J. Microbiol. Biotechnol. (1998), 8(4), 422-425

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY
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NOTE

Hyphal Growth Inhibition by Deer Antler Extract Mimics the Effect of Chitin Synthase Deletion in *Candida albicans*

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Received: May 11, 1998

Abstract Chitin synthase null-mutants propagate in yeast form in RPMI medium with suppression of hyphal growth. This hyphal suppression is also observed in the wild type culture grown in RPMI medium supplemented with deer antler extract. To identify the possible target of deer antler extract, the enzymatic activities of chitin synthases were examined. The enzymatic activities of three chitin synthases, CAChs1, CAChs2, and CAChs3, were found to be differentially inhibited by deer antler extract. Of them, CAChs1, was the most sensitive to the extract. These results indicate that deer antler extract causes hyphal suppression, which resembles the effects of chitin synthase deletion, probably through direct inhibition of chitin synthases.

Key words: Chitin synthase, deer antler extract, *Candida albicans*, antifungal activity, hyphal transition

Deer antler is known to be a stimulator for many cellular physiological functions. However, no information had been available on the effects for the microbes until we reported that deer antler extract suppressed hyphal transition of *Candida albicans* [12]. *C. albicans* mostly inhabits the vagina as a normal flora in the yeast form, but when invading the host cell, the morphological change to the hyphal form occurs especially in immunocompromised patients [10]. The hyphal form is a major pathogen and is widely believed to be responsible for tissue invasion [10]. During hyphal transition from the yeast form, the chemical composition of the newly formed cell wall is changed significantly in the dimorphic fungi. In *C. albicans*, the chitin level in the cell wall of hyphae is increased three to five fold [4, 14], and the specific activity of chitin synthase in hyphae is twice of that found in yeast cells [2]. The expression of the *Candida* chitin synthase 3 (*CACHS3*) gene is dramatically increased during the transition to the hyphae form [5]. These observations suggest that chitin is crucial in *Candida* morphogenesis and in the transition to the hyphae form.

C. albicans has three chitin synthases named CAChs1, CAChs2, and CAChs3 [1, 5, 8]. CAChs1 is involved in the septum formation, and CAChs3 is responsible for the chitin in the cell wall [8]. Homozygous null mutants of chitin synthase 2 (*chs2△::HisG/chs2△::HisG*, referred to as *chs2△* mutant hereafter), chitin synthase 3 (*chs3△::HisG/chs3△::HisG*, referred to as *chs3△* mutant hereafter), and double null mutant of chitin synthases 2 and 3 (*chs2△::HisG/chs2△::HisG chs3△::HisG/chs3△::HisG*, referred to as *chs2△chs3△* mutant hereafter) have been isolated [8]. The homozygous deletion of the CAChs1 gene has never been successful, probably due to its lethality ([9], personal communication with Dr. M. Sodoh, Nippon Roche Research Center, Japan). Accordingly, neither *chs1△chs3△* mutant nor *chs1△chs2△* mutant is available. The *chs3△* mutant and *chs2△chs3△* mutant show attenuated virulency [3] and grow somewhat slowly in rich media such as YPD, whereas the *chs2△* mutant exhibits a similar growth pattern to the wild type (Fig. 1, E and F, Fig. 2, E and F) [8].

One of the pathogenic mechanisms in *C. albicans* is the morphology transition from yeast to hyphae. Since chitin synthases seem to play important roles in pathogenicity [2] and morphogenesis, the morphologies of null mutants of chitin synthases in Sabouraud media for the yeast form and RPMI for the hyphal form were compared in this study. The stationary culture of the *chs3△* mutant and *chs2△chs3△* mutant grew as a cluster (Fig. 1, lanes H and J). When clusters were lightly sonicated, most of the cells turned out to be in yeast form (data not shown).

Transfer of stationary cultures of the mutants above from Sabouraud media to RPMI induced the suppression

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of hyphal growth (Fig. 1, H and J). Most of the cells lost the ability to germinate and to form hyphae, even though some cells formed one or two branches. These phenomena were confirmed by calcofluor staining where the bud scar and chitin ring of the yeast form were clearly visible (Fig. 2). These results are contrary to the previously published report, in which normal hyphal formation occurred in mutants [8, 9].

Hyphal suppression was also observed in the wild type of *C. albicans* transferred from Sabouraud to RPMI supplemented with deer antler extract (Fig. 1, C and D, Fig. 2, C and D) [12]. The similarity in growth between chitin synthase-deleted mutants and the wild type treated with deer antler extract suggested a possibility that deer antler extract might affect chitin synthases. To test this possibility, the most inhibitory hydrophobic fraction of deer antler extract was tested on the enzymatic activities of CAChs1, CAChs2, and CAChs3.

The effect of deer antler on the CAChs1 activity can be easily checked using the *chs2△chs3△* mutant containing CAChs1 activity only. The *chs3△* and *chs2△* mutants can be used for the examination of CAChs2 and CAChs3 activities, respectively. The coexisting CAChs1 activity in these mutants is suppressed to a larger extent under the optimal enzymatic conditions for CAChs2 and CAChs3 activities [6]. The CAChs1 activity is optimal at pH 7.5, and is stimulated by Co^{2+}, but is strongly inhibited by Ni^{2+}. 80% of CAChs1 activity is inhibited by 10 mM Ni^{2+}. The CAChs2 activity is optimal at pH 6.5, and is stimulated by Mg^{2+}, but is strongly inhibited by Ni^{2+} and Co^{2+}. 75% and 50% of activities were inhibited by 2 mM Ni^{2+} and 5 mM Co^{2+} respectively. The CAChs3 activity is optimal at pH 8.5, and is synergistically stimulated by Co^{2+} and Mg^{2+} [6]. The CAChs1 activity was measured directly from the *chs2△ chs3△* mutant. For the CAChs2 activity, the enzymatic preparation from the *chs3△* mutant was incubated at pH 6.5 in the presence of Mg^{2+}. For the CAChs3 activity, the enzymatic preparation from the *chs2△* mutant was incubated at pH 8.5 in the presence of Co^{2+} and Mg^{2+} as stimulators and Ni^{+2} as an inhibitor of the CAChs1 activity. Up to 80% of the CAChs1 activity was repressed by the addition of 1 mg of deer antler extract to the reaction mixture. In contrast, 40% of CAChs2 and CAChs3 activities were inhibited by the 1.4 mg of deer antler extract. The inhibition effect was proportional to the concentration of deer antler extract (Fig. 3).

Here, we demonstrated that chitin synthase-deleted mutants suppressed hyphal transition in a similar manner to the effect of deer antler extract. The mechanism of hyphal transition is not well understood. A signal transduction pathway is likely to be involved, even though an alternative pathway may be in effect [7]. It is worth clarifying in the future study whether the final

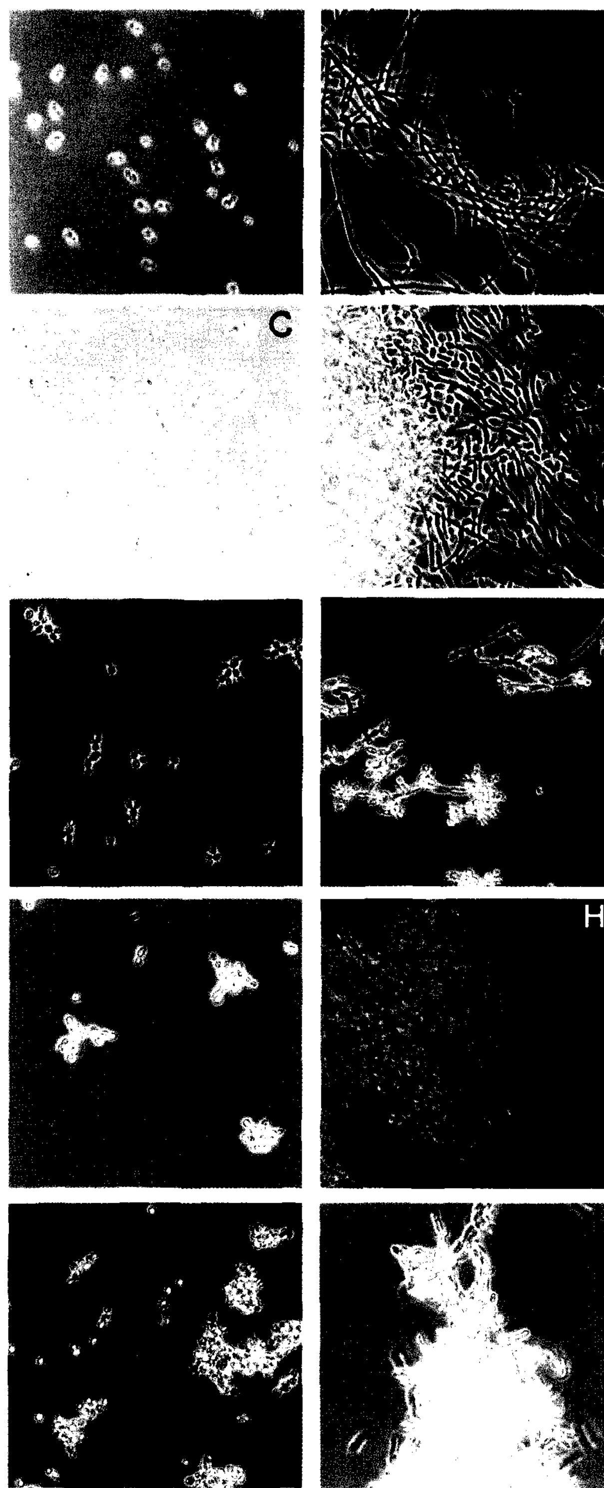

Fig. 1. The morphology of chitin synthase-deleted mutants and wild type.

The cultures were either grown at 30°C in Sabouraud-Dextrose media (2% dextrose, 1% peptone) for the yeast form (A, C, E, G, I) or at 37°C in RPMI 1640 (GIBCO, Gaithersburg, MD, U.S.A.) for the hyphae form (B, D, F, H, J): CAI-4 (A and B), CAI-4 with 250 μg/ml of deer antler extract (C and D), *chs2△* mutant (E and F), *chs3△* mutant (G and H), *chs2△chs3△* mutant (I and J).

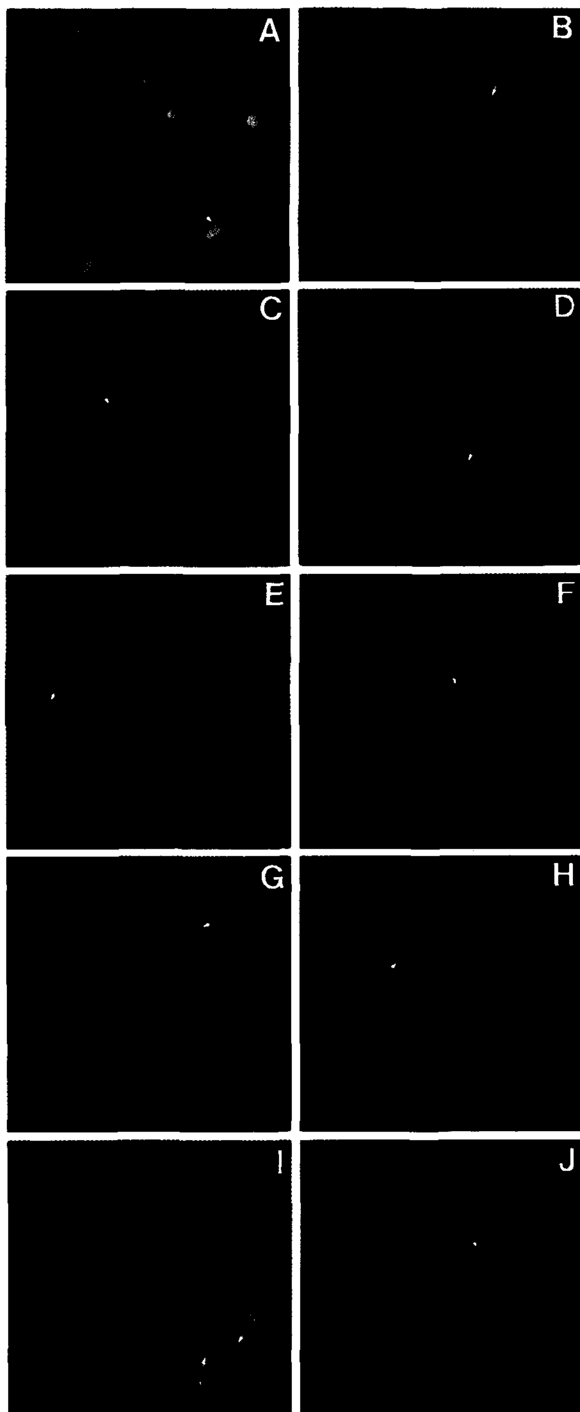

Fig. 2. Calcofluor staining. Washed cells were suspended in 0.01% calcofluor and the fluorescence was observed with an excitation filter at 365 nm.

The cultures were either grown at 30°C in Sabouraud-Dextrose media (2% dextrose, 1% peptone) for the yeast form (A, C, E) or at 37°C in RPMI (GIBCO, Gaithersburg, MD, U.S.A.) for the hyphae form (B, D, F, G, H, I, J): CAI-4 (A and B), CAI-4 with 250 μg/ml of deer antler extract (C and D), *chs2*△ mutant (E and F), *chs3*△ mutant (G and H), *chs2*△*chs3*△ mutant (I and J). Cluster part of culture (G and I). Single cell of mutants (H and J). Arrows indicate chitin ring (A, C, E, G, Ia) and septum (B, D, F, H, Ib, J).

destination of a signal transduction pathway in hyphal transition is the regulation of chitin synthases.

The CAChs1 activity is the most affected by deer antler extract. As mentioned above, the homozygous deletion mutant of *CACHS1* has never been obtained, while the haploid deletion mutant of *S. cerevisiae* chitin synthase 2 gene (*SCCHS2*) is isolated [13]. Possibly, CAChs1 possesses other essential function than chitin synthesis of the septum. The *Candida* wild type culture treated with deer antler extract forms a cluster as does the haploid deletion mutant of *SCCHS2* (Choi, W. and E. Cabib, unpublished result). Thus, inhibition of CAChs1

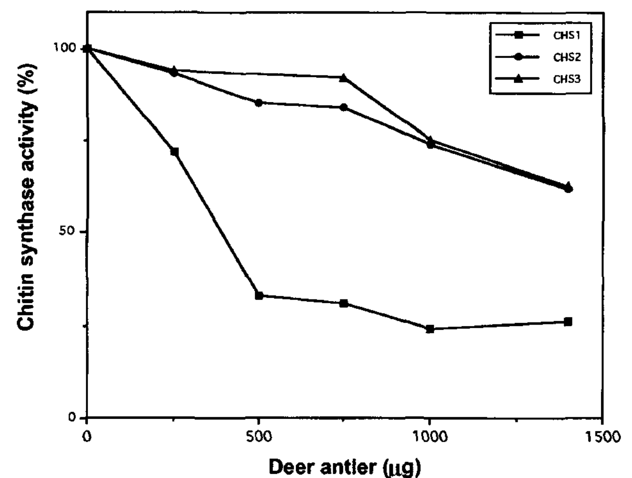

Fig. 3. The inhibitory effects of deer antler extract on three chitin synthase isozymes.

The solid squares, circles, and triangles indicate Chs1, Chs2, and Chs3 activities, respectively, at different concentrations of hydrophobic fraction of deer antler extract prepared as previously described [12]. Chitin synthase activities were measured from membrane preparations elsewhere [11]. For the activity of CAChs1, reaction mixtures contained 32 mM Tris-chloride, pH 7.5, 2 mM cobalt acetate, 1.1 mM UDP-[U-^{14}C] N-Acetylglucoamine (UDP-[U-^{14}C]GlcNAc, 400,000 cpm/μmol, Dupont, Boston, MA, U.S.A.), 2 μl of trypsin at the optimal concentrations for activation (0.25, 0.5, 0.75 mg/ml), and 20 μl of membrane suspension in a total volume of 46 μl. For CAChs2, the reaction mixture contained 32 mM 2-(N-morpholino) ethane sulfonate (MES), 4 mM magnesium acetate, 10 mM nickel acetate, 2 μl of trypsin at the optimal concentration for activation (0.25, 0.5, 0.75 mg/ml), and 10 μl of membrane suspension. For CAChs3, the reaction mixture contained 32 mM Tris-chloride, pH 8.5, 4 mM magnesium acetate, 1.1 mM UDP-[U-^{14}C]GlcNAc (400,000 cpm/μmol), 2 μl of trypsin at the optimal concentration for activation (0.25, 0.5, 0.75 mg/ml), and 20 μl of membrane suspension in a total volume of 46 μl. Mixtures were incubated for 15 min at 30°C. Proteolysis was stopped by adding 2 μl of a soybean trypsin inhibitor solution at a concentration 1.5 times that of the trypsin solution used, and tubes were placed on ice. N-Acetylglucoamine (GlcNAc) was added to a final concentration of 32 mM, followed by incubation at 30°C for 90 min. In the case of CAChs2, 1.1 mM UDP-[U-^{14}C]GlcNAc (400,000 cpm/μmol) was added after proteolytic activation. In all cases, the insoluble chitin formed was assayed by measurement of radioactivity after addition of 10% trichloroacetic acid and filtration through glass fiber filters. The percent activity is expressed as ratio of with to without deer antler extract.

activity, which in turn blocks the septum formation, is likely to be crucial in hyphal transition.

Hyphal formation is also blocked in the *chs3*△ or *chs2*△*chs3*△ mutants (Fig. 1, H and J), whereas the *chs2*△ mutant grows like a wild type (Fig. 1, E and F). This observation implies that CAChs3 is one of the key enzymes in hyphal transition. The effects of deer antler extract on chitin synthases of *C. albicans* is not correlated with the degree of hyphal suppression in mutants. Deer antler extract inhibits the CAChs2 activity to the same degree as the CAChs3 activity, although hyphal transition is not blocked in the *chs2*△ mutant. One possibility is that the effect of deer antler extract *in vivo* may be different from that *in vitro*.

In this study, it is shown that chitin synthases play important roles in the transition to a hyphal form. It is apparent that septum formation mediated by the CAChs1 activity is the crucial step in the transition. However, a proper explanation for that is not feasible until an antifungal agent to inhibit CAChs1 activity in a specific way is available. Identification of a component(s) in deer antler extract which inhibits chitin synthases will lead to the development of a new antifungal agent.

Acknowledgments

We appreciate Dr. Masayuki Sudoh (Department of Mycology, Nippon Roche Research Center, Kanagawa, Japan) for the strains; *Candida albicans*, CAI-4(*ura3*△:: *imm34*/*ura3*△::*imm34*,) and chitin synthase △ null mutants derived from CAI-4. This work was supported by Korea Research Foundation made in the program year 1997.

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