STUDIES ON SUBSPORIN. I

ISOLATION AND CHARACTERIZATION OF SUBSPORINS A, B AND C

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From the culture of *Bacillus subtilis* PCI 219, three new peptide antifungal substances were isolated. Based on the physicochemical properties of the three antibiotics, they were named subsporins A, B and C. Subsporin A, the main component of the antibiotic mixture, consisted of fourteen amino acid residues and of a nitrogen-containing long chain compound. Constituent amino acids of subsporin A were L-Asp_4, D-Asp_2, D-Tyr_2, L-Glu_2, L-Pro_2 and D-Ser_2. Subsporins are active against filamentous fungi and yeast, while no activity is observed against bacteria.

It has been known for many years that *Bacillus subtilis* produces peptide antifungal substances when the organism is cultivated at relatively low temperature. More than ten antifungal substances have been isolated from cultures of *B. subtilis*. Among them, mycosubtilin[1], mycobacillin[2], bacillomycin A[3], porcillin[4], and bacillocin[5] have been relatively well characterized. It has been shown that they resemble each other in amino acid composition, all containing aspartic acid, glutamic acid, tyrosine and proline with the exception of bacillomycin A[3]. Elucidation of their structures has not yet been achieved except for mycobacillin due to the presence of D-amino acids.

Recently we have found that *B. subtilis* PCI 219 produces three mycosubtilin-like antifungal substances when the organism is cultivated in a medium designed for the production of mycosubtilin[1]. A preliminary characterization, however, has revealed that all three can be distinguished from mycosubtilin by nitrogen content, UV absorption and by antibiotic activity. Since they are apparently new substances, the three antibiotics have been designated as subsporins A, B and C.

The present paper deals with isolation and characterization of subsporins A, B and C. These antibiotics consist of fourteen amino acid residues and of a nitrogen-containing long chain compound. Subsporins are active against many filamentous fungi and yeast, while no activity is observed against most bacteria.

Experimental

Organisms and Cultural Medium

Bacillus subtilis PCI 219 was obtained from the Antibiotics Division of our laboratory. This organism has been used for assay of erythromycin and siomycin. Some of the *Trichophyton* species were also supplied by the Antibiotics Division.

The ammonium citrate-sucrose medium for mycosubtilin production described by WALTON and WOODRUFF[1] was used for the preparation of subsporins.

Production of Subsporins A, B and C

B. subtilis PCI 219 was cultivated in the ammonium citrate-sucrose medium at 28°C in all cases. For vegetative inoculum, the organism was grown in shake flasks on a reciprocal shaker for 48 hours. The resultant culture was used to provide a 2% inoculum for fermentation flasks. Incubation of the 2-liter Erlenmeyer fermentation flasks (each containing 800 ml) was carried out on a rotary shaker operating at 190 rpm for 4 days. Jar fermentors (20 liters) were also used for the fermentation. In this case, incubation was also at 28°C for 4 days.

Production of subsporins reached 400~600 μg/ml in the ammonium citrate-sucrose medium. Other media were not suitable for subsporin production. In the case of the glucose-glutamate medium used for bacillomycin production[3], only 78 μg/ml of subsporin were produced. When a peptone-beef extract medium was used, no subsporin production occurred.

Analytical Methods

Ultraviolet absorption spectra were measured in 70% ethanol, with a Perkin-Elmer Type 202 Spectrophotometer. Infrared spectra were taken with a Nihon Bunko DS-201B Spectrophotometer by the nujor method. Optical rotations were measured in pyridine, with a Perkin-Elmer Polarimeter, Type 141. Optical configuration of the constituent amino acids was also determined by the aid of gas chromatography of the applied N-trifluoroacetylamino acid-D-*sec*-butyl ester[6].

Isolation and Purification of Subsporins A, B and C

Crude Subsporin Preparation

Ten liters of the harvested culture of *B. subtilis* PCI 219 were adjusted to pH 2.5 by adding 180 ml of 9 N HCl. The precipitates were collected by centrifugation and extracted with 1 liter of 95% ethanol. Extraction was repeated twice more each with 800 ml of 70% ethanol. The extracts were combined and two volumes of water were added. After standing overnight, the resultant precipitates were collected by centrifugation and were lyophilized. The dried solids were washed with a small volume of ethanol to remove impurities such as pigments. Yield was 7.12 g.

Subsporin A

Pure subsporin A could be prepared from the crude preparation in 60~70% yield by the following procedures.

Step 1. Recrystallization from the mixed solvent of chloroform, methanol and 70% ethanol (7 : 3 : 5).

Step 2. A diluted solution of subsporins in the above mixed solvent was passed through a silica gel G (for TLC use) layer. Then the solution was evaporated.

Step 3. Recrystallization from the mixed solvent. The subsporin A was further recrystallized from 70% ethanol.

Purity was more than 99% based on TLC.

Subsporin B

Subsporin B could be prepared only from shake flask fermentation where it was

found together with subsporin A. Mother liquors from streps 1 and 3 described above were combined and evaporated. One gram of the dried residue was dissolved in 25 ml of the mixed solvent (described above). This solution was fractionated on a silica gel column (20×600 mm) which was equilibrated with the same solvent. Elution was carried out with the same solvent and fractions (5 g) were collected. Subsporin B (25 mg, tube Nos. 65~72), subsporins A and B (150 mg), and subsporin A (500 mg), were obtained according to TLC.

Subsporin C

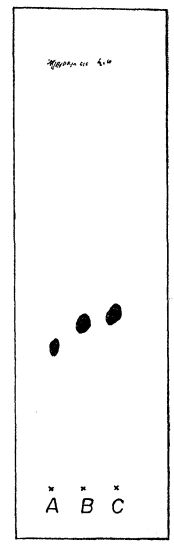
Subsporin C was obtained from the culture which was grown in jar fermentors. Five grams of the dried residue derived from the mother liquors of steps 1 and 3, were fractionated on a silica gel column (22×600 mm) as described above. Subsporin C rich fractions so obtained were evaporated (dry weight, 200 mg) and further purified by the preparative thin-layer chromatographic technique (silica gel GF_{254}-chloroform, methanol, 70 % ethanol, 7 : 3 : 5). The ultraviolet-sensitive bands on thin-layer chromatogram corresponding to subsporin C were eluted with 70 % ethanol and were evaporated. Yield was 20 mg.

The proportions of each component in the usual crude subsporin preparations were approximately 97 % (A) and 3 % (B) in the case of flask culture, and approximately 99 % (A) and 0.5 % (C) in samples from the jar fermentor.

A typical thin-layer chromatogram of these antifungal substances is depicted in Fig. 1.

From the present culture of *B. subtilis* PCI 219, other minor substances such as peptides X, Y, C′ and substance B′ (a long-chain fatty acid) and W (a mucopeptide) were also isolated by the aid of a silica gel column chromatography using the same solvent mixture as described above. They were, however, all inactive. From a culture which was incubated at 37°C instead of 28°C, the antibiotic subtilin was obtained, while subsporins were no longer produced at this temperature.

Fig. 1. Thin layer chromatogram of subsporins A, B and C. Thin layer plate, silica gel G; solvent, chloroform-methanol-70 % ethanol (7 : 3 : 5).



Physicochemical Properties of Subsporins A, B and C

Some of the physicochemical properties of subsporins A, B and C are listed in Table 1. Molecular weight of each subsporin was determined. Amino acid analysis of subsporin A gave 957 as a minimum molecular weight, and the osmotic method gave 1,766. From the result of preliminary amino acid sequence analysis[7], it is probable that the molecular weight of subsporin A is twice 957. Based upon this molecular weight, the formula $C_{88}H_{148}O_{26}N_{20}$ (molecular weight, 1,902) comes closest to the values of the elemental analysis of subsporin A. A final formula for the antibiotic, however, can be obtained only after complete structural elucidation.

Subsporins are negative in the ninhydrin test due to the

Table 1. Some physicochemical properties of subsporins A, B and C

	Subsporin A	Subsporin B	Subsporin C
Appearance	Colorless crystalline	Colorless crystalline	Colorless crystalline
M. p. (°C)	259~260.5°	272~273.5°	182~186°
Elementary analysis (%)	C 53.91 H 7.61 N 14.65 O 20.88 S —	C 54.56 H 7.68 N 14.16 O 19.67 S 0.22	
Estimated formula	$C_{88}H_{148}N_{20}O_{26}$ (1,902)	$C_{88}H_{148}N_{20}O_{24}$ (1,870)	
Molecular weight UV (Tyr)	1,168	1,150	1,140
Amino acid analysis (minimum)	957	971	1,230
Titration	1,859	—	—
Osmotic method	1,766	—	—
$[\alpha]_D^{23}$ (c 0.5, pyridine)	+ 20.5	+ 16.8	+ 13.0
λ_{max} (mμ)	278	278	278
$E_{1cm}^{1\%}$ (70 % EtOH)	12.58	12.60	12.80

presence of a nitrogen-containing long chain compound, probably C_{19} fatty acid-like compound, at the nitrogen terminal of the peptide moiety. The structure of the compound is still unknown.

The ultraviolet absorption spectra of approximately 0.1 % solution of subsporins A, B and C are shown in Fig. 2. Absorption maxima were observed at 278 mμ indicating the presence of tyrosine residues. Fig. 3 shows infrared absorption spectra of the three antibiotics. The large absorption band between 1,600 and 1,700 cm^{-1} indicates that subsporins are typical peptide substances. No meaningful difference between spectra was observed.

The amino acid compositions of subsporins A, B and C were determined from several runs by extrapolating to the zero time of hydrolysis. Optical configurations of the constituent amino acids were determined mainly by the gas chromatographic

Fig. 2. Ultraviolet absorption spectra of subsporins A, B and C in 70 % ethanol. Concentrations: subsporin A 0.116 %; subsporin B 0.100 %; subsporin C 0.099 %.

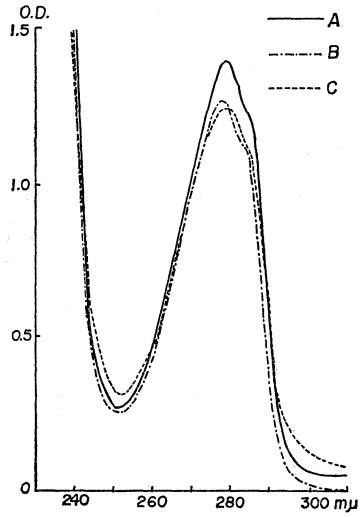
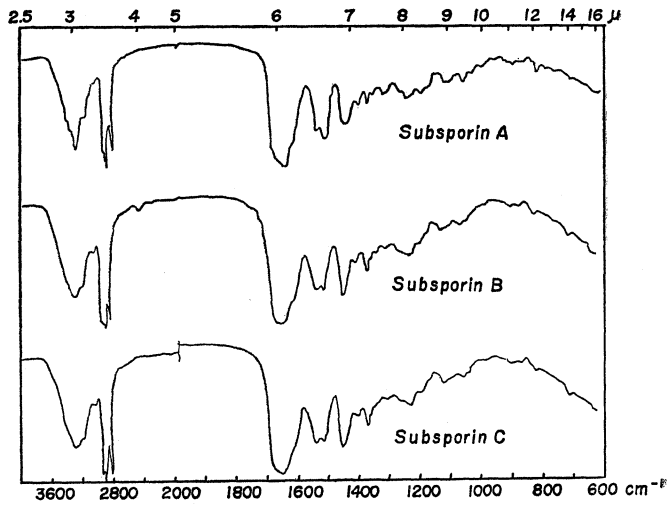


Fig. 3. Infrared absorption spectra of subsporins A, B and C (nujol method).



technique and are shown in Table 2.

Specific optical rotations of the tyrosine and serine isolated from the acid hydrolysate of subsporin A were +11.0° (*c* 0.5, N HCl) and −13.8° (*c* 1.2, 2 N HCl), respectively.

Subsporins were soluble in methanol, 70 % ethanol, aqueous acetone, dioxane, pyridine and 0.1 N NaOH. They are almost insoluble in water, chloroform, benzene, ether and 0.1 N HCl.

Subsporin A was not hydrolyzed by chymotrypsin, pepsin or carboxypeptidase A. Subsporin A did not loss its antifungal activity by heating at 120°C for 20 minutes.

Table 2. Amino acid composition of subsporins A, B and C

	Subsporin A	Subsporin B	Subsporin C*
L-Aspartic acid	4	4	4
D-Aspartic acid	2	2	4
L-Glutamic acid	2	2	4
D-Tyrosine	2	2	2
D-Serine	2	0	2
D-Alanine	0	2	0
L-Proline	2	2	2
Ammonia	5	(8)	—

* Optical configurations were not estimated except for proline (L-form).

Table 3. Antibiotic activity of subsporins A, B and C (MIC, μg/ml)

	Subsporin A	Subsporin B	Subsporin C
Aspergillus niger	10	50	—
Trichophyton rubrum	10	10	—
Trichophyton mentagrophytes	5	5	—
Trichophyton interdigitale	5	10	12
Epidermophyton floccosum	10	5	—
Penicillium notatum Westling	7.5	>20	10
Penicillium digitatum	20	>50	—
Pericularia oryzae	5	2	—
Fusarium oxysporum	>50	>50	—
Fusarium sp.	5	>20	20
Candida albicans M-9	5	50	—
Candida pseudotropicalis var. *lactosa* No. 616	5	>20	10
Rhodotorula minuta Saito No. 0387	5	15	10
Shigella dysenteriae	>50	>50	
Salmonella typhosa	>50	>50	
Escherichia coli B	>20	>20	
Pseudomonas aeruginosa	>50	>50	
Bacillus subtilis	>50	>50	
Bacillus anthracis	>50	>50	
Micrococcus lysodeikticus	>18	—	
Mycobacterium tuberculosis H 37 Rv	>50	>50	
Trichomonas vaginalis	25	12.5	

Biological Activity

Antibiotic activities of subsporins A, B and C are summarized in Table 3. Subsporins are active not only on many filamentous fungi but also on some yeasts. Antibacterial activity, however, was not demonstrated. Subsporins A and B were moderately active against *Trichomonas vaginalis*.

The intraperitoneally acute LD_{50} for mice was found to be 73±5.4 mg/kg body weight.

Hemolytic activity of subsporins A and B for rabbit erythrocytes was not demonstrated until the concentrations of the antibiotics reached to 100 μg/ml.

Discussion

On the basis of physicochemical and biological properties of subsporins A, B and C, the three antibiotics are clearly new peptide antifungal substances. They can be differentiated from mycobacillin[2], bacillomycin A[3], B[8] and C[9], B-456[10] and bacillocin[5] by amino acid composition. They also differ from porcillin[4] by melting point, and from iturin[11] by molecular weight and stability, and further from mycosubtilin[1] by stability against serum enzymes.

Subsporins A, B and C were shown to be different from each other in amino acid composition (Table 2). D-Aspartic acid, D-serine (or D-alanine) and D-tyrosine were found in the antibiotics by the aid of the GLC technique and optical rotation measurements. Among these, D-tyrosine is a rare amino acid residue in antibiotics produced by *B. subtilis*, tyrosine usually occurring as the L-form[2,12]. This finding, however, is not the first cases; the occurrence of D-tyrosine in the antifungal substance B-456 had been pointed out by TANAKA in 1956[10] The occurrence of these D-amino acids in subsporin A might account for the resistance of the antibiotic toward the action of several enzymes.

The biological activities of the subsporins (Table 3) show that they have relatively wide spectra against fungi as compared with griseofulvin. These spectra resemble that of mycosubtilin except that mycosubtilin is active against *M. lysodeikticus*[1].

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References

1) WALTON, R. P. & H. B. WOODRUFF: A crystalline antifungal agent, mycosubtilin, isolated from subtilis broth. J. Clin. Invest. 28 : 924~926, 1949.
2) MAJUMDAR, S. K. & S. K. BOSE: Amino acid sequence in mycobacillin. Biochem. J. 74 : 596~599, 1960.
3) TURNER, R. A.: Chemical studies on bacillomycin. Arch. Biochem. & Biophys. 60 : 364~372, 1956.
4) FUKUMOTO, J.; U. SATOMURA, K. HASHIMOTO, S. IWAI & U. TANIGUCHI: Method of production of an antifungal antibiotic. Japanese Patent 36-1,149, Mar. 7, 1961.
5) SAWADA, J.; H. NAKAYOSHI, K. OKUMURA & S. OMURA: Method of production of a new antibiotic polycillin. Japanese Patent 40-12,476, June 18, 1965.
6) POLLOCK, G. E. & V. I. OYAMA: Resolution and separation of racemic amino acid by gas-chromatography and the application to protein analysis. J. Gas Chromatogr. 4 : 126~131, 1966.
7) EBATA, M.; K. MIYAZAKI & Y. TAKAHASHI: Studies on subsporin. II. J. Antibiotics (in press)
8) SHIBAZAKI, I. & G. TERUI: Antifungal substances. II. Antifungal substance produced by a strain of *Bacillus subtilis*. (in Japanese) J. Ferment. Technol. 31 : 339~344, 1953.
9) SHIBAZAKI, I. & G. TERUI: Antifungal substances. III. Antifungal substances produced by a strain of *Bacillus subtilis*. (in Japanese) J. Ferment. Technol. 32 : 115~118, 1954.
10) TANAKA, Y.: Studies on an antifungal antibiotic produced by *B. subtilis*. J. Antibiotics, Ser. B 9 : 1~8, 1956.
11) DELCAMBE, L: Research for constitution of iturin. Compt. Rend. Soc. Biol. 146 : 1808~1812, 1952.
12) ROGERS, H. J.; N. LOMAKINA & E. P. ABRAHAM: Observations of the structure of bacilysin. Biochem. J. 97 : 579~586, 1965.