The Effect of Chitinase Inhibitors, Cyclo(Arg-Pro) against Cell Separation of *Saccharomyces cerevisiae* and the Morphological Change of *Candida albicans*

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In a previous paper[1], we reported a new chitinase inhibitor, cyclo(L-Arg-D-Pro) from marine bacteria. At the budding cell stage of *Saccharomyces cerevisiae*, chitin is known to be a main constituent of the primary septum between the mother and daughter cells[2]. Among the chitin metabolite enzymes, chitin synthase is essential for the septum formation[3]. It is known that polyoxin D inhibits chitin synthase of *S. cerevisiae* leading to cell death[4]. A pair of cells were observed with an extrusion of cytoplasmic material at their interface[5]. In the final fission of the septum, chitinase promotes cell separation[6]. Chitinase inhibitors are expected to inhibit the cell separation of yeasts. SAKUDA *et al.* have shown that the chitinase inhibitor demethylallosamidin inhibited cell separation of *S. cerevisiae*[7].

On the other hand, *Candida albicans* is a yeast that exhibits dimorphism, growing as either a yeast form or a filamentous form. In the yeast form, the *C. albicans* cells are not pathogenic, but they do exhibit pathogenic characteristics in the filamentous form[8]. *C. albicans* grown in the presence of polyoxin D formed chains of swollen bulbous cells[9]. Chitinase is also thought to play an important role during this morphological change and chitinase inhibitor is expected to inhibit morphological changes of yeast directly. However, there is no report about the effect of chitinase inhibitor against morphological change of *C. albicans*.

In this paper we describe the effects of the chitinase inhibitors, cyclo(L-Arg-D-Pro), cyclo(L-Arg-L-Pro) and cyclo(D-Arg-L-Pro) against 2 kinds of yeast, *S. cerevisiae* and *C. albicans*.

Materials and Methods

Observation of Cell Separation of *Saccharomyces cerevisiae*

A 0.05 ml sample of pre-incubated cells suspension of *Saccharomyces cerevisiae* JCM1499 was inoculated at concentration of about 1×10^8 cells/ml in 0.9 ml of Difco YM broth containing 0.1 ml of test sample and incubated at 30°C for 8 hours on the reciprocating shaker. After incubation, the cell suspension was observed with an optical microscope. The cells were also observed with a fluorescence microscope after staining the chitin layer in the cell wall with Calcoflour white M2R[10]. For fluorescence microscopic observation, 100 μl of culture broth was suspended in 100 μl of 0.01% Calcoflour white M2R solution in phosphate-buffered saline pH 7.0.

Observation of Morphological Changes of *Candida albicans*

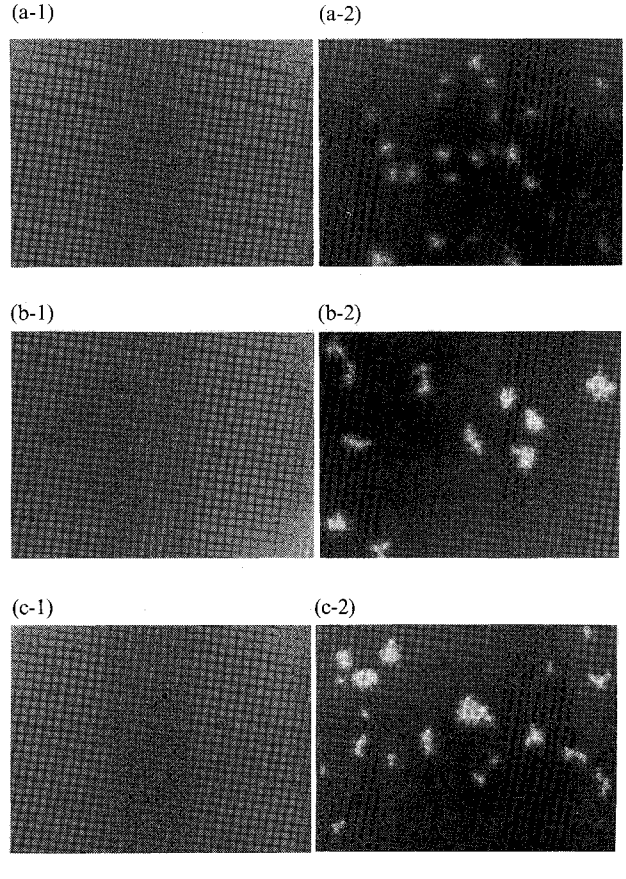
A 1.0 ml of sample pre-incubated cell suspension of *Candida albicans* IFO1060 was mixed in 50 ml of Difco YM agar medium at a concentration of about 1×10^8 cells/ml at 40°C. Paper disks (5 mm diameter) were impregnated with the test sample dissolved in 50 μl of water, put on agar plates and incubated at 30°C for 5 days. An incubated agar (2.0 mm × 2.0 mm) adjacent to a paper disk was cut and rapidly frozen in liquid nitrogen. The morphology of *C. albicans* on the surface of frozen agar was observed with a cryo scanning electron microscope.

Results and Discussion

Fig. 1 shows the state of the cells of *Saccharomyces cerevisiae* after 4 hours of cultivation. As shown in photograph-(a-1), normal cell separation was observed

Fig. 1. Effects of allosamidin and cyclo(L-Arg-D-Pro) against cell separation of *Saccharomyces cerevisiae*.

Microphotograph: (a-1) control, (b-1) allosamidin (250 μg/ml), (c-1) cyclo(L-Arg-D-Pro) (250 μg/ml); Fluorescence microphotograph: (a-2) control, (b-2) allosamidin (250 μg/ml), (c-2) cyclo(L-Arg-D-Pro) (250 μg/ml).



without allosamidin or cyclo(L-Arg-D-Pro). When the *S. cerevisiae* was cultured with 250 μg/ml of allosamidin or cyclo(L-Arg-D-Pro), the yeast cells could not separate, and were observed as grape-like clusters as shown in photograph-(b-1, c-1). Similar morphological features of *S. cerevisiae* were observed at a concentration of more than 100 μg/ml of cyclo(L-Arg-D-Pro). Result of calcofluor staining were shown in Fig. 1 [photograph-(a-2, b-2, c-2)]. The septums of yeast cells were observed more brightly, and as shown in photograph-(b-2, c-2), each cell of grape-like clusters was confirmed to link without separation. Growth curves of *S. cerevisiae* with allosamidin and cyclo(L-Arg-D-Pro) were shown in Fig. 2. Allosamidin and cyclo(L-Arg-D-Pro) did not inhibit growth of *S. cerevisiae* in the 250 μg/ml concentrations. After 4 hours of cultivation, average cell numbers per cell groups were counted to be 2.65 in control culture, 4.92 with allosamidin (250 μg/ml), and 3.90 with cyclo(L-Arg-D-Pro). Both allosamidin and cyclo(L-Arg-D-Pro) inhibited cell separation of *S. cerevisiae*. Cyclo(L-Arg-L-Pro) and cyclo(D-Arg-L-Pro) also showed the clustered in either the 250 μg/ml concentrations, and did not inhibit growth. L-Arg, D-Arg, L-Pro and D-Pro showed no effect at a concentration of 1000 μg/ml. It is presumed that cyclo(L-Arg-D-Pro) inhibited chitinase, which inhibited normal cell separation of *S. cerevisiae* resulting in grape-like clusters.

As shown in Fig. 4-(a), *Candida albicans* changed to the filamentous form under conditions without cyclo(L-Arg-D-Pro) after 1 day. But very little of the filamentous forms were observed at the area adjacent to the paper disk with 6 μg/disk of cyclo(L-Arg-D-Pro) even after 5 days (Fig. 4-(b)). When the amount of cyclo(L-Arg-D-Pro) was increased to 50 μg/disk, almost all cells maintained the yeast form as shown in Fig. 4-(c). Cyclo(L-Arg-L-Pro) and cyclo(D-Arg-L-Pro) also showed a similar phenomenon at the same concentration. To the best of our knowledge this is the first time such observation is reported. Growth inhibition was not observed by these compounds within 50 μg/disk. L-Arg, D-Arg, L-Pro and D-Pro did not show inhibition of morphological change at a concentration of 50 μg/disk.

This observation implies the role of chitinase at the elongating points of filaments of *C. albicans*, and cyclo(L-Arg-D-Pro) seems to inhibit this morphological change through the inhibition of the chitinase.

Fig. 2. Effects of allosamidin and cyclo(L-Arg-D-Pro) on growth of *S. cerevisiae*.

Symbols: ●, control culture; □, culture with allosamidin (250 μg/ml); ○, culture with cyclo(L-Arg-D-Pro) (250 μg/ml).

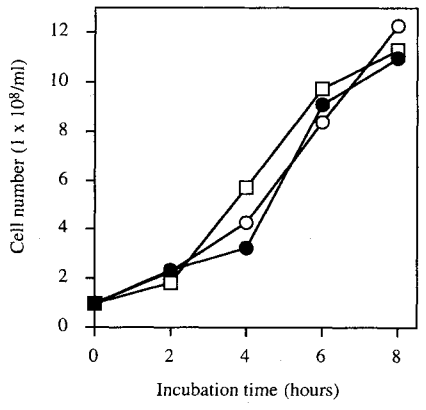


Fig. 3. Effects of allosamidin and cyclo(L-Arg-D-Pro) on the cell ceparation of *S. cerevisiae*.

Symbols: ●, control culture; □, culture with allosamidin (250 μg/ml); ○, culture with cyclo(L-Arg-D-Pro) (250 μg/ml).

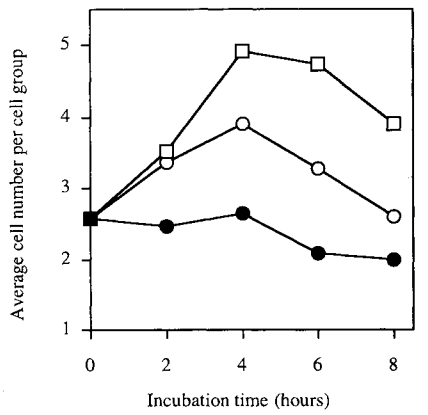
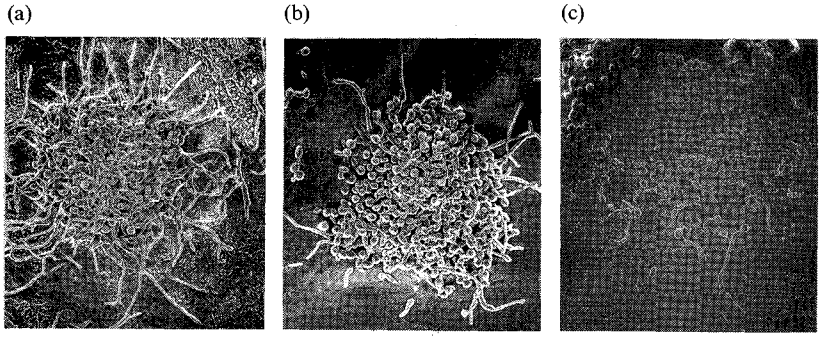


Fig. 4. Morphological change of *Candida albicans*.

(a) Control; (b) 6 μg/disk; (c) 50 μg/disk.



Acknowledgments

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References

1) IZUMIDA, H.; N. IMAMURA & H. SANO: A novel chitinase inhibitor from a marine bacteria *Pseudomonas* sp. J. Antibiotics 49: 76~80, 1996
2) CABIB, E. & R. ROBERTS: Synthesis of the yeast cell wall and its regulation. Ann. Rev. Biochem. 51: 763~793, 1982
3) SILVERMAN, S. J.; A. SBURLATI, M. L. SLATER & E. CABIB: Chitin synthase 2 is essential for formation and cell division in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. 85: 4735~4739, 1988
4) KELLER, F. A. & E. CABIB: Chitin and yeast budding. Properties of chitin synthetase from *Saccharomyces cerevisiae*. J. Biol. Chem. 246: 160~166, 1971
5) BOWERS, B.; G. LEVIN & E. CABIB: J. Bacteriol. 119: 564~575, 1974
6) CORREA, J. U.; N. ELANGO, I. POLACHECK & E. CABIB: Endochitinase, a mannan-associated enzyme from *Saccharomyces cerevisiae*. J. Biol. Chem. 257: 1392~1397, 1982
7) SAKUDA, S.; Y. NISHIMOTO, M. OHI, M. WATANABE, S. TAKAYAMA, A. ISOGAI & Y. TAMADA: Effect of demethylallosamidin, a potent yeast chitinase inhibitor, on the cell division of yeast. Agric. Biol. Chem. 54: 1333~1335, 1990
8) TANAKA, Y: "Antifungal Agents" in The Search for Bioactive Compounds from Microorganisms. *Ed.*, ŌMURA, S. pp. 30~44, Springer-Verlag, New York, 1992
9) HILENSKI, L. L.; F. NAIDER & J. M. BEKER: Polyoxin D inhibits colloidal gold-wheat germ agglutinin labelling of chitin in dimorphic forms of *Candida albicans*. J. Gen. Microbiol. 132, 1441~1451, 1986
10) HAYASIBE, M. & S. KATOHDA: Initiation of budding and chitin-ring. J. Gen. Appl. Microbiol. 19: 23~39, 1973