Antimicrobial Polypeptide Synthesized by *Mucor pusillus* NRRL 2543 (34563)

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Of the hundreds of antibiotics known today, only a few are synthesized by microorganisms that belong to the class Phycomycetes. This group of fungi, as evidenced by the scarcity of published information, has not been regarded as a potential source of antibiotics. Within the class Phycomycetes, members of the genus *Mucor* often display inhibitory activity against a variety of microorganisms (1). This activity, however, is the result of the accumulation of relatively simple organic acids in the fermentation medium, and the manifestation of activity can be obliterated by neutralizing the medium with alkali.

The only antibiotic that has been isolated from this genus is ramycin, which is a steroid compound (2, 3). Another substance, which is produced by *Mucor racemosus* and shows toxic activity against several bacteria, has not been characterized (4).

It has been reported previously that *Mucor pusillus* NRRL 2543 frequently produced in liquid cultures a substance that showed inhibitory activity against gram-positive bacteria (5). The activity of the culture filtrates was not lost on neutralization with alkali, indicating that activity was not due to the presence of organic acids. This suggested that the fungus produced an antibacterial compound that may be novel to the genus *Mucor*. Certain properties of the crude preparation, e.g., nonextractibility by organic solvents, chromatographic behavior, etc., were suggestive of a large peptide-type molecule.

This report describes the isolation, purification, and some of the properties of the antibacterial peptide synthesized by *M. pusillus* NRRL 2543.

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Materials and Methods. Culture. Mucor pusillus NRRL 2543 was a gift from Dr. C. W. Hesseltine. The mold was maintained on Difco potato malt agar slants at 34° and transferred weekly.

Production and recovery of active material. The conditions for the submerged cultivation of *M. pusillus* NRRL 2543 were described earlier (6). Flasks inoculated with the spore suspension of the mold were usually agitated for 6–10 days at 37° on a gyrotory shaker. At the end of the incubation period, the fermentation medium was filtered through Whatman No. 1 filter paper. The pH of the combined filtrates was adjusted to 7.0 with 2 *N* KOH. To the mycelium-free filtrate, an equal volume of acetone was added. The mixture was heated to 55° in a water bath and maintained at this temperature for 20 min. After cooling the mixture to room temperature, the precipitate was removed by filtration. The clear, amber-colored filtrate was evaporated to dryness in a flash evaporator. The dry residue was washed with 80% (v/v) aqueous acetone and then with ethyl ether. The lipid-free residue was suspended in distilled water and stirred for 30 min. Insoluble matter was removed by centrifugation at 3000*g* for 15 min in an International Model HT centrifuge. This crude preparation was stored in the freezer compartment.

Purification of antibiotic. A carboxymethyl-derivative of Sephadex, CM-Sephadex C-50 cation exchanger (Pharmacia Fine Chemicals, Inc.) was used to build a 2.7-50-cm column which was equilibrated with 0.02 *M* sodium phosphate buffer (pH 6.0). Twenty milliliters of the crude preparation were percolated through the column with phosphate buffer as the eluant. After all visi-

ble traces of pigmented material passed through the gel bed, a stepwise gradient elution was carried out according to the following schedule: 200 ml of 0.02 *M* sodium phosphate–0.07 *M* NaCl, 300 ml of 0.02 *M* sodium phosphate–0.14 *M* NaCl. and 200 ml of 0.02 *M* sodium phosphate–0.2 *M* NaCl. The pH of each buffer solution was pH 6.0. An automatic fraction collector was used to collect 8-ml fractions at room temperature. The absorbance of each fraction was checked at 280 nm in a Zeiss M4QIII spectrophotometer. Antibiotic activity was determined by dipping 12.7-mm antibiotic sensitivity discs into each fraction and placing them on nutrient agar plates inoculated with *B. subtilis*. Active fractions were combined and evaporated to 4–5 ml under reduced pressure. The concentrated solution was passed through a 2.7-X 100-cm column of Sephadex G-15, using distilled water as the eluant. The distribution of active material was established by the paper-disc method with *B. subtilis* as the test organism. Phosphate and chloride ions were detected by mixing one drop of each fraction (5 ml) with one drop of a saturated aqueous solution of $AgNO_3$. The desalted antimicrobial compound was lyophilized in a Virtis freeze-drying apparatus. The dry powder was stored in the freezer compartment over $CaSO_4$.

Electrophoretic analysis. The electrophoretic analysis of the desalted active material was carried out in a modified Beckman Model R electrophoretic cell, employing different buffer systems. Sepraphore III (Gelman Instruments Co.) cellulose acetate strips were used as the support. All buffer systems had an ionic strength of 0.1. Buffers with pH 4.15, 5.05, and 7.95 were prepared according to the method of Miller and Golder (7). Buffers with pH 10.00, 11.30, and 12.30 were prepared by a modification of the above method which involved the replacement of glycine in the buffer mixtures, since the presence of glycine interfered with the location of ninhydrin-positive material on the cellulose acetate strips. Ten microliters of the aqueous solution of the purified antibiotic (100 μg) were spotted on the membranes and electrophoresis was conducted for 2 hr at 0.5 mA cm^{-1} constant current. The developed strips were dried in air and dipped into an alcoholic ninhydrin solution (8). The dried strips were heated at 100° for 3–5 min.

Ultraviolet spectrum. Purified antibiotic was dissolved in 0.01 *N* HCl and 0.01 *N* NaOH at 1 mg/ml final concentration. The spectral behavior of the compound in acid and alkali was established between 220 and 340 nm using a Zeiss M4 QIII spectrophotometer.

Amino acid analysis. A sample of electrophoretically pure antibiotic (5 mg) was placed in a screw-cap vial and subjected to acid hydrolysis in 6 *N* HCl, for 18 hr, in a 105° mineral oil bath. After cooling to room temperature, the hydrolyzate was evaporated to dryness under reduced pressure. In order to remove acid, the residue was taken up in distilled water and evaporated. After repeating this treatment three times, the residue was dissolved in 2 ml of distilled water. An aliquot of the acid hydrolyzate was analyzed in a Beckman Spinco Model 120 amino acid analyzer, according to the method of Spackman *et al.* (9).

Biological evaluation of the purified antibiotic. The activity of the purified preparation was tested against gram-positive and gram-negative bacteria and also against yeasts. All cultures were obtained from the Midwest Culture Service (Indianapolis, Ind.). Bacteria were grown in nutrient broth (Difco) except streptococci, which were cultured in dextrose broth (Difco). Yeasts were propagated in nutrient broth supplemented with 0.4 % yeast extract. The test cultures were incubated for 18 hr prior to use, at 28°. Spread plates were prepared using three types of media: dextrose agar for culturing streptococci, nutrient agar for growing all other bacteria, and nutrient agar supplemented with yeast extract for propagating yeasts. Wells, 5 mm in diameter, were cut into the agar layer by means of the large orifice of a Pasteur pipette. The agar plugs were lifted out with a pair of forceps. The purified antibiotic was dissolved in distilled water at 1 mg/ml concentration and serial

dilution was carried out with distilled water. Exactly 0.1 ml of the test solutions containing varying amounts of the polypeptide was delivered into the wells of the spread plates. Plates were incubated at 28° for 24 hr. The concentration of the antibiotic at which inhibition of growth was still observed was taken as the minimum inhibitory concentration (MIC) of the antibiotic for a given test organism.

Effect of acid and alkali on the antibiotic. The electrophoretically pure antimicrobial compound was dissolved in 0.01 *N* HCl and 0.01 *N* NaOH at 500 μg/ml concentration and the mixtures were allowed to stand at room temperature. At 0, 2, 8, and 24 hr, aliquots (0.5 ml) were withdrawn and neutralized with an equivalent amount of acid or alkali. The amount of intact antibiotic was estimated by bioassay using *B. megaterium* as the test organism. In order to establish the degree of destruction of the antibiotic by either acid or alkali, a control experiment was set up to correlate the size of the zone of inhibition obtained against *B. megaterium* with the log concentration of the antibiotic in the test solution. Spread plates of *B. megaterium* were prepared as described in the preceding section, and 0.1 ml of the test solution was pipetted into wells cut into the agar. The diameter of the zones of inhibition (mm) was plotted against the log antibiotic concentration (μg/ml). Between 7 and 250 μg/ml antibiotic concentration a straight line was obtained. The use of this plot permitted not only the estimation of antibiotic left intact following acid and alkali treatment but also the efficiency of the recovery process in each stage of isolation.

Results. A time study conducted over a period of 8 days showed that the antibiotic titer of the fermentation medium reached a maximum after 5–6 days of incubation at 37°. The activity of the aseptically withdrawn, mycelium-free culture filtrates was determined against *B. megaterium* and potency was expressed in terms of biounits. One biounit was arbitrarily defined as the amount of antibiotic needed to give a 10-mm zone of inhibition after 18 hr of incubation at 28°.

TABLE I. Recovery of Antibiotic by Acetone Precipitation and Cation-Exchange Chromatography.

Step	Antibiotic concentration (μg/ml)	Total antibiotic yield (mg)	(%)
Crude filtrate	174	278.4	100
Acetone treatment	317	79.2	28.5
CM-Sephadex C-50	218	70.0	25.1

It was found that the attained maximum antibiotic titer (17 units/ml) did not change during the rest of the incubation period. The results of large scale experiments in which usually 1600–2000 ml of fermentation broth were processed showed that antibiotic yields by acetone precipitation were low (Table I). Approximately 28.5 % of the antibiotic present in the crude filtrate was recovered. The precipitate brought down by acetone also showed some activity against *B. megaterium*; however, the amount of lost activity was not estimated. Cation-exchange column chromatography provided an excellent means to free the active compound from pigments and inert material. During stepwise gradient elution the antibiotic was displaced from the cation-exchanger at 0.14 *M* NaCl concentration (Fig. 1). By establishing the antibiotic concentration of each fraction showing activity against *B. megaterium*, it was calculated that 87.5 % (14 mg) of the antibiotic was recovered following cation-exchange chromatogra-

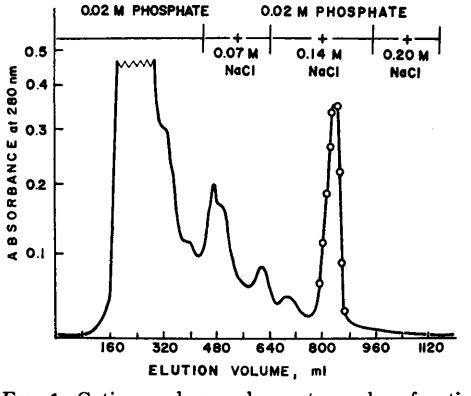

FIG. 1. Cation-exchange chromatography of antimicrobial peptide on Sephadex CM-50. Load: 16 mg antibiotic. O, fractions showing inhibitory activity against *B. subtilis*.

phy. The relatively small loss (12.5 %) was probably due to the inability of the sensitivity test to detect very low concentrations of the antibiotic in fractions corresponding to the two sides of the active peak. The overall recovery of the antimicrobial peptide was about 25 %. Evidently, the greatest loss occurred at the acetone precipitation step (Table I). Gel filtration of the antibiotic on Sephadex G-15 completely removed chloride ions. Phosphate, on the other hand, was only partially separated from the antibiotic as evidenced by matching the results of bioautography and tests with $AgNO_3$.

After electrophoresis, cellulose acetate strips treated with ninhydrin showed the presence of a single component in the purified preparation. The identity of the ninhydrin-positive material with the active compound was confirmed by means of bioautography against *B. subtilis*. Cellulose acetate strips that were not developed with ninhydrin were left in contact with agar plates seeded with *B. subtilis*. After a 15-min-long contact, the strips were removed, and the plates were incubated overnight. The R_f value of the zone of inhibition coincided with that of the ninhydrin-positive spots.

The results of spectral analysis in the ultraviolet region showed that in acid medium (0.01 *N* HCl), the antibiotic had maximum absorbance near 280 nm. By taking optical density values between 270 and 285 nm at 2-nm intervals, it was found that the compound had a maximum absorbance at 278 nm. In alkaline medium (0.01 *N* NaOH) the peak was much less distinct.

The results of amino acid analysis indicated the presence of the following amino acids in the antibacterial compound: glycine, alanine, leucine, isoleucine, valine, serine, threonine, cystine, cysteine, aspartic acid, glutamic acid, lysine, arginine, tyrosine, proline, and, traces of histidine. The exact amount of each amino acid and the molar ratio of the residues were not calculated.

Acid treatment with 0.01 *N* HCl did not affect the antibiotic. Even after a 24-hr exposure the antimicrobial compound retained 100% of its activity. On the other hand, alkali treatment resulted in 55 and 74 % destruction of the antibiotic, after 2 and 8 hr incubation, respectively. Longer exposures to alkali (24 hr), did not yield a higher degree of destruction of the antibiotic.

The results of *in vitro* studies on the antimicrobial activity of the purified peptide antibiotic are summarized in Table II. As a rule, only gram-positive microbes were inhibited. The only gram-negative organism showing any degree of sensitivity was *Alcaligenes faecalis* (MIC 50 μg/ml). Staphylococci were the most resistant and were inhibited only at 250 μg/ml antibiotic concentration. The two yeast cultures were insensitive to the preparation over the entire concentration range tested.

Discussion. The synthesis of a biologically active substance by *M. pusillus* was evidenced by the inhibition of growth of gram-positive microorganisms by neutralized filtrates of 5–6-day-old cultures. The estimated yield of the peptide antibiotic after 6 days of incubation (174 μg/ml) was low compared with the yield of some other peptide antibiotics (10). It is conceivable that the yield could be improved substantially by selecting a more suitable medium for cultivating the mold. Recovery of the antibacterial compound by acetone precipitation was partially successful. The fact that activity was found not only in the aqueous phase but also in the precipitate could account for the low yield (Table I). Possibly, the antibiotic was either inactivated by the solvent or strongly attracted by proteins that were precipitated by acetone. A third possibility, which should not be overlooked, is that acetone precipitated other active compounds present in the crude extract whose solubility properties were different from the compound finally isolated.

Cation-exchange chromatography was a singularly successful method for purifying the active compound because in this step pigments and inert contaminants were removed. Electrophoretic analysis of the purified antibiotic showed that in all buffer systems employed the compound behaved as a cation. The distance traveled by the peptide in the direction of the cathode decreased

TABLE II. *In Vitro* Activity of Peptide Antibiotic of *Mucor pusillus* NRRL 2543.

Test organism	Concentration of antibiotic inhibitory to test organism, μg/ml								
	250	200	100	50	25	10	5	2.5	1
Gram-positive bacteria									
Streptococcus lactis	+	+	+	+	+	+	+	+	−
Streptococcus agalactiae	+	+	+	+	+	+	+	+	+
Streptococcus pyrogenes	+	+	+	+	+	+	+	+	+
Streptococcus mitis	+	+	+	+	+	+	+	+	−
Streptococcus faecalis var. *liquefaciens*	+	+	+	+	+	−	−	−	−
Streptococcus durans	+	+	+	+	+	+	+	−	−
Micrococcus agilis	+	+	+	+	+	−	−	−	−
Staphylococcus albus	+	−	−	−	−	−	−	−	−
Staphylococcus aureus	+	−	−	−	−	−	−	−	−
Bacillus subtilis	+	+	+	+	+	−	−	−	−
Bacillus megatherium	+	+	+	+	+	+	+	−	−
Sarcina subflava	+	+	+	+	+	−	−	−	−
Sarcina lutea	+	+	+	+	+	−	−	−	−
Corynebacterium xerosis	+	+	+	+	−	−	−	−	−
Gram-negative bacteria									
Escherichia coli	−	−	−	−	−	−	−	−	−
Aerobacter aerogenes	−	−	−	−	−	−	−	−	−
Proteus vulgaris	−	−	−	−	−	−	−	−	−
Alcaligenes faecalis	+	+	+	+	−	−	−	−	−
Pseudomonas fluorescens	−	−	−	−	−	−	−	−	−
Pseudomonas aeruginosa	−	−	−	−	−	−	−	−	−
Serratia marcescens	−	−	−	−	−	−	−	−	−
Yeasts									
Candida utilis	−	−	−	−	−	−	−	−	−
Saccharomyces cerevisiae	−	−	−	−	−	−	−	−	−

with the increase in the alkalinity of the buffer system. At pH 12.30, no migration of the compound was observed. The electrophoretic behavior of the peptide, *i.e.*, migration toward the cathode at very high pH values, suggested the presence of arginine in the compound. This was confirmed by the results of amino acid analysis.

The peptide antibiotic was acid resistant but lost over 50 % of its activity after a 2-hr exposure to 0.01 *N* alkali. Peptide antibiotics have been reported to vary in their resistance to acid and alkali treatment. For example, antibiotic 4025, which is a polypeptide produced by a soil bacterium, was reported to be acid resistant but unstable in basic solutions (11). Matamycin, produced by *Streptomyces matansis*, is very unstable in acid medium (12).

The antimicrobial compound of *M. pusillus* may be regarded unusual because of the presence of arginine and possibly, histidine in the compound. These two amino acids have rarely been detected in biologically active peptides (13). Some of the peptide antibiotics that contain arginine are cinnamycin (14), licheniformin (15), and mycosubtilin (16). Other peptide antibiotics contain histidine, *e.g.*, bacitracins (17) and nisins (18), and very few peptide antibiotics contain both of these amino acids, *e.g.*, alpha-sarcin (19) and malanomycin (20).

As mentioned before, in general, Phycomycetes are not thought of as good sources of antibiotics. In this connection it is of interest to mention a recent report on an antibacterial compound synthesized by *Rhizopus oligosporus* (21), an organism that belongs to

the other commonly found genus of the class Phycomycetes. There is clear indication that the question of antibiotic production by phycomycetes merits further consideration.

Summary. The results of this investigation confirmed that *M. pusillus* NRRL 2543 synthesized an antibiotic substance which restricted the growth of gram-positive microorganisms. The analytical data showed that the antibiotic, which was isolated in the electrophoretically pure form, was a polypeptide that on acid hydrolysis released 16 different amino acids commonly found in proteins.

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