Mechanism of Antifungal Action of Citrinin

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The mycotoxin citrinin had antifungal activity under acidic conditions. At the minimum inhibitory concentration, it completely inhibited cellular respiration and partially inhibited the incorporation of radioactive precursors into macromolecules in *Saccharomyces cerevisiae*. It had no effect on cell permeability. In mitochondrial preparations, it significantly inhibited succinate oxidase and NADH oxidase. *Rhizopus chinensis* was more sensitive than *S. cerevisiae*; its growth and mycelial respiration at acidic pH were completely inhibited by lower concentrations of citrinin. The pH-dependent antifungal activity of citrinin seems to be associated with its uptake by fungi. Approximately half of the citrinin taken up was found in mitochondria. The main site of the antifungal action of citrinin, therefore, appears to be the mitochondrial electron transport system.

In screening for bioactive metabolites, we found a filamentous fungus that produced large amounts of a yellow antimicrobial pigment. The fungus, isolated from a soil sample collected on our university campus, was found to be a strain of *Penicillium oxalicum*. The pigment was identified as citrinin on the basis of various spectral data.

Citrinin is known to be a secondary metabolite of several penicillia and aspergilli. It is nephrotoxic¹⁾ and was first implicated in disease outbreaks in Japan associated with moldy rice shortly after World War II.²⁾ Later studies on the toxic properties of citrinin disclosed that it widely inhibits the growth, biosynthesis, and enzyme activity of animals,^{3,4)} plants,^{5,6)} and microorganisms.^{7,8)} However, its common mechanism of action through these organisms has not been described.

This report describes the antifungal activity of citrinin at acidic pH and its mode of action mainly in the yeast *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Preparation of citrinin. A strain of *P. oxalicum* was

cultured with shaking at 25°C in Stone and Farrell's medium.⁹⁾ After cultivation for 15 days, the broth filtrate was extracted with ethyl acetate at pH 2.5. Concentration of the extract *in vacuo* gave a crude yellow powder, which was chromatographed on a silica gel column and recrystallized from a methanol solution to obtain citrinin as yellow needles.

[¹⁴C]Citrinin was produced by adding sodium [¹⁴C]acetate (1.8 mCi/mmol) obtained from New England Nuclear, Boston, Mass. to the cultivation medium on day 5 and harvesting on day 15. The radioactivity of crystalline [¹⁴C]citrinin was 2.1×10^6 dpm/mg.

Antimicrobial assay. The antibacterial activity was measured by a two-fold serial broth dilution method using peptone medium after incubation at 37°C for 2 days, and the antifungal activity was done using malt wort after incubation at 25°C for 2 days.¹⁰⁾ To assess the influence of the pH of the assay medium, the pH was adjusted with 0.5 N HCl and 0.5 N NaOH.

Incorporation of radioactive precursors into cellular constituents. Radioactive precursors were obtained from New England Nuclear, Boston, Mass.; [¹⁴C]uracil (53.8 mCi/mmol) was labeled at position C₂, [¹⁴C]adenine (45.0 mCi/mmol) at C₆, D-[¹⁴C]mannose (49.3 mCi/mmol) and sodium [¹⁴C]acetate (2.3 mCi/mmol) at C₁, and L-[¹⁴C]leucine (346.0 mCi/mmol) and D-[¹⁴C]glucose (3.5 mCi/mmol) uniformly. Radioactive uracil, adenine, L-leucine, D-mannose, or sodium acetate was added to a mid-logarithmic phase culture of *S. cerevisiae* IFO 0203

(1 mg dry cells/ml) in GYE medium¹¹⁾ (0.8% glucose and 0.4% yeast extract, pH 3.5) at the respective final radioactivity of 0.0024, 0.013, 0.012, 0.02, and 0.02 $\mu\text{Ci/ml}$. In the experiment on radioactive glucose incorporation, 0.02 $\mu\text{Ci/ml}$ was added to a culture in modified GYE medium (0.2% glucose and 0.4% yeast extract, pH 3.5). After further cultivation at 25°C for 10 min, citrinin was added and, at intervals of 10 min, 1-ml samples of the culture were treated with 1 ml of ice cold 10% (w/v) trichloroacetic acid (TCA). To measure adenine incorporation into DNA (alkali-resistant fraction), samples were incubated with 0.1 N NaOH at 30°C for 30 min before TCA precipitation.¹²⁾ The acid-insoluble fractions were collected on glass fiber filters (Whatman GF/C) and washed with 5 ml of 5% (w/v) TCA. Radioactivities on the filters were counted with a liquid scintillation spectrometer (Packard Tri-Carb 460) in 10 ml of scintillation fluid in 1 liter of toluene, 5 g of 2,5-diphenyloxazol and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene.

Leakage of cellular constituents. *S. cerevisiae* was cultured in YPG medium¹³⁾ (1% glucose, 0.35% yeast extract, 0.35% peptone, 0.2% KH_2PO_4 , 0.1% $(\text{NH}_4)_2\text{SO}_4$, and 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) with shaking at 25°C for 15 hr. The cells were harvested and washed with 1% NaCl by centrifugation. The cells were then suspended in 100 mM citrate- Na_2HPO_4 buffer (pH 3.5) at a final cell concentration of 3 mg dry weight/ml. The suspension was shaken in the presence or absence of citrinin at 25°C. At various times, portions were withdrawn and the cells were removed by centrifugation to examine the phenol-sulfuric acid-positive substances of the supernatant at OD 480 nm by the method of Dubois *et al.*¹⁴⁾

Oxygen consumption. The washed yeast cells prepared as described above were suspended in 100 mM citrate- Na_2HPO_4 buffer (pH 3.5 and 5.3) containing 28 mM glucose, 10 mM KCl, and 0.1 mM EDTA at a final cell concentration of 3 mg dry weight/ml, and incubated with shaking at 25°C. After 10 min, citrinin was added and, with 10-min intervals, portions of the incubation mixture were withdrawn. The respiratory activity of yeast cells in the mixture was measured polarographically at 25°C with a Yanagimoto PO-100A oxygen electrode.¹⁵⁾

When mycelia of *Rhizopus chinensis* IFO 4745 were used in place of yeast cells, the fungus was cultured in malt wort with shaking at 25°C for 2 days. Mycelia were harvested and washed with 1% NaCl on filter paper, and suspended in the above incubation solution at a final mycelial concentration of 3 mg dry weight/ml.

[^{14}C]Citrinin uptake. The washed yeast cells were suspended in 100 mM citrate- Na_2HPO_4 buffer (pH 3.5, 4.4 and 5.3) containing 0.1 mM [^{14}C]citrinin at a final cell concentration of 1 mg dry weight/ml, and incubated with shaking at 25°C. At various times, the cells contained in 1-ml samples were collected on glass fiber filters (Whatman

GF/C) and washed three times with 5 ml of same buffer. Radioactivity was measured as described above.

Intracellular distribution of [^{14}C]citrinin. Yeast cell suspensions [25 mg dry cells/ml of 100 mM citrate- Na_2HPO_4 buffer (pH 3.5) containing 0.5 mM [^{14}C]citrinin] were incubated with shaking at 25°C for 30 min. The cells were harvested by centrifugation, washed with ice-cold 20 mM Tris-HCl buffer (pH 7.4) containing 1 M sucrose and 1 mM EDTA, and resuspended in the same cold solution. The cells were disrupted by ultrasonication and fractionated by successive centrifugations at $5,000 \times g$ for 10 min and $15,000 \times g$ for 15 min.¹⁶⁾ Appropriate amounts of samples were taken from each fraction for radioactivity assay.

The protein of each fraction was measured by the biuret method after the addition of an equal volume of 5% Triton X-100,¹⁷⁾ bovine serum albumin being used as a standard.

Mitochondrial enzyme assays. The yeast cells suspended in 20 mM Tris-HCl buffer (pH 7.4) containing 1 M sucrose and 1 mM EDTA were disrupted using glass beads.¹⁸⁾ The filtrate of the disrupted cells was centrifuged at $5,000 \times g$ for 10 min, and the supernatant was further centrifuged at $15,000 \times g$ for 15 min. Mitochondria in the $15,000 \times g$ pellet were then suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 M sucrose and 0.5 mM EDTA,¹⁹⁾ re-centrifuged, and resuspended in the same solution.

Succinate oxidase activity was measured polarographically. The reaction mixture contained 66 mM sodium phosphate buffer (pH 7.4), 33 mM sodium succinate, 2 mg cytochrome *c*, and mitochondria (equivalent to 0.66 mg protein) in a total volume of 3 ml.²⁰⁾

NADH oxidase activity was assayed by measuring the decrease in the absorbancy at 340 nm. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.4), 0.1 mM NADH and mitochondria (equivalent to 0.17 mg protein) in a total volume of 3 ml.²⁰⁾

RESULTS

Antimicrobial activity

Citrinin had weak activity against Gram-positive bacteria, including *Staphylococcus aureus*, *Bacillus subtilis*, and *Micrococcus luteus*; the minimum inhibitory concentration (MIC) ranged from 50 to 100 $\mu\text{g/ml}$. It was almost or totally ineffective against Gram-negative bacteria, yeasts, and molds.

Interestingly, however, citrinin tended to inhibit the growth of some yeasts and molds in malt wort adjusted to acidic pH. As shown in Table I, the antifungal activity was apparently dependent upon the pH of the assay medium.

TABLE I. EFFECTS OF pH OF THE ASSAY MEDIUM ON ANTIFUNGAL ACTIVITY OF CITRININ

Microorganism tested	MIC ($\mu\text{g/ml}$)			
	pH			
	6.0	5.0	4.0	3.0
<i>Saccharomyces cerevisiae</i> IFO 0203	> 100	> 100	50	6.25
<i>Candida utilis</i> ATCC 42402	> 100	> 100	50	25
<i>Schizosaccharomyces pombe</i> IFO 0342	> 100	> 100	100	25
<i>Hansenula anomala</i> IFO 0136	> 100	> 100	100	12.5
<i>Mucor mucedo</i> IFO 7684	> 100	> 100	12.5	3.13
<i>Rhizopus chinensis</i> IFO 4745	> 100	50	6.25	1.56
<i>Aspergillus niger</i> ATCC 6275	> 100	> 100	50	25
<i>Penicillium crustosum</i> Thom	> 100	> 100	100	25

FIG. 1. Effects of Citrinin on Incorporation of Radioactive Precursors into Acid-insoluble Fraction of *S. cerevisiae* Cells.

○, control; ▲, 0.1 mM; ●, 0.5 mM. The arrows indicate the time of addition of citrinin.

The effect of pH differed according to the fungal species. *R. chinensis* and *Mucor mucedo* were very sensitive to citrinin at pH 3.0. *S. cerevisiae* was fairly sensitive.

Effects on synthesis of major cellular constituents

The effects of citrinin on DNA, RNA, protein, polysaccharide, and lipid synthesis in *S.*

cerevisiae at pH 3.5 were examined (Fig. 1). In the control cultures, the incorporation of radioactive adenine, uracil, L-leucine, D-glucose, D-mannose, and sodium acetate into acid-insoluble fractions started instantaneously and the counts of each fraction increased almost linearly up to 60 min after the onset of incubation. Citrinin at 0.1 mM [equivalent to MIC (25 $\mu\text{g/ml}$) at pH 3.5] in-

hibited the incorporation of all species of precursors employed by 30~50% compared with the control. Moreover, no significant difference was observed in the sensitivity of the synthesis of DNA, RNA, protein, polysaccharide, and lipid.

Effects on cell permeability

As shown in Fig. 2, citrinin at 0.5 mM had little effect on leakage of phenol-sulfuric acid-positive substances from resting cells of *S.*

FIG. 2. Effects of Citrinin and Amphotericin B on Leakage of Phenol-Sulfuric Acid Positive Substances from *S. cerevisiae* Cells.

○, control; ●, 0.5 mM citrinin; △, 10 µg/ml amphotericin B.

cerevisiae during incubation for at least 2 hr at pH 3.5. Amphotericin B, on the other hand, readily caused leakage. Similar results were obtained with leakage of UV (260 and 280 nm)-absorbing materials and Folin reagent-positive substances (data not shown), though citrinin itself absorbs in the UV at 260 and 280 nm and reacts with Folin reagent.

Effects on cellular and mycelial respiration

As shown in Fig. 3, when cell suspensions of *S. cerevisiae* were incubated with citrinin at pH 3.5, the respiratory activity rapidly decreased; incubation periods resulting in 50 and 100% inhibition by citrinin at 0.1 mM were 7 and 50 min, respectively. Almost complete inhibition became obvious immediately after the addition of 0.5 mM of citrinin, suggesting direct action on endogenous respiration. On the other hand, the cellular respiratory activity at pH 5.3 was hardly affected by citrinin even at 0.5 mM. In addition, citrinin greatly inhibited the mycelial respiration of *R. chinensis* at pH 3.5, as shown in Fig. 4. The inhibition was complete at 0.1 mM within 10 min.

Cellular uptake of [14 C]citrinin

[14 C]Citrinin uptake by cell suspensions of *S. cerevisiae* in citrate- Na_2HPO_4 buffer (pH 3.5, 4.4 and 5.3) was examined. As shown in

FIG. 3. Effects of Citrinin on Respiratory Activity of *S. cerevisiae* Cells at pH 3.5 and 5.3.

○, control; ▲, 0.1 mM; ●, 0.5 mM. The arrows indicate the time of addition of citrinin.

FIG. 4. Effects of Citrinin on Respiratory Activity of Mycelia of *R. chinensis* at pH 3.5.

○, control; △, 0.05 mM; ▲, 0.1 mM; ●, 0.5 mM. The arrow indicates the time of addition of citrinin.

FIG. 5. Course of Uptake of [^{14}C]Citrinin by *S. cerevisiae* Cells under Acidic Conditions.

○, pH 3.5; ▲, pH 4.4; ●, pH 5.3.

Fig. 5, the uptake at pH 3.5 started quickly and proceeded rapidly for about 5 min after the addition of [^{14}C]citrinin. A plateau was reached within 30 min. The rate of initial uptake at pH 4.4 was slower than one-fourth of that at pH 3.5, but the cell-associated amount of [^{14}C]citrinin continued to rise gradually. The uptake at pH 5.3 was very small. Similar pH-dependent uptake was also observed in *R. chinensis* (data not shown).

TABLE II. DISTRIBUTION OF [^{14}C]CITRININ IN *S. cerevisiae* CELLS

Fraction	Percent of total yield	nmol citrinin per mg protein
Precipitate of $5,000 \times g$	39.6	38.2
Precipitate of $15,000 \times g$	46.1	547.8
Supernatant of $15,000 \times g$	14.3	67.7

TABLE III. EFFECTS OF CITRININ ON SUCCINATE OXIDASE AND NADH OXIDASE OF *S. cerevisiae* MITOCHONDRIA

Citrinin (mM)	Succinate oxidase (nmol O_2 /mg protein/min)	NADH oxidase (ΔOD at 340 nm/mg protein/min)
0	33.8	3.53
0.05	32.9	3.06
0.1	25.0	1.94
0.25	15.9	1.64
0.5	2.8	0.24

Subcellular localization of [^{14}C]citrinin

Cells of *S. cerevisiae* were incubated together with 0.5 mM of [^{14}C]citrinin at pH 3.5 for 30 min, washed, and disrupted by ultrasonication. The distribution of radioactivity in the three fractions obtained after differential centrifugation (Table II) showed that 39.6 and 46.1% were associated with the $5,000 \times g$ and $15,000 \times g$ pellets, respectively. A lower level was recovered from the $15,000 \times g$ supernatant fluid. All fractions contained citrinin according to the results of high performance liquid chromatography.

Interestingly, the amount of [^{14}C]citrinin per mg protein of the fraction sedimented at $15,000 \times g$, which contains mitochondria, was much larger than those of the other fractions.

Effects on respiratory chain enzymes

Citrinin significantly inhibited both succinate oxidase and NADH oxidase of mitochondria isolated from *S. cerevisiae* at 0.1–0.5 mM (Table III). This indicated that citrinin blocks electron transport in mitochondria.

DISCUSSION

Although the mycotoxin citrinin has been reported to have antibiotic activity toward Gram-positive bacteria,²¹⁾ this is the first report of its antifungal activity under acidic conditions. This pH-dependency was found to be associated with the uptake of citrinin by fungi.

In whole cells of *S. cerevisiae*, citrinin inhibited both respiration and macromolecular synthesis. Betina *et al.* reported that citrinin caused leakage of riboflavin from the mycelium of *Eremothecium ashbyi*²²⁾ and anthocyanins from root slices of *Beta vulgaris*.²³⁾ They believed that it is an inducer of permeability changes in cytoplasmic membranes of fungi and plants. In our experiments on *S. cerevisiae*, however, citrinin did not stimulate leakage of any cellular constituents.

The growth of *S. cerevisiae* at pH 3.5 was completely inhibited by citrinin at 25 µg/ml (0.1 mM). Under these conditions, the inhibition of DNA, RNA, protein, polysaccharide, and lipid synthesis was partial; irrespective of the precursors, synthetic activity were reduced to 50~70% of the control levels. The extent of inhibition appeared to be independent of the incubation periods. The inhibition of cellular respiration at pH 3.5 by citrinin at 0.1 mM was complete within 50 min. In addition, citrinin significantly inhibited succinate oxidase and NADH oxidase in mitochondrial preparations. Interestingly, 46.1% of citrinin taken up by yeast cells was found in the mitochondria. Therefore, the antifungal activity of citrinin seems to be closely related to respiratory inhibition. However, the *S. cerevisiae* used in this study can grow under anaerobic conditions, though the growth rate is very slow.

Among the fungi used in the antimicrobial assay, *R. chinensis* which cannot grow under anaerobic conditions, was most susceptible to citrinin at acidic pH. Its respiratory activity completely disappeared immediately after addition of 0.1 mM citrinin to the mycelial suspension at pH 3.5.

Based on these observations, the primary

site of antifungal action of citrinin is probably the mitochondrial electron transport system, with the other effects being secondary. Details of the site of inhibition in this system will be reported in our next paper.²⁴⁾

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