ON THE MODE OF ACTION OF A NEW ANTIFUNGAL ANTIBIOTIC, ACULEACIN A: INHIBITION OF CELL WALL SYNTHESIS IN *SACCHAROMYCES CEREVISIAE*

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The mode of action of a new antifungal antibiotic, aculeacin A, was studied with the cells of *Saccharomyces cerevisiae*. In the presence of aculeacin A, the distinct decrease of viable cells was observed. The most of cells treated with aculeacin A lysed with releasing intracellular substances at the tips of their buds. This lysis was considered to be due to the inhibition of cell wall synthesis, because the incorporation of glucose into the cell wall glucan was significantly reduced. Aculeacin A also had a weak activity to burst the protoplasts of *S. cerevisiae* at a relatively high concentration.

Aculeacin A is a new antifungal antibiotic produced by *Aspergillus aculeatus*, and was discovered in our laboratory.[1] Its chemical structure is considered to be an oligopeptide containing a fatty acid. Aculeacin A is active against fungi and yeasts, but does not inhibit the growth of bacteria.

Action mechanisms of several antifungal substances have been studied. Polyene antibiotics and imidazolyl methane antibiotics bind to membrane lipids and induce the destruction of the cell membrane[2,3]. 5-Fluorocytosine inhibits the synthesis of DNA or RNA[4]. In the studies of the mode of action of aculeacin A, several interesting results different from those of these antibiotics were obtained.

This paper reports the effect of aculeacin A on the cell wall synthesis and on the membrane of *S. cerevisiae*. Then, the site of glucan synthesis and the relationship between glucan synthesis and membrane are discussed.

Methods and Materials

Yeast and Cultivation

Saccharomyces ceresiae strain Hansen 0209, was grown aerobically in a medium containing 4% glucose, 1% peptone and 0.2% yeast extract (glucose-peptone medium) on a reciprocating shaker at 30°C. The growth was chased by measuring the absorbance at 550 nm of cell suspensions. The viability of cells was determined by counting the colony numbers grown on SABOURAUD glucose agar plates which were incubated at 30°C for 48 hours after planting the cell suspensions serially diluted with 0.9% saline.

Incorporation of Radioactive Substances into Acid-insoluble Fraction of Growing Cells

Yeast cells in the exponential phase were transferred to fresh media supplemented with 5 μg of aculeacin A. After incubation for 10 minutes at 30°C, 2.5 μCi of labeled L-leucine, uridine or D-glucose was added to each medium. Samples were taken at 30, 60 and 90 minutes and suspended in ice-cold 5% trichloroacetic acid (TCA). The acid-insoluble precipitates were collected on glass filter (Whatman, GF/C) and washed with ice-cold 5% TCA. The radioactivity was counted with a liquid scintillation counter after addition of 10 ml of toluene containing POPOP (0.3 g per liter) and PPO (4 g per liter).

Incorporation of Labeled Glucose into Cell Wall Glucan and Mannan

Yeast cells were incubated under the conditions similar to those described above. After 30 minutes the cells were collected by centrifugation. Wall polysaccharides were extracted according to the method described by ELORZA[5]. The cells were digested with 2 N sodium hydroxide at 100°C for 2 hours in a sealed ampoule. The supernatant obtained by centrifugation was treated with FEHLING reagent to form the Cu^{++}-mannan complex. The precipitate was extracted with 0.5 N acetic acid at 100°C for 2 hours to remove glycogen. The radioactivities of the Cu^{++}-mannan complex and the insoluble glucan residue after acetic acid extraction were counted with a liquid scintillation counter.

Preparation of Protoplast

Exponential phase cells were suspended in 0.8 M mannitol in 1/15 M phosphate buffer (pH 7.2). Zymolyase-5000[6] was added to make final concentration of 1 mg/ml, and the suspension was incubated at 30°C for 2 hours. The protoplasts thus prepared were washed three times with 0.8 M mannitol in the same buffer and used immediately for further experiments.

Electron Microscopy

Cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 hours at 2°C and washed three times with the same buffer. The cells were then dehydrated through a series of acetone-water mixture and embedded into a butyl- and methyl-metacrylate mixture. The sections were stained with uranyl acetate and examined with an electron microscopy (JEM-100B).

Chemicals

L-[1-^{14}C] Leucine (384 mCi/m mole), [2-^{14}C] uridine (57 mCi/m mole) and D-[U-^{14}C] glucose (255 mCi/m mole) were purchased from New England Nuclear. Amphotericin B (Fungizone) was from the Squibb & Sons Corp. Zymolyase-5000 was from the Research Laboratories of Kirin Brewery Co., Ltd.

Results

Effect on Growth and Viability of *S. cerevisiae*

The effect of aculeacin A on growth of *S. cerevisiae* in a glucose-peptone medium is shown in Fig. 1.

In the culture with aculeacin A at 0.5 μg/ml, the growth was completely inhibited, whereas at 0.2 μg/ml the growth was partially affected.

The effect of aculeacin A on viability of *S. cerevisiae* was studied at three levels of concentrations (Fig. 2A). Aculeacin A had a fungicidal effect at all levels of concentrations tested. The distinct decrease of viable cells took place within two hours, then the rate of the decrease dropped gradually. While, the decrease of viable cells was not seen in the buffer solution where no growth was permitted (Fig. 2B). So, the proliferation of cells should be necessary in order to exhibit the killing effect of aculeacin A.

In the presence of 5 μg of aculeacin A per ml the morphological change of cells was observed by light microscope. The most of them lysed with releasing intracellular substance, and the site of lysis was at the tips of their buds (Plate 1). This observation suggested that the fungicidal action of aculeacin A was mainly owing to this cell lysis.

Effect on Macromolecular Synthesis of *S. cerevisiae*

The incorporation of radioactivity from [1-^{14}C] leucine and [2-^{14}C] uridine into acid-insoluble fraction of growing cells were not affected by 5 μg of aculeacin A per ml, however, the incorporation of [U-^{14}C] glucose was inhibited (Fig. 3).

This results and the morphological change described above suggested that aculeacin A might affect the cell wall synthesis of *S. cerevisiae*. To clarify this point, the incorporation of [U-^{14}C] glucose into the cell wall fraction was examined.

[U-^{14}C] Glucose was added to growing culture of the organisms with or without aculeacin A, and the cells were harvested after 30 minutes. Then, the polysaccharides of the cell wall were fractionated into mannan and glucan. The incorporations of the glucose into the mannan fraction prepared from antibiotic-treated cells was about the same as that in the control, while, that into the glucan fraction was about a half of that of the control cells (Table 1). This suggests that aculeacin A affects the glucan synthesis rather than mannan synthesis in the cells.

Effect on Cell Membrane

The most of cells treated with 5 μg of aculeacin A per ml for one hour lysed, but some of them remained unlysed. Thin sections of remaining cells were examined by electron microscope. Several invaginations originating from the surface of the cell membrane are shown in the electron micrograph (Plate 2), and invaginations frequently formed small pockets between cell wall and cytoplasm. Within these pockets, membranous globular bodies were located, and this feature in shape and localization looks closely like the membrane structure found in cells treated with clotrimazole.[7]

To examine directly the effect of aculeacin A on the cell membrane, protoplasts of *S. cerevisiae* were used. The protoplasts slightly lysed at 100 μg of aculeacin A per ml, but no lysis occurred with the drug at 10 μg/ml. While the protoplasts lysed completely within 10 minutes when they were incubated with 10 μg of am-

Fig. 1. Effect of aculeacin A on growth of *S. cerevisiae*.

The cells (1.0×10^5 cells/ml) were grown in a glucose-peptone medium with varying concentrations of aculeacin A.

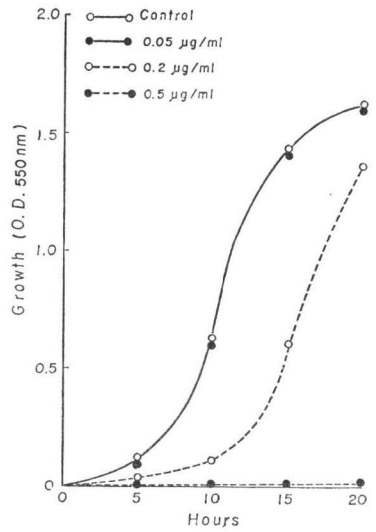


Fig. 2. Effect of aculeacin A on viability of *S. cerevisiae*.

The cells were grown in a glucose-peptone medium (A), or incubated with 0.1 M phosphate buffer (B).

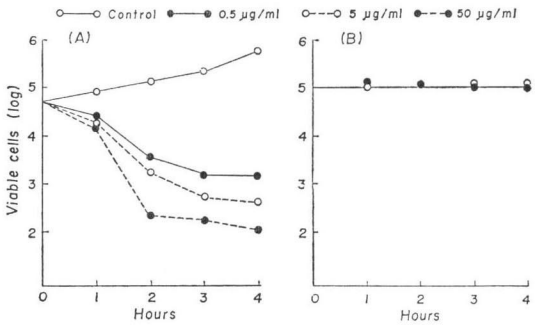


Plate 1. Effects of aculeacin A on the cell morphology of *S. cerevisiae*.

The cells were incubated in a glucose-peptone medium with 5 μg of aculeacin A per ml for 1 hour. The cell lyses with releasing intracellular materials (single-shafted arrow), and the site of lysis is at its bud (double-shafted arrow). ×1,500.

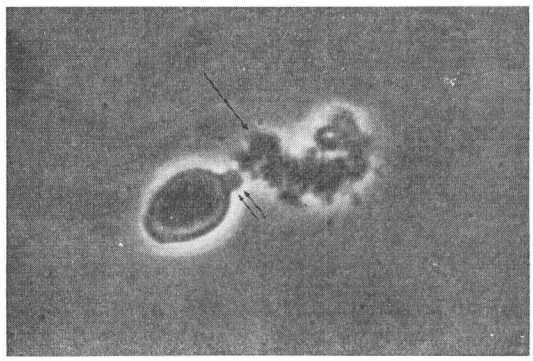


Fig. 3. Incorporation of radioactivity from labeled compounds into acid-insoluble fraction of cells of *S. cerevisiae*.

The cells were incubated in the medium containing 5 μg of aculeacin A per ml and after 10 minutes, 2.5 μCi of L-[1-^{14}C] leucine, [2-^{14}C] uridine and D-[U-^{14}C] glucose were added. Then, incorporation of radioactivity into the acid-insoluble material was measured.

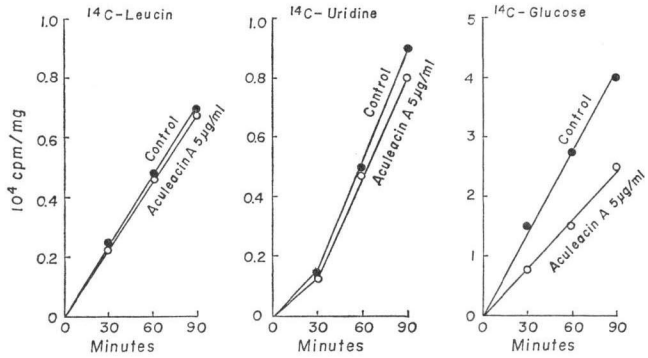


Table 1. Incorporation of radioactivity from D-[U-^{14}C] glucose into cell wall polysaccharides of *S. cerevisiae*.

	Control (cpm/mg)	5 μg of aculeacin A per ml	
		(cpm/mg)	% Control
Glucan	14,595	6,671	45.7
Mannan	3,561	3,190	89.6

The cells were incubated in a medium containing 5 μg of aculeacin A per ml and after 10 minutes, 2.5 μCi of D-[U-^{14}C] glucose was added. After incubation for 30 minutes at 30°C, the cells were harvested and fractionated as described in Materials and Methods.

photericin B per ml which had been known to have a destructive influence on the membrane (Fig. 4).

Discussion

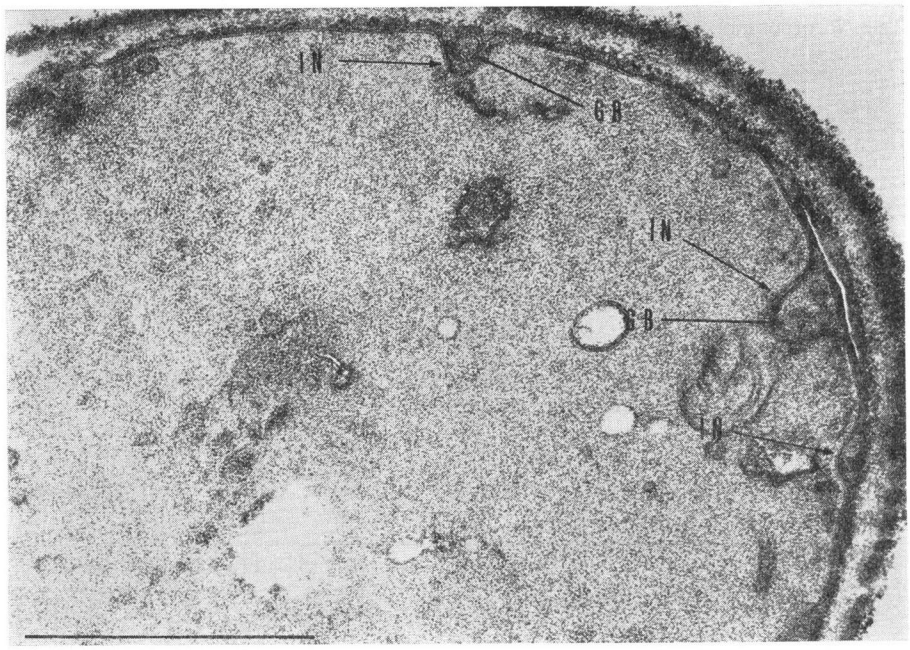
Aculeacin A selectively acted on the growing cells, but it was inactive against the resting cells. In other words, the growing cells were the target for the antibiotic, and the death of cells was caused by resulting leakage of intracellular substances.

The specific site of the leakage was a tip of bud, and this site had been known as the active locus for glucan synthesis[8]. In the experiments of macromolecular biosynthesis aculeacin A inhibited the synthesis of the cell wall glucan. Therefore, the leakage of intracellular substances might be caused by the inhibition of the cell wall synthesis.

In respect to the leakage of *S. cerevisiae* owing to the inhibition of cell wall synthesis, similar result has been reported by JOHNSON using 2-deoxyglucose which has been known as an inhibitor of mannan and glucan synthesis[9,10]. These facts suggest that the site of glucan synthesis is located at the tip of bud and cell wall glucan serves to maintain the cell shape and its rigidity.

Recently, biosynthesis of cell wall polysaccharides has been elucidated in relation to membrane and lipids. In mannan synthesis, the transfer of mannose from GDP-mannose proceeds through a lipid carrier[11], which has been characterized as a phosphodiester of mannose and isoprenol alcohol[12]. In glucan synthesis, SENTANDREU reported using toluene-treated cells of *S. cerevisiae* that UDP-glucose was a possible precursor of glucose into a particulate membrane fraction[13]. However, the involvement of a lipid intermediate is yet obscure.

Plate 2. Thin section of *S. cerevisiae*.
The cells were incubated in a glucose-peptone medium with 5 μg of aculeacin A per ml for one hour. Invaginations (IN) of the cell membrane and membranous globular bodies (GB) are seen. ×12,000.



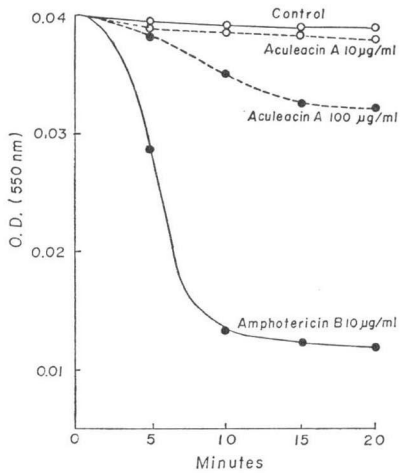
On the other hand, aculeacin A also slightly affected the membrane as observed in protoplast bursting experiment and the electron microscope. However, as seen in Fig. 4, amphotericin B bursted rapidly the protoplast at lower concentration than those of aculeacin A.

Insofar as our findings, the direct action of aculeacin A is quite different from the polyene[2] or imidazolyl methane antibiotics[13], and at present time it is interpreted as follows: aculeacin A made an attack against the growing cells, then inhibited the glucan synthesis at the tips of buds.

Although a possible relationship between glucan synthesis and membrane or lipid as a function is not clear, for the killing of cells the immediate participation of membrane or lipid in the aculeacin A action might be scanty if any.

Fig. 4. Effect of aculeacin A or amphotericin B on the absorbancy of a suspension of *S. cerevisiae* protoplasts.

Protoplasts of *S. cerevisiae* were suspended in 0.8 M mannitol in 1/15 M phosphate buffer (pH 7.2), and each drug was added to the suspension at zero time. The burst of protoplasts was measured by the decrease of absorbancy at 550 nm.



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