose, maltose, raffinose, rhamnose, starch, sucrose, and xylose	A, G
Slow fermentation of: adonitol, glycerol, and lactose	A, G
Fermentation of: dextrin and salicin	A
Fermentation of: dulcitol and inositol	−
Sensitive to: chloramphenicol, colistin, neomycin, polymyxin B, dihydrostreptomycin, oxytetracycline, and tetracycline	+
Resistant to penicillin G	+
Sensitive to ampicillin	±

[a] Symbols: +, positive; −, negative; ±, variable; A, acid; G, gas; C, coagulation; P, peptonization.

The observations presented here suggest that H_2S formation in homogenates of papaya seeds and BITC-enriched papaya pulp resulted from the dissimilation of BITC by *E. cloacae*. Based on our results, and on available knowledge concerning the chemical hydrolysis of isothiocyanates generally (13), we propose the following overall reaction to describe the biodegradation of BITC:

$$\underset{\textit{BITC}}{C_6H_5{-}CH_2{-}N{=}C{=}S} + 2H_2O \xrightarrow{\text{Bacterial enzyme(s)}} \underset{\textit{Benzylamine}}{C_6H_5{-}CH_2{-}NH_2} + H_2S + CO_2$$

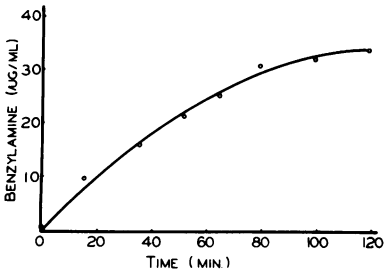

FIG. 3. *Kinetics of benzylamine formation through degradation of BITC (100 μg/ml) at room temperature by cells of Enterobacter cloacae strain P6A.*

Additional studies with cell-free extracts of *E. cloacae* strain P6A are in progress to determine the optimum conditions for this biodegradative system and to characterize the enzyme(s) involved.

A number of workers have found that gram-positive bacteria (22, 23), yeasts (11), and fungi (3, 22, 23) were inhibited by various isothiocyanates, whereas Zsolnai (23) reported that several gram-negative bacteria were resistant to isothiocyanates. This could mean that cell wall composition plays an important role in determining the permeability of cells to isothiocyanates. Whether biodegradation of isothiocyanates is necessary for resistance to the antimicrobial effects of mustard oils is not known but appears unlikely.

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