

## Antimicrobial Peptides of Thermophilic *Mucor* Fungi

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Thermophilic species of the genus *Mucor* have become important sources of proteases used as milk coagulants in cheese production. A survey of these fungi indicated that the potential for the synthesis of antimicrobial peptides was widespread among different strains of *Mucor pusillus* and *Mucor miehei*. Maximum antimicrobial peptide titers were reached after about 7 days in wheat bran medium and under submerged conditions of incubation. The antibiotic peptides were isolated and purified by cation-exchange and gel-permeation chromatography. Different strains of *M. pusillus* synthesized sillucin, a 30-residue peptide, which was distinguished by the absence of methionine, phenylalanine, and histidine residues. Strains of *M. miehei* synthesized mieheins, a family of related peptides varying in size from 56 to 74 residues. Mieheins also uniformly lacked methionine but all had phenylalanine and some contained histidine. Sillucin and miehein peptides were characterized by high cystine contents, indicative of highly structured, compact molecules. The antimicrobial peptides inhibited the growth of gram-positive bacteria, possibly by interfering with ribonucleic acid metabolism.

### INTRODUCTION

The antibiotics synthesized by fungi account for less than 25% of the more than 3,000 antibiotics described since the discovery of penicillin. Of these, only 10 have acquired commercial significance: penicillins G, V, and O, cephalosporin, griseofulvin, fumagillin, variotin, siccanin, xanthocillin, and fusidic acid (Berdy 1974; Hopwood and Merrick 1977). Furthermore, in terms of clinical significance, only the  $\beta$ -lactam group, griseofulvin, and fusidic acid merit special recognition.

The distribution of fungal antibiotics among the various groups of true fungi is a matter of some interest. About 39% are produced in the class Ascomycetes, 18% in the class Basidiomycetes, 41% in the class Deuteromycetes (Fungi Imperfecti), and 2% in the class Phycomycetes (Berdy 1974).

The lack of widespread antibiotic-producing potential was noticed long ago in the class Phycomycetes that includes many genera of saprophytic fungi. In a systematic study, Miller and Porter (1957) surveyed over a hundred species in 22 different genera and concluded that the biological activity displayed by less than 16% of the species tested resulted from high titers of common organic acids that accumulated in the growth medium. Later, Van Dijck and deSomer (1958) discovered ramycin in *Mucor ramannianus*, a steroid antibiotic, which subsequently was shown to be identical with fusidic acid (Vanderhaeghe et al. 1965).

More recent investigations indicated that the biosynthesis of antimicrobial metabolites by Phycomycetes may not be as rare as had once been believed. Several species in the genus

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*Rhizopus* (*R. arrhizus*, *R. chinensis*, *R. chinensis* var. *liquefaciens*, *R. javanicus*, *R. niveus*, *R. oryzae*, and *R. chlamydosporus*), *Actinomucor elegans*, and *Chlamydomucor oryzae* produced antibacterial compounds, the yield of which varied markedly with the composition of the growth media (Wang et al. 1969, 1972; Ellis et al. 1974).

The thermophilic species of the genus *Mucor*, *M. pusillus* and *M. miehei*, were reported to synthesize antibacterial peptides, active only against gram-positive bacteria, that were isolated, partially characterized, and named sillucin and miehein, respectively (Somkuti et al. 1969; Somkuti and Walter 1970; Somkuti 1973). Since acid proteases isolated from both *M. pusillus* and *M. miehei* have found widespread commercial acceptance as milk coagulants in cheese production (Sardinas 1969, 1972) and the presence of antimicrobial agents in coagulants may deleteriously affect the performance of lactic starter cultures, the development of nonantibiotic synthesizing strains or the removal of sillucin and miehein from enzyme preparations was recommended (Somkuti and Babel 1968; Somkuti 1973).

The original work on sillucin was carried out with *M. pusillus* NRRL 2543, a homothallic organism that was later reclassified as *M. miehei* (Somkuti 1974), according to the species concepts of Cooney and Emerson (1964). Therefore, a renewed effort was made to extend the work on the characterization of the peptide antibiotics to include other strains of *M. pusillus* and *M. miehei*.

This report describes results of research on the purification, chemical composition, and other properties of peptide antibiotics isolated from different strains of thermophilic *Mucor* fungi.

#### MATERIALS AND METHODS

**Microorganisms.** Cultures originated from the following sources: *M. pusillus* 410, Purdue University Culture Collection; *M. pusillus* 6 and 8, and *M. miehei* 21, D. J. Cooney, University of Nevada; *M. pusillus* 1426, 3638, and 3639, and *M. miehei* 2543, 3169, 5282, 5283, and 5284, from J. J. Ellis, U.S. Department of Agriculture. Stock cultures were maintained on 10% (w/v) potato malt agar (Difco) slants at 37 C and transferred weekly.

**Medium for antibiotic production.** A wheat bran infusion medium was prepared as described by Somkuti (1974). The volume of medium per 2-liter Erlenmeyer flask was 400 ml. Inoculation with spore suspension was followed by incubation at 37 C for 7 days on a Psychrotherm G26 incubator shaker (New Brunswick Scientific Co., Inc.) set at 180 rpm.

**Antibiotic isolation and purification.** Mycelial growth was removed from the fermentation medium by filtration through gauze. To the filtrate 40 g per liter of IR401 (Amberlite) ion exchange resin (Cl<sup>-</sup>) was added. After stirring for 30 min at room temp, the supernatant was decanted and centrifuged at 30,000 g at 4 C. The clear supernatant was adjusted to pH 6.0 and charged on a column of Carboxymethyl-Sephadex 25 (CMS-25)-Na<sup>+</sup> (Pharmacia Fine Chemicals), equilibrated with 10 mM phosphate buffer, pH 6.0. This was followed by washing with phosphate buffer containing 5 mM NaCl. The antibiotic peptides were eluted with a linear gradient of NaCl (5–400 mM) in phosphate buffer. Acetate buffer (10 mM, pH 6.0) could also be used in the column chromatography procedure. Active fractions were combined, desalted on Sephadex G-10, and lyophilized. Frequently, peptide preparations were rechromatographed on CMS-25 and Biogel P-60 (Bio-Rad Laboratories).

*Analytical methods.* Acrylamide gel electrophoresis was carried out in several buffer systems, according to Smith (1968) in the presence and absence of 6 M urea. Antibiotic peptides were stained with Coomassie Brilliant Blue or Naphthalene Black.

Amino acid composition of sillucin and miehein peptides was determined according to the procedures of Moore and Stein (1963). Samples were hydrolyzed in sealed evacuated tubes at 110 C for 24 h with 5.7 N HCl containing phenol (10 µg/ml). Air oxidation of hydrolyzed samples was carried out before analysis. Tryptophan was determined on separate samples on a 0.9 × 14 cm basic column at 50 C, using 0.35 M sodium citrate buffer, pH 5.26, following peptide hydrolysis with mercaptoethanesulfonic acid (Penke et al. 1974). Tryptophan was also determined according to the reversible formylation procedure of Previero et al. (1967). Free sulfhydryl groups were titrated by the method of Ellman (1959) with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as the reagent.

*Antimicrobial assays.* A filter paper plate diffusion assay was employed with streptomycin assay agar (BBL) at 10 ml per plate, seeded with *Bacillus subtilis*. Paper discs, 9.52 mm diameter (Schleicher and Schuell), were impregnated with culture fluid or an appropriate amount of the purified peptide and placed in a random position on the agar plates conforming to a preset design. Plates were stored for 4 h at 4 C and diameters of the zones of inhibition were measured after 18 h of incubation at 30 C. Antibiotic titers were estimated with a standard curve which was constructed by plotting diameters (mm) of inhibition zones versus log concn (µg/ml) of electrophoretically pure sillucin or miehein peptides.

*Studies on macromolecular synthesis in target cells.* *Bacillus megaterium* KM was cultivated in Antibiotic Medium 3 (Difco), 17.5 g per liter. Isotopic incorporation studies were performed by addition of <sup>14</sup>C-isoleucine (specific activity, 201 µC/umole), 4 µC; <sup>14</sup>C-uracil (specific activity, 55 µC/umole), 4 µC; or <sup>3</sup>H-thymidine (specific activity, 51.8 µC/umole), 4 µC, to growth flasks containing 20 ml of medium, which were inoculated with 1 ml of an overnight culture. The growth flasks were agitated in a rotary action shaker-incubator at 37 C. At appropriate times, 1-ml samples were mixed with 1 ml of cold 10% trichloroacetic acid (TCA), filtered through HAWP-02500 membrane (Millipore Corporation), and washed with 10 ml of cold 5% TCA. The membrane filters were dried and assayed for radioactivity in a liquid scintillation counter.

## RESULTS AND DISCUSSION

### *Antibiotic Production*

The available strains of *M. pusillus* and *M. miehei* were examined for their dynamics of antibiotic production. Fungi in liquid wheat bran medium (4% w/v) were agitated at 35 C up to 10 days. Aliquots aseptically withdrawn from flasks at 24-h intervals were tested for antibiotic activity with *B. subtilis* as the target organism. Antibiotic titer calculations were based on standard curves obtained with pure sillucin and miehein-21 which showed a linear relationship between the dose of the peptides and the response in the test organism (Fig. 1). A similar linear relationship was observed with *B. megaterium* KM as the test organism. Fig. 2 shows typical antimicrobial peptide production curves of *M. pusillus* 410 and *M. miehei* 21. Other strains of either species showed similar antibiotic production patterns. Depending on the culture used, at the end of the 7-day incubation period the amount of antimicrobial peptide varied from 8.5–120 µg/ml.

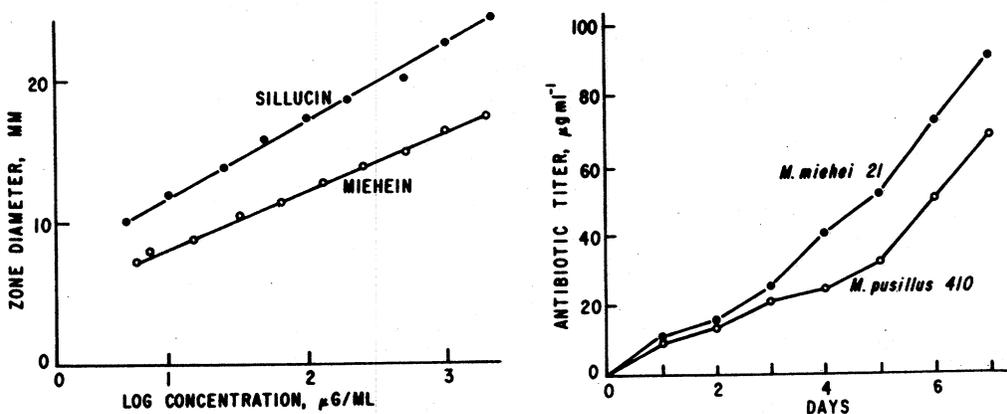


FIG. 1. Linear relationship between log concn of sillucin and miehein-21 peptides and diameter of the zone of inhibition with *Bacillus subtilis* as test organism.

FIG. 2. Synthesis of sillucin and miehein peptides in wheat bran medium.

#### Purification of Sillucin and Miehein Peptides

Gradient chromatography on CMS-25 provided an excellent method for the purification of the antimicrobial peptides (Fig. 3). The cation exchanger selectively adsorbed the highly basic peptides from mycelium-free culture liquids. Testing the fractions for biological activity showed the distribution of antimicrobial activity exactly under the ultraviolet absorbing peak. Peptides of all *M. pusillus* strains and *M. miehei* 2543 showed a single active peak. Elution of these peptides commences at approx. 0.14 M NaCl. The elution pattern of miehein peptides of various *M. miehei* strains characteristically yielded two biologically active peaks (A and B). The relative sizes of peaks A and B in miehein preparations varied

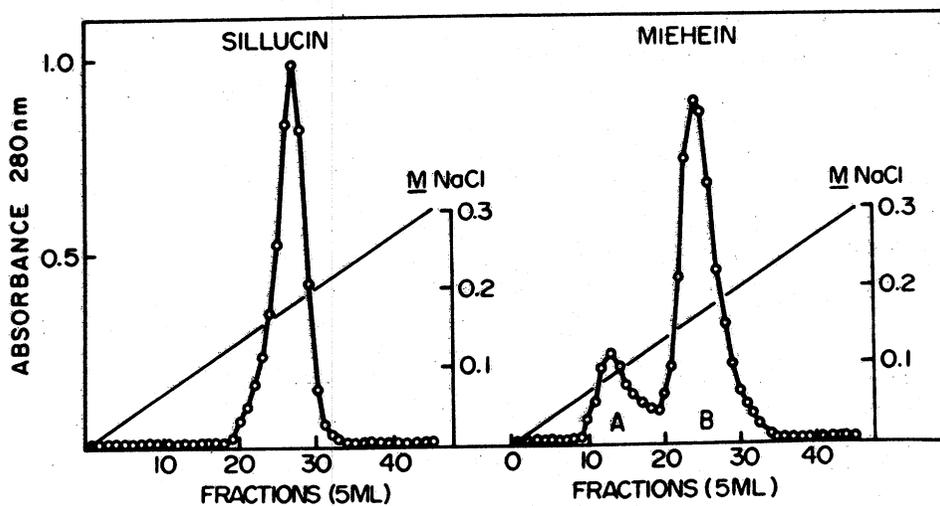


FIG. 3. Purification of sillucin and miehein peptides on Carboxymethyl Sephadex 25. Column, 2 × 20 cm; fractions, 5 ml; buffer, 10 mM phosphate or acetate, pH 6.0; gradient, 5-400 mM NaCl.

with the individual strains of *M. miehei*. All results and discussion on miehein peptides in this paper pertain to material found under peak B from gradient chromatography. Studies on material found under peak A are now in progress.

Active fractions were pooled, concentrated under reduced pressure, and desalted on Sephadex G-10 gels equilibrated with 10 mM acetic acid. Salt-free fractions were pooled and lyophilized. Lyophilization of each preparation yielded a snow-white, powdery product.

Purity of the peptide preparations was checked by rechromatography on CMS-25, Biogel P-60, and acrylamide disc gel electrophoresis. Gel electrophoresis was carried out under both nondenaturing and denaturing (6 M urea) conditions, in various buffer systems. All sillucin and miehein peptide preparations migrated toward the cathode over the entire pH range (pH 2.5–10), reflecting the highly basic nature of the peptides. The electrophoretic behavior of sillucin (*M. pusillus* 410) and miehein (*M. miehei* 21) is shown in Fig. 4. All other sillucin as well as miehein peptide preparations showed an identical pattern.



FIG. 4. Acrylamide gel electrophoresis of sillucin and miehein-21. Buffer, acetic acid-glycine, pH 4.0; current, 4 ma/tube; time, 120 min; stain, Coomassie Brilliant Blue.

#### *Chemical Properties of Sillucin and Miehein Peptides*

The amino acid composition of the antimicrobial peptides produced by different strains of *M. pusillus* is shown in Table 1. Strain 2543 synthesized this peptide and was included although the organism had been reclassified as *M. miehei*. Peptides isolated from all the strains studied exhibited identical compositions. The total of 30 residues yielded a calculated minimum molecular weight of 3200 daltons. This was confirmed by analysis in the ultracentrifuge. Purified sillucin from strain 410 was subjected to ultracentrifugation at 36,000 rpm. The analysis of sedimentation pattern by Archibald's method (1947) indicated a molecular weight for sillucin of about 3400 (average of 3 consecutive runs).

TABLE 1. Amino acid composition of sillucin peptides from various strains of *Mucor pusillus*

Amino acid	Residues/mole <sup>a</sup>							Integer values
	1426	410	3638	Strain 3639	6	8	2543 <sup>b</sup>	
Asp	2.1	2.0	2.0	2.1	1.9	2.0	2.0	2
Thr	1.0	1.0	0.9	1.0	0.9	1.0	1.0	1
Ser	2.8	2.7	2.5	2.6	2.4	2.5	2.5	3
Glu	1.1	1.1	1.0	1.1	1.0	1.1	1.0	1
Pro	1.0	1.0	1.3	1.1	0.9	1.0	1.3	1
1/2Cys	7.5	7.8	7.9	8.2	8.3	7.5	7.5	8
Gly	4.2	4.0	3.9	4.2	3.7	3.8	4.0	4
Ala	1.1	1.1	1.0	1.1	1.1	1.0	1.0	1
Val	1.2	0.9	0.9	1.1	1.0	0.9	1.0	1
Met	—	—	—	—	—	—	—	—
Ile	0.8	1.0	0.9	0.9	1.0	0.9	0.9	1
Leu	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1
Tyr	2.0	2.0	2.0	1.9	2.0	1.9	1.9	2
Phe	—	—	—	—	—	—	—	—
Lys	2.0	2.0	2.3	1.8	2.0	2.0	2.1	2
His	—	—	—	—	—	—	—	—
Arg	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1
Trp <sup>c</sup>	0.9	0.9	1.0	0.9	1.0	1.0	0.9	1
	Total residues							30

<sup>a</sup>Calculated from mean molar ratios using arg = 1, asp = 2, and tyr = 2.

<sup>b</sup>Now classified as *M. miehei*.

<sup>c</sup>See text for method of analysis.

Sillucin peptides were devoid of methionine, phenylalanine, and histidine and contained an inordinately high content of cystine (4 residues/mole). Free sulfhydryl content of sillucin peptides was estimated by the DTNB reagent. In the assays, native sillucin at 0.5 mg/ml concn in 0.5 M phosphate buffer, pH 8.0, or modified forms of the peptide in the same buffer but made 6 M and 8 M with respect to guanidine, HCl, or urea, in 3 ml total volume was mixed with 0.2 ml DTNB (12.5 mg/ml) and incubated for 60 min at room temp. There was no color change monitored at 412 nm, indicating the absence of free, reactive sulfhydryl groups in sillucin. Since no free sulfhyryl was detected, the four disulfide bonds represented by the cystinyl residues indicated a rather high degree of intramolecular crosslinking for a peptide chain of this size. This "knotlike" structure probably contributes toward the molecule's predicted resistance to proteolytic attack and also accounts in part for its marked thermal stability. The probability of a highly organized tertiary structure was also supported by the results of tryptophan determination according to the formylation process of Previero et al. (1967). This method is based on the specific reaction of tryptophan residues with formic acid in a HCl-saturated medium. The formation of formyl-tryptophan was followed spectrophotometrically at 298 nm. The formylation of the native peptide was only partial, amounting to 43%, if one residue of tryptophan per mole of sillucin was assumed. In the presence of 8 M urea, 56% formylation was achieved. Pretreatment of sillucin with mercaptoethanol raised the degree of formylation to 89% of theoretical value.

This indicated that tryptophan was located in a highly cross-linked portion of the antimicrobial peptide.

In the case of miehein, there appeared to be not a single peptide, but a family of biologically active molecules (Table 2). These ranged in size from 56 to 74 amino acid residues and were identified by the number of the producing strain of *M. miehei*. As sillucin, miehein peptides were devoid of methionine. However, several of these peptides contained histidine and all contained two residues of phenylalanine. Other amino acids were apparently also conserved, namely, glutamic acid, valine, leucine, tyrosine, lysine, and tryptophan. The remaining integer values varied somewhat from strain to strain but close compositional relationship was quite evident. The Ellman procedure (DTNB) for free sulfhydryl groups was carried out only on miehein-21, with negative results, indicating a high degree of cross-linking in the molecule. The Previero tryptophan assay resulted in 43% formylation in 8 M urea medium and 93% formylation following the pretreatment of miehein-21 with mercaptoethanol.

The amino acid profiles of sillucin and miehein peptides were not mutually exclusive. The entire 30 residue composition of the smaller molecule was contained within each of the larger peptides. Speculation indicates that the 3200 dalton molecular weight entity represented by sillucin was sufficient to specify biological activity and that the miehein

TABLE 2. Amino acid composition of miehein peptides from various strains of *Mucor miehei*

Amino acid	Residues/mole <sup>a</sup>				
	3169	21	Strain 5282	5283	5284
Asp	3.9 (4) <sup>b</sup>	3.9 (4)	4.0 (4)	3.8 (4)	4.1 (4)
Thr	4.2 (4)	3.9 (4)	2.0 (2)	2.0 (2)	3.7 (4)
Ser	5.1 (5)	6.0 (6)	3.1 (3)	3.8 (4)	8.3 (8)
Glu	2.5 (3)	2.6 (3)	2.6 (3)	2.7 (3)	3.0 (3)
Pro	1.7 (2)	1.9 (2)	2.0 (2)	2.8 (3)	2.3 (2)
1/2Cys	4.8 (5)	7.9 (8)	12.7 (13)	13.4 (13)	15.4 (15)
Gly	6.6 (7)	6.6 (7)	9.1 (9)	9.3 (9)	9.8 (10)
Ala	4.6 (5)	4.7 (5)	3.8 (4)	4.8 (5)	5.2 (5)
Val	3.1 (3)	3.0 (3)	2.6 (3)	2.5 (3)	3.3 (3)
Met	—	—	—	—	—
Ile	1.0 (1)	1.4 (1)	2.2 (2)	2.7 (3)	2.0 (2)
Leu	2.5 (3)	2.7 (3)	2.8 (3)	3.0 (3)	2.9 (3)
Tyr	3.8 (4)	3.6 (4)	3.7 (4)	3.7 (4)	3.9 (4)
Phe	2.2 (2)	2.3 (2)	1.8 (2)	1.9 (2)	2.3 (2)
Lys	4.5 (4)	4.4 (4)	3.9 (4)	3.6 (4)	4.2 (4)
His	1.3 (1)	1.0 (1)	—	—	0.6 (1)
Arg	0.9 (1)	1.4 (1)	2.1 (2)	2.3 (2)	2.0 (2)
Trp <sup>c</sup>	1.6 (2)	1.9 (2)	d (2)	d (2)	2.3 (2)
Total residues	56	60	62	66	74

<sup>a</sup>Calculated from mean molar ratios using asp = 4, tyr = 4.

<sup>b</sup>Residues in parenthesis indicate probable integer values.

<sup>c</sup>See text for method of analysis.

<sup>d</sup>Not determined.

family of peptides have been further elaborated while retaining the same active site configuration. Future comparisons of this nature must await sequence and tertiary structure examinations.

The typical ultraviolet absorption spectrum of sillucin isolated from *M. pusillus* 410 is shown in Fig. 5. The sillucin of other *M. pusillus* strains as well as the miehein peptides of various *M. miehei* strains showed identical characteristics. The UV spectrum in 0.1 N HCl was characterized by a maximum at 276 nm and shoulders at 282 nm and 288 nm. In 0.1 N NaOH, two absorption maxima were found, at 282 nm and 288 nm.

The thermal stability of the antibacterial peptides was also tested. Sillucin was dissolved in distilled water, diluted to 1000  $\mu\text{g/ml}$  and adjusted to various pH levels ranging from pH 2.0 to pH 11.0, with glycine-HCl, sodium phosphate, and citrate buffers and the solutions were heated at 100 C for various lengths of time. Each sample was diluted with phosphate buffer (pH 6.0) to provide a suitable dilution for bioassay. A greater loss of activity was determined in alkaline solution than in acid (Table 3). Similar studies on miehein-21 showed a lower degree of heat stability. Approximately 75% activity remained associated

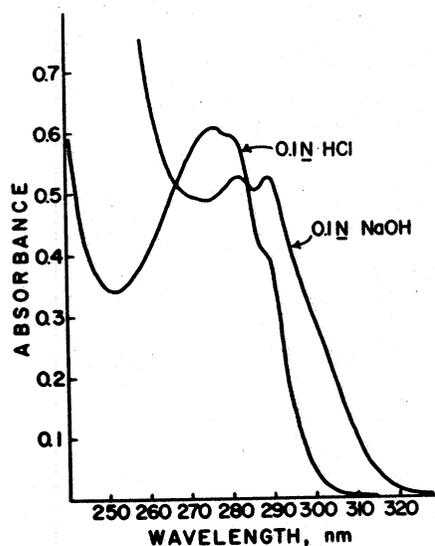


FIG. 5. Ultraviolet spectrum of sillucin and miehein peptides.

TABLE 3. Stability of sillucin solution (1000  $\mu\text{g/ml}$ ) at various pH levels at 100 C

pH	Percent Activity after Heating	
	5 min	30 min
2.0	96	95
5.0	95	95
7.0	95	90
8.0	85	55
11.2	15	5

with miehein-21 after heating at 100 C for 30 min in pH 7.0 buffer. Storage studies indicated no loss of antibiotic activity of sillucin stored over CaSO<sub>4</sub> at room temp during a period of 2 yr.

#### Antimicrobial Properties of Sillucin and Miehein

The effect of the peptide antibiotics on bacterial growth and macromolecular synthesis was studied with sillucin (*M pusillus* 410) and miehein-21 (*M. miehei* 21) as model compounds. The growth of a log-phase culture of *B. megaterium* was arrested following the addition of sillucin to the culture medium (Fig. 6). As a rule, a greater effect was shown when the peptide was added early during the log phase. The culture showed a similar response in the presence of miehein-21 in the culture medium. Growth experiments also were carried out with *Escherichia coli*, a typical gram-negative organism. To increase the permeability of the more complex gram-negative cell envelope, cells were pretreated with ethylenediaminetetra acetic acid (EDTA) used at  $5 \times 10^{-3}$  M. This procedure is known to partially remove the lipopolysaccharide from the outer layers of the cell wall (Leive 1965). However, the growth of *E. coli* was not affected, indicating the inability of the cationic peptides to penetrate the gram-negative cell envelope even under destabilizing conditions.

Among the antibiotics synthesized by *Phycomycetes*, only fusidic acid has been subjected to detailed mode of action studies. Fusidic acid, a steroidal antibiotic, was shown to inhibit the activity of transfer factor G, which is required for peptide chain elongation (Tanaka et al. 1969). More recently fusidic acid was reported also to inhibit peptide chain initiation in *E. coli* (Sala and Ciferri 1970).

The effect of sillucin on macromolecular synthesis in *B. megaterium* was studied by measuring the uptake of labeled thymidine, uracil, and isoleucine (Fig. 7). Upon addition of

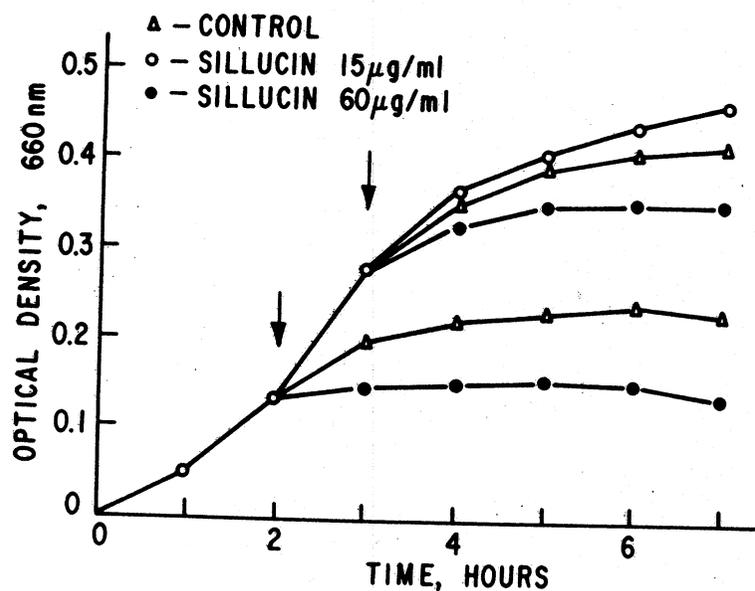


FIG. 6. Effect of sillucin on growth of *Bacillus megaterium* in trypticase soy broth. Arrow indicates time of sillucin addition.

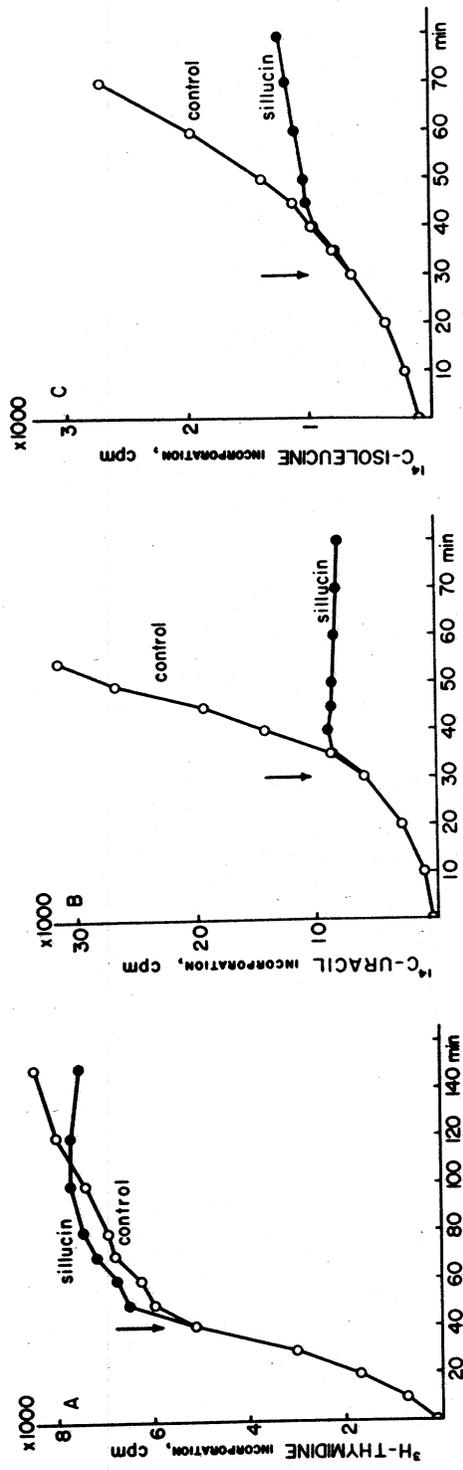


FIG. 7. Effect of sillucinin (70  $\mu\text{g}/\text{ml}$ ) on incorporation of (A)  $^3\text{H}$ -thymidine, (B)  $^{14}\text{C}$ -uracil, and (C)  $^{14}\text{C}$ -isoleucine by intact cells of *Bacillus megaterium*. Time of addition of sillucinin is indicated by the arrow. See text for details of experimental conditions.

70  $\mu\text{g}$  of sillucin per ml to the growth medium,  $^3\text{H}$ -thymidine incorporation continued nearly at its original rate for at least 40 min.  $^{14}\text{C}$ -isoleucine incorporation proceeded at its former rate for about 10-15 min and then stopped, whereas  $^{14}\text{C}$ -uracil incorporation stopped within 5 min. At 10 min and 20 min after the addition of sillucin, the amount of uracil incorporated by treated cells was less than 65% and 30% of the control, respectively. Similar results were obtained with miehein-21 as the antibacterial agent. Inference was made from these data that the primary inhibitory effect of sillucin and miehein was on uracil utilization, presumably RNA synthesis. The lag of inhibition of isoleucine uptake could be attributed to utilization of messenger RNA formed prior to the addition of antibiotic peptides to the culture. However, the data on isotope uptake trials were insufficient to define the exact mode of action of sillucin and miehein.

The information presented here does not imply that milk-clotting enzymes of *M. pusillus* and *M. miehei* produced under industrial conditions necessarily contain antimicrobial agents that may affect the synthesis of lactic acid by lactic starter cultures. However, the data indicated a widespread potential for antimicrobial peptide synthesis among *M. pusillus* and *M. miehei* cultures. Therefore, genetic selection of fungal strains, fermentation medium development, or fractionation procedures should be regarded as desirable means to prevent the occurrence of these peptide antibiotics in milk-clotting enzyme preparations.

#### ACKNOWLEDGMENT

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