A CRYSTALLINE ANTIFUNGAL AGENT, MYCOSUBTILIN, ISOLATED FROM SUBTILIN BROTH [1]

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The presence of an antifungal agent in the broth of subtilin cultures has been reported (1). It is not unusual in cultures of aerobic spore formers to find several different antibiotics present. Often, these have been related polypeptides, which have different biological spectra of antibiotic action (gramicidin, tyrocidine, etc.).

This paper reports the isolation and properties of a fungistatic substance from the cells of a culture of *Bacillus subtilis* 370, originally obtained from the Western Regional Research Laboratory of the United States Department of Agriculture, where it was used for studies on subtilin (2). Under certain cultural conditions, fungistatic activity is produced which is not due to subtilin. The standard assay organism for the fungistatic agent, *Trichophyton* sp. MF 301 was not inhibited by 200 μgm. of subtilin per ml. of agar. The antifungal agent has tentatively been named Mycosubtilin.

METHODS AND RESULTS

Conditions for production

Submerged aerated cultures of *B. subtilis* 370 were grown in 250 ml. flasks containing 50 ml. of 20% beet molasses with 0.8% $(NH_4)_2HPO_4$ and 0.005% $MnSO_4 \cdot 4H_2O$ on a shaker rotating at 220 RPM (3). The production of subtilin proceeds at optimum rate at a temperature of 34° C., but a temperature of 25° C. was found more suitable for the fungistatic substance. Yields were better at this lower temperature and filtration and extractions were conducted with greater ease. Fungistatic activity was produced in five days under the foregoing conditions.

Fermented cultures produced in a semi-synthetic medium containing 0.5% yeast extract [2] gave broth activity equal to that in cultures prepared with beet molasses medium. Broth samples were usually mixed with three volumes of ethanol to aid extraction of the antibiotic and to sterilize the culture for assay. The agar streak-plate method was used, with *Trichophyton* sp. MF 301 as the test organism, with two-fold dilution levels.

Extraction

The harvested broth was adjusted to about pH 2.5 with concentrated hydrochloric acid to precipitate the cellular material, which was separated by centrifugation. The first extraction of the moist cell solids was made overnight with 95% ethanol, which resulted in a final concentration of approximately 70% ethanol. Subsequent extractions of the cells were made overnight with 70% ethanol solutions. The solids were separated from the ethanol suspensions by filtration. The active fraction was precipitated quantitatively by the addition of two volumes of water and was separated by centrifugation. The solids were successively extracted with 95% ethanol. Material of increased purity was obtained with each successive extract. The ethanol was evaporated to dryness and all of the resulting solids dissolved in a very small quantity of pyridine. White crystalline material was separated during a period of several hours at 10° C. following addition of 10 volumes of water. The crystals were washed with water and recrystallized from 70% ethanol.

It was later observed that the solids from the 70% ethanol extracts of the cells could be directly dissolved in pyridine, thus eliminating the 95% EtOH stages, with better yields. Several recrystallizations were necessary to obtain material which showed a constant melting point and analysis.

The results in Table I represent a typical extraction from a culture produced in beet molasses medium, which had a moist cell volume of 15%.

Properties

The white crystals are practically insoluble in reagents, with the exception of pyridine and 70% ethanol. They are very soluble in the former but show only limited solubility in warm 70% EtOH. The antifungal agent crystallized readily from the latter upon cooling. The crystals are soluble in

[1] Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11–12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

[2] The semi-synthetic medium contained sucrose, 100 gm.; citric acid, 11.7 gm.; Na_2SO_4, 4 gm.; yeast extract, 5 gm.; $(NH_4)_2HPO_4$, 4.2 gm.; KCl, 0.76 gm.; $MgCl_2 \cdot 6H_2O$, 0.42 gm.; $ZnCl_2$, 0.0104 gm.; $FeCl_3 \cdot 6H_2O$, 0.0245 gm.; $MnCl_2 \cdot 4H_2O$, 0.0181 gm., dissolved in water, adjusted to pH 6.8–6.9 with NH_4OH and brought to one liter volume. The formula for the semi-synthetic medium and details of the procedures used at the Western Regional Research Laboratory for production of subtilin were disclosed to us by personal communication.

TABLE I

Isolation data for antifungal agent

	Volume	Solids dry wt.	Activity dilution	Total activity
	ml.	*mgm./ml.*	*units/mgm.*	
Original whole broth	1080	79.3	1.9	162,000
Cell extraction				
First (95% EtOH)	103	36.4	19.2	72,000
Second (70% EtOH)	96	21.5	46.5	96,000
Third (70% EtOH)	100	12.9	54.3	70,000
Extraction of solids				
First (95% EtOH)	17	28.6	35.0	17,000
Second (95% EtOH)	19	9.9	101	19,000
Third (95% EtOH)	19	2.4	208	9,500
Fourth (95% EtOH)	15	1.0	400	6,000
Fifth (95% EtOH)	19	0.6	416	4,750
Crystallization from pyridine	—	88.9 (total)	700	62,000

dilute cold NaOH but insoluble in dilute cold HCl and cold $NaHCO_3$.

No precipitate was obtained with 2,4-dinitrophenylhydrazine. There was no reduction of Fehling solution nor color development with ferric chloride solution. Millon's reagent produced a temporary pink coloration and nitration with conc. HNO_3 formed a pale yellow pigment. Acid hydrolyzates gave a strongly positive ninhydrin reaction.

Following recrystallization three times from 70% ethyl alcohol, the crystals melted with decomposition at 256°–257° C. (corr.) on a micro block.[3] The crystals gave an analysis of C—55.31, 55.12; H—7.61, 7.33; N—15.15, 15.18. Sulfur was not present. The ultra-violet absorption spectrum of alcohol-water solution consisted of a band at 2770 Å with an $E\frac{1\%}{1\text{ cm.}} = 14.6$. Potentiometric titration showed an apparent combining weight by alkali titration of 1980. General insolubility made the compound unsuitable for molecular weight determination.

Ten mg. of crystalline mycosubtilin was hydrolyzed in a sealed tube with 3 ml. of 6 *N* HCl at 120° C. for 16 hours. Paper strip chromatography with a phenol-water system, followed by development with ninhydrin, yielded four distinct zones, with observed R_f values presented in Table II, and an indistinct zone at R_f 0.97, close to the advancing solvent boundary. Amino acids which have characteristic R_f values in the region of the zones observed with mycosubtilin are listed in Table II. Aspartic acid, tyrosine and proline were found to be present by microbiological assay. The natural isomers of the other amino acids listed, with the exception of norvaline and norleucine for which no assays were available, were not found to be present by microbiological assay in concentrations exceeding 5%. The microbiological assay for aspartic acid and alanine is not specific for the natural form. Therefore, it is not certain which isomer of aspartic acid was present and it was demonstrated that the D form of alanine was not present. The tyrosine content is adequate to account for the ultra-violet absorption band described above. It is interesting to note that tyrosine has not been found in subtilin, the other amino acid-containing antibiotic produced by *B. subtilis* 370.

Aspartic acid accounts for nearly one-half the molecule and one-third of the nitrogen. An ad-

TABLE II

Amino acid composition of mycosubtilin and subtilin HCl hydrolyzate

Mycosubtilin			Subtilin composition†
Observed R_f	Literature values*	Observed microbiological assay	
.19	Aspartic acid (.19)	45%	Aspartic acid Lanthionine
.34	Glutamic acid (.32) Serine (.37)	— —	L-glutamic acid Glycine L-lysine
.62	Alanine (.63) Tyrosine (.63) Arginine (.66)	— 5.4% —	Alanine Tryptophane
	Valine (.82) Norvaline (.84)	—	L-valine
.85	Isoleucine (.88) Leucine (.88) Norleucine (.89)	— —	L-isoleucine L-leucine
	Phenylalanine (.90)	—	L-phenylalanine
.97	Proline (.90)	4.2%	L-proline

* Pratt, J. J. and Auclair, J. L. Science, 1948, *108*, 213.
† Lewis, J. C. and Alderton, G. A.C.S. Abstracts, 1948.

[3] We are indebted to Dr. E. F. Rogers for helpful suggestions and criticisms concerning the chemical characterization. Mr. R. N. Boos performed the elemental analysis and Dr. Charles Rosenblum the UV spectrum and potentiometric titration.

TABLE III

Antibiotic activity of mycosubtilin against yeasts

	Complete inhibition μgm./ml.
Candida guilliermondii, 488	N.A. at 20
Debaryomyces gruetzii, 4144	N.A. at 20
Schwanniomyces occidentalis	N.A. at 20
Zygopichia californica	N.A. at 20
Mycoderma valida	7.5
Saccharomyces carlsbergensis, 9080	7.5
Rhodotorula rubra	Ca. 5.0
Sporobolomyces roseus	5.0
Torula cremoris	5.0
Dipodascus uninucleatus	3.75
Hansenula anomala, 4104	3.75
Torulopsis delbruckii	3.75

(N.A. indicating no inhibition)

TABLE IV

Fungistatic activity of mycosubtilin

	Complete inhibition μgm./ml.
Aspergillus niger	N.A. at 20
Trichoderma sp.	N.A. at 20
Mucor flavus	N.A. at 20
Rhizopus javanicus takeda	N.A. at 20
*Microsporum lanosum**	10.0
*Trichophyton mentagrophytes**	10.0
Fusarium moniliforme	7.5
Nematospora coryli	7.5
Penicillium notatum	7.5
Chaetomium bostrychodes	5.0
*Microsporum audouini**	5.0
*Achorion schöenleinii**	5.0
*Cryptococcus neoformans**	5.0
*Epidermophyton inguinale**	5.0
Sclerotinia fructicola	2.5
Ustilago zeae	1.5
Trichophyton sp.	1.5

(N.A. indicating no inhibition)

* We are indebted to Dr. Morris Solotorovsky for the assay of these particular pathogenic fungi and for toxicity determinations.

ditional one-third of the nitrogen, 5.1% N, was liberated as NH_3 by acid hydrolysis. The speculation arises that aspartic acid is present in the molecule as the amide.

The substance is heat stable, resisting autoclaving in agar for 15 minutes at 120° C. and 15 lbs. pressure.

A concentration of 0.0015 mgm./ml. of agar inhibited the growth of the test organism *Trichophyton* sp. (MF 301) by the streak-plate method on yeast extract dextrose agar. However, the addition of 10% horse serum incorporated in the agar completely neutralized the activity.

Antibiotic spectrum

Micrococcus lysodeikticus was inhibited by 0.001 mgm./ml. of nutrient agar by the streak-plate method. But 0.016 mgm./ml. failed to inhibit *Staphylococcus aureus* (Smith), *Streptococcus pyogenes* C–203, *Streptococcus viridans*, *E. coli*, *Eberthella typhosa*, *Klebsiella pneumoniae*, *Hemophilus pertussis*, *Corynebacterium diphtheriae* (gravis), *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* 607, *Salmonella aertrycke*, *Salmonella paratyphi* A, *Salmonella schottmuelleri*, *Alcaligenes faecalis*, and *Bacillus megatherium*.

The yeast spectrum, Table III, and the fungus spectrum, Table IV, were streaked on yeast extract dextrose agar. The results indicate the smallest amount of crystalline material required to cause complete inhibition. Although mycosubtilin is insoluble in water, it does not precipitate when a 10 mgm./ml. solution in hot 70% alcohol is added to liquid agar to make a concentration of 1 mgm./ml.

Toxicity

Toxicity was determined by the subcutaneous injection of mycosubtilin into white Swiss mice weighing approximately 20 gms. each. For injection into mice, a solution containing 2 mgm. of agent per ml. was prepared by mixing an alcoholic solution of the crystalline material with 20% aqueous gelatin. Deaths were observed following single doses of 1 or 0.5 mgm., but single doses of 0.25 mgm. or less were tolerated.

It was impossible to demonstrate the agent in the blood of injected animals because of the neutralizing effect of blood serum on the antifungal activity.

SUMMARY

A fungistatic substance has been isolated from the cells of *B. subtilis*, the subtilin producing microorganism. A method of extraction has been suggested by which crystalline material has been obtained. The white crystals, having a melting point of 256°–257° C. with decomposition, yield amino acids following acid hydrolysis. The new antibiotic has been named mycosubtilin.

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