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Effects of chelators (EGTA, EDTA) and calcium ions on nematode capture by the nematode-trapping fungus *Arthrobotrys ellipsospora*

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The nematode-trapping fungus *Arthrobotrys ellipsospora* developed an adhesive knob and trapped nematodes when cultured on a low-nutrient medium. It also trapped polystyrene beads in the same way. The adhesive knob produced mucus that was stained with alcian blue, while mycelium of the fungus was stained with periodic acid/Schiff (PAS). The amount of mucus increased with in days after culturing in the low-nutrient media. The fungus completely lost its ability to trap nematodes when treated with EDTA and EGTA, but it recovered the ability after incubation in the presence of a low concentration of Ca (10^{-6}–10^{-7} M) for 1 h. Calmodulin inhibitor W-7 also inhibited the trapping ability of the fungus, and there was a significant ($p<0.05$) difference between the effects of W-7 and W-5. Ca-binding protein was also detected in the fungus.

Key Words——Ca-binding protein; Ca ions; nematode-trapping fungus.

Nematode-trapping fungi develop trapping organs of various types, including constricting rings, nonconstricting rings, adhesive nets, and adhesive knobs. The factors that influence fungal attachment are important to understand the mechanism of trapping nematodes. However, knowledge is still limited (Kennedy, 1990), and no fungal adhesive compound has been fully characterized (Nicholson and Epstein, 1991). Carbohydrate-binding protein (lectin) has been reported to mediate adhesion in a nematode-trapping fungus, *Arthrobotrys oligospora* Fresenius (Nordbring-Hertz and Mattiasson, 1979; Barron, 1977), and the lectin has been isolated and partially characterized (Borrebaeck et al., 1984). In this fungus, trapping was induced by a low-nutrient medium containing peptides or amino acids (Nordbring-Hertz, 1973; Friman et al., 1985), and phosphate inhibited the trap formation (Nordbring-Hertz, 1973; Friman, 1996). *Arthrobotrys ellipsospora* Tubaki & Yamanaka Y4007 is also a nematode-trapping fungus isolated from healthy pine trees (Yoneda et al., 1980). It traps pine-wilt nematode, *Bursaphelenchus xylophilus* Steiner & Buhrer, within a short period using adhesive trapping knobs (Tubaki and Yamanaka, 1984). This fungus also produced a mucin-specific hemagglutinating substance, which was detected in the mycelia and cultural medium. However, although the proportion of nematodes trapped by the fungus decreased when it was treated with mucin, the purified exocellular hemagglutinin showed no inhibitory effect on the trapping (Yamanaka et al., 1988). The mechanisms behind the regulation of trap formation in this fungus are still unknown, as in other nematophagous fungi. Therefore, we studied the phenomenon of attachment by trapping knobs, and the effects of Ca on nematode trapping, which was found to be inhibited by chelating reagents.

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Materials and Methods

Culture of *Arthrobotrys ellipsospora* Y4007 and nematodes *A. ellipsospora* Y4007 was originally obtained from a pine tree (Tubaki and Yamanaka, 1984). About 1.2×10^6 spores were spread onto 15 ml of Bacto agar medium (1.5% w/v with distilled water, DW) in a culture dish and cultured for 7 d at 25°C. The fungus on the Bacto agar was removed with a cork borer of 18 mm in diam and placed in a petri dish, then immediately soaked with various solutions to examine their effect on its trapping ability. The nematode *B. xylophilus* was isolated from an infected pine tree and maintained in axenic potato-dextrose agar (Yamanaka et al., 1988) cultures of a non-predatory fungus, *Botrytis cinerea* Persoon ex Fries. Nematodes from the *B. cinerea* cultures were obtained using a Baermann funnel (Yamanaka et al., 1988) and washed with DW containing 100 ppm of chloramphenicol before the experiment.

Staining of fungus with alcian blue and periodic acid/Schiff (PAS) The fungus was soaked in 3% (v/v) acetic acid for 3 min and stained with 0.1% (w/v) alcian blue 8GS diluted with 3% acetic acid solution (pH 2.5) for 30 min. After washing with DW, the fungus was incu-

bated with 0.3% (w/v) sodium carbonate solution for 30 min, washed with DW two times, soaked in 0.5% (w/v) HIO_4 solution for 10 min, and then washed with DW. After washing with DW, it was stained with Schiff reagent (Hot-Schiff) for 30 min, washed with solution of 10% $NaHSO_4$: 1 N HCl : H_2O (6 : 5 : 100) three times (each for 3 min), and then washed with tap water. The stained fungus was observed under a microscope.

Trap experiments The time course of the trapping ability of *A. ellipsospora* was examined by incubating the fungus in the presence of the nematode (*B. xylophilus*). To study the effects of nutrients on trapping ability, NH_4Cl (0.2%), $NaNO_3$ (0.2%), urea (0.2%), glucose (3.0%), and mannose (3.0%) were each added to Bacto agar as N or C sources. PDA medium (potato, 200 g; glucose, 20 g; agar, 15 g/L) was also used for experiments. The fungus was cultured on these plates for 7 d, then an excess of nematodes was added, and incubation was continued for 30 min. Trapped nematodes were counted after washing with DW three times. Various microorganisms obtained from soil were added to the fungus cultured on Bacto agar medium to investigate its host specificity. Polystyrene beads (5 μm, Funakoshi) were added to study whether a chemical interaction is needed to trap nematodes. The beads were added, incubated for 10 min and washed with DW three times, and attached beads were observed.

Effects of chelating reagents on nematode trapping The fungus cultured on Bacto agar medium was treated with various concentrations of EDTA for 2 min, then washed with DW. After incubation for 10 min in the presence of *B. xylophilus*, the fungus was again washed with DW, and the effects of EDTA concentration were examined. To examine chelating effects in more detail, the fungus was also treated with 50 mM EDTA (pH 7.5) or EGTA (pH 7.5) for 3 min, washed with DW three times, and incubated for 1 h with various concentrations of Ca buffer (Ogawa, 1968). It was then washed with DW three times, and incubated with nematodes for 10 min. After removal of untrapped nematodes by washing with DW three times, the trapped nematodes were counted under a microscope. As controls, the fungus was treated with DW in place of the chelating agent in order to determine the trapping ability before chelating treatment, and with only EDTA or EGTA in order to determine the effects of these reagents on trapping.

Effects of various concentrations of calmodulin inhibitor W-7 The fungus was incubated in the presence of various concentrations (10^{-5}–10^{-3} M) of calmodulin inhibitor W-7 for 3 and 10 h. W-5 was used as a control to examine the structure-activity relationship of W-7 (Hidaka et al., 1981). After removal of the inhibitors, the cultures were washed with DW three times, then incubated with nematodes for 10 min. Trapped nematodes were counted after washing with DW three times. The fungus treated with these inhibitors was also incubated with DW for 10 h to check if these concentrations were adequate to inhibit calmodulin activity.

Extraction of Ca-binding protein and electrophoresis The fungus (30 g wet) was disrupted with 90 ml of a solution (pH 8.0) containing 9 M urea, 75 mM tris-HCl, 1 mM $CaCl_2$, and 15 mM β-mercaptoethanol, then centrifuged at 15,000 × g for 30 min. The supernatant was transferred to a new tube, and ethanol was added to a concentration of 55% (v/v) (Roger et al., 1979). The supernatant was centrifuged at 15,000 × g for 30 min, then transferred to a new tube. Ethanol was added to a concentration of 80% and the solution was centrifuged at 15,000 × g for 30 min. The supernatant was discarded, the pellet was dissolved in a small amount of DW, and the resulting solution was dialyzed against DW for 12 h at 4°C, then freeze-dried. For polyacrylamide gel electrophoresis on 10% Tris-glycine alkaline glycerol gel (Perrie and Perry, 1970), the freeze-dried sample was dissolved in 30 μl of sample buffer (2.5 mM Tris-HCl, 8 mM glycine) containing 100 μM $CaCl_2$ or 10 or 50 mM EGTA and applied to the gel. Proteins were stained with CBB (Wako Chemicals Tokyo).

Statistical analysis Percentages of trapped nematodes were expressed as means +/− SE. Statistical significance was determined by one-way analysis of variance followed by a posteriori comparisons on the significant ANOVA results using Fisher PLSD. Statistical significance was set at $p < 0.05$.

Results and Discussion

Nematode trapping ability of *A. ellipsospora* Although nematophagous fungi have been reported to release adhesive materials upon stimulation by the presence of the nematode host (Nordbring-Hertz, 1988), *A. ellipsospora* trapped nematodes by the adhesive knob within a few minutes. The number of trapped nematodes after 10 min of incubation reached 92% of that after 1 h (Fig. 1). These results indicate that the adhesive materials had already been released before nematode addition, and the capture occurred by chance in this fungus.

The trapping ability was decreased by adding sup-

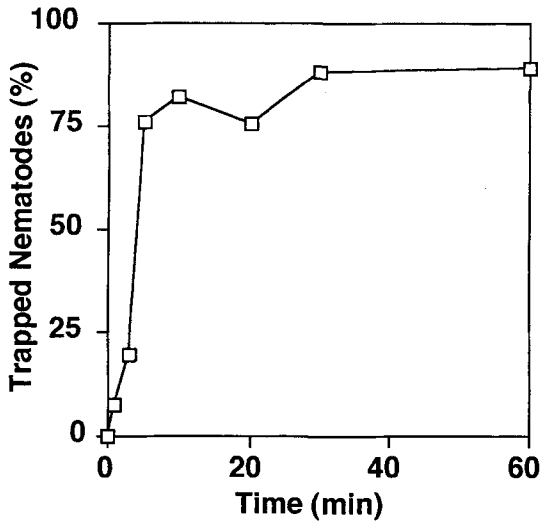
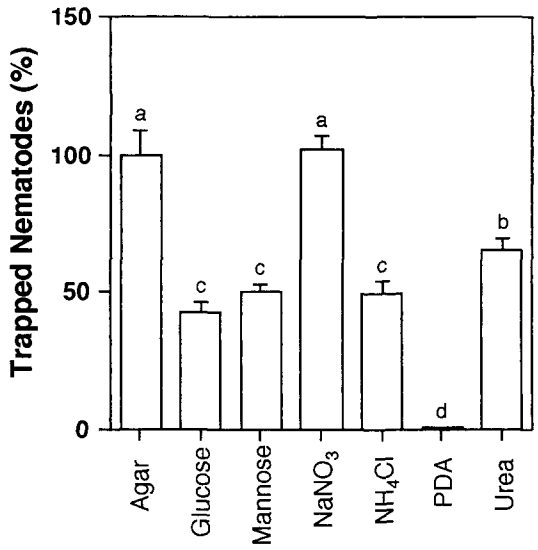


Fig. 1. Time course of trapping of the nematode (*Bursaphelenchus xylophilus*) by *A. ellipsospora*.



plementary nutrients to the culture medium, and it almost disappeared when the fungus was cultured on PDA culture medium (Fig. 2). *Arthrobotrys ellipsospora* formed trapping knobs only when cultured in media low in nutrients, suggesting that these organs were an adaptation to starvation. In addition to nematodes, it also trapped such microorganisms as *Helicotylenchus*, Rabditida, Tardigrada, and Acarina within a few minutes (Figs. 3a, b). To test the mechanism of interaction between adhesive knobs and nematode surfaces, polystyrene beads were added to the fungus. The beads attached to the surface of the adhesive knobs (Figs. 3c, d), and a

Fig. 2. Effects of supplementary nutrients in culture medium on nematode trapping. Various nutrients were added to agar medium, and the agar medium without supplement was used as a control. Vertical bars represent the standard error of five replicates. a–d, columns marked with the same letter do not show significant difference at $P<0.05$.

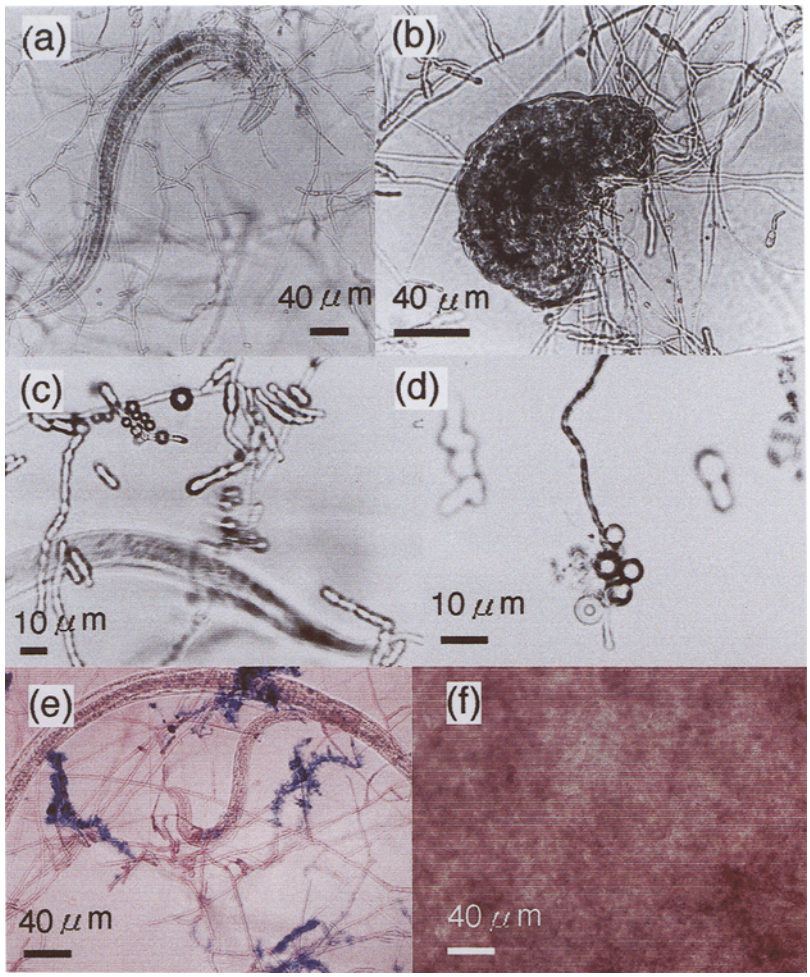


Fig. 3. Trapping of various microorganisms and polystyrene beads by *A. ellipsospora*. (a): Dorylaimida sp. (b): Tardigrada sp. (c, d): polystyrene beads and *Bursaphelenchus xylophilus* (e). Double stain of the fungus by alcian blue and PAS under nematode-trapping conditions on water agar medium and (f) non-trapping conditions on PDA.

mucus-like substance was also observed on the surface of the knobs (Fig. 3e). The beads were trapped at almost the same positions as the nematodes (Fig. 3c), and they remained attached to the trapping knob after washing with DW many times. Although it was difficult to compare the binding strengths of the beads and nematodes, there was no distinct difference in the number of trapped beads between polystyrene beads and mucin-coated beads using carbodiimide binding on the knobs (data not shown). The mucus on the knobs was stained by alcian blue (Fig. 3e), while hyphae were stained by PAS, suggesting that the mucus is acid mucicpolysaccharide. Acid mucicpolysaccharide was observed only when the fungus was cultured on low-nutrient media, and the stained mucus disappeared when the fungus was cultured on PDA medium (Fig. 3f). The mucus was produced parallel to the time when fungus started to trap nematodes. Although it is well known that the development of trapping structures can be affected by the nutrient composition, the trapping knob formation and mucus production only in low nutrient culture suggest that these two systems might be interconnected in *A. ellipsospora* Y4007.

Although adhesive compounds are generally classified into two types by composition, namely, polysaccharides and proteins or glycoproteins (Bonfante-Fasolo and Perotto, 1986; Onyile et al., 1982), most nematophagous fungi are known to trap nematodes through the binding of lectin to specific haptens. *Arthrobotrys ellipsospora* also produces mucin-specific hemagglutinin (Yamanaka et al., 1988), but nematode trapping through lectin alone in a mucin-lectin chemical interaction seems to be difficult. The mucus might also be involved in nematode trapping, and if so would account for the fact that various microorganisms could be captured regardless of various types of host skin (Fig. 3).

Effects of chelating reagents and Ca ion on nematode trapping Ca ions play important roles in the phenomena of life of organisms from prokaryotes to mammals. However, the direct involvement of Ca ions in the nematode trapping of nematophagous fungi has not been reported. Interestingly, *A. ellipsospora* lost the ability to trap nematodes when treated with EDTA for 2 min, and treatment with 0.1 M EDTA completely inhibited the trapping (Fig. 4). Of the chelating agents tested, EDTA had a significantly ($p<0.05$) greater inhibitory effect on the trapping than EGTA (Fig. 5a). After treatment with these reagents (50 mM) for 3 min, the fungus recovered

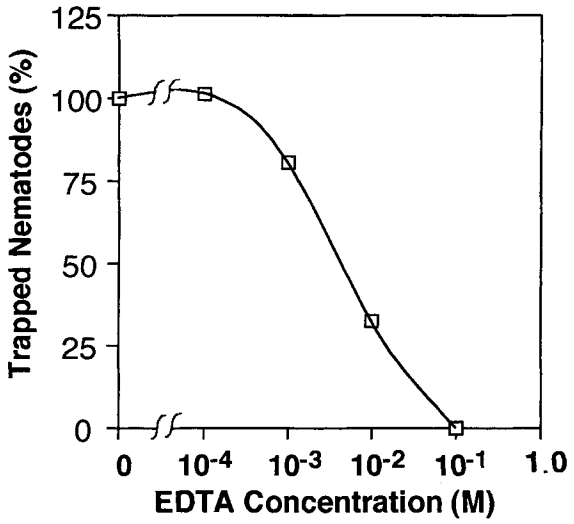


Fig. 4. Effects of various concentrations of EDTA on nematode trapping.

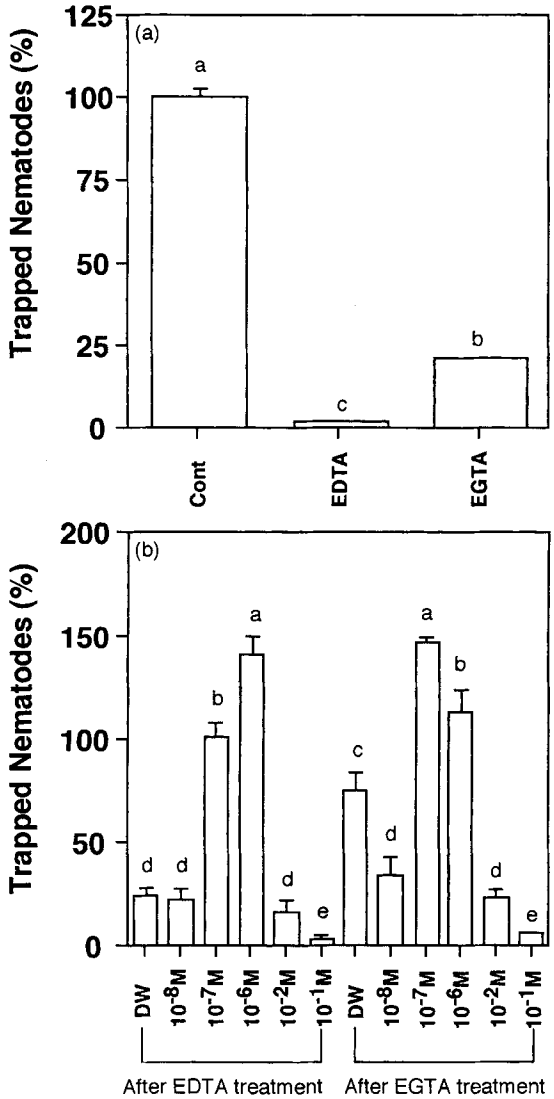


Fig. 5. Effects of EDTA and EGTA on nematode trapping. (a) The fungus was treated with a chelating agents (50 mM) for 3 min, then nematodes were added. DW treatment was used as a control. (b) After treatment with a chelating agent, the fungus was incubated with the indicated various concentrations of Ca-buffer for 1 h, then nematodes were added. Trapped nematodes are shown by percentages, and the control was taken as 100%. Vertical bars represent the standard error of five to six replicates. a–e, columns marked with the same letter do not show significant difference at $P<0.05$.

its trapping ability upon incubation with a low concentration of Ca (Fig. 5b). No such recovery was seen with other metal ion solutions, namely, Mn, Mg, Al, Zn, Cu, Fe, Cr, Cd, and Ni, at various concentrations (data not shown). This result implied that EDTA and EGTA worked as chelating agents for Ca ions. The greatest number of nematodes was trapped following incubation with a Ca ion concentration of 10^{-6} M after EDTA treatment (141.3%) and of 10^{-7} M after EGTA (146.6%). However, the numbers of trapped nematodes were significantly ($p<0.05$) different at same concentration of EDTA and EGTA. This might suggest that not only Ca but some cations are required for nematode trapping, and that lectins are usually metalloproteins containing cations such as Ca^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} (Borrebaeck et al., 1981). Although the percentage of trapped nematodes was increased to almost 150% of control by incubating the fungus with Ca buffer after EDTA or EGTA, the reason for this is not known.

Since calmodulin purified from barley and Basidiomycete fungi (Grand et al., 1980) is a Ca binding protein involved in various physiological functions, the effect of calmodulin inhibitor W-7 was examined on trapping

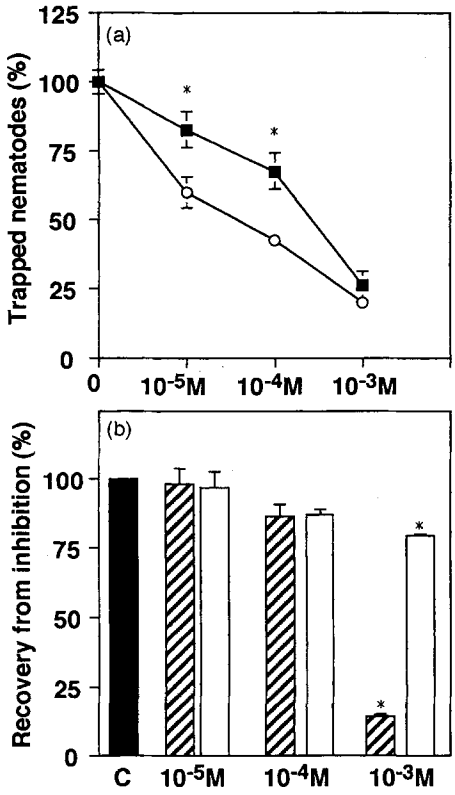


Fig. 6. Effects of calmodulin inhibitors W-7 and W-5 on nematode trapping.
(a) Trapped nematodes were counted after incubation with the inhibitors for 10 h. ■, W-5; ○, W-7. (b) Recovery of nematode trapping upon removal of the inhibitors. ▨, W-7; □, W-5; C, control without calmodulin inhibitor. Vertical bars represent the standard error of five replicates. *, differences significant at $P<0.05$ between W-7 and W-5 (a), and against control (b).

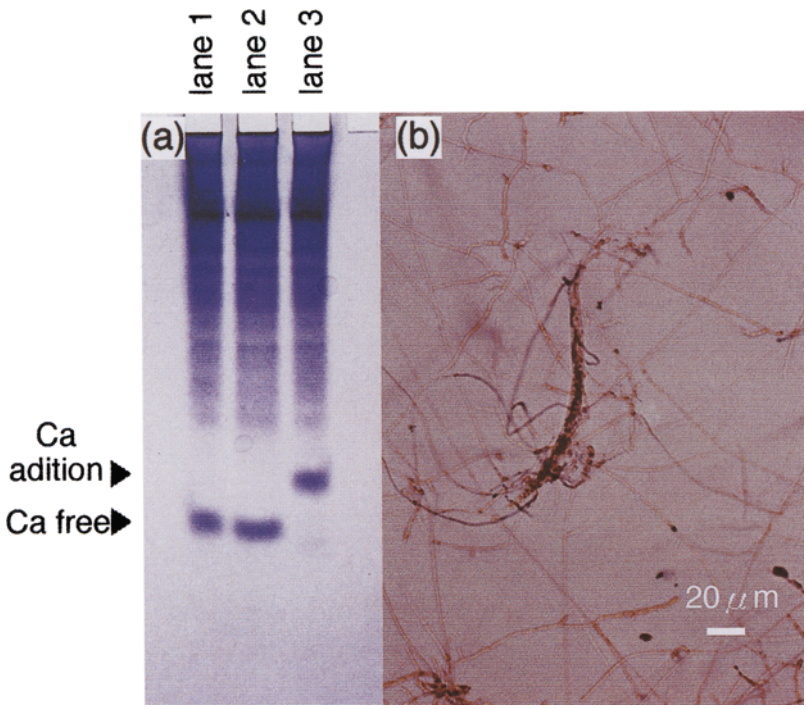


Fig. 7. Electrophoresis of Ca-binding protein extracted from *A. ellipsospora*, and PAS and Alcian blue staining of W-7 treated fungus. (a) Ca binding protein in 10% alkali-glycerol polyacrylamide gel. Sample was applied on the gel with 10 mM EGTA. (lane 1), 50 mM EGTA. (lane 2), or 100 μM $CaCl_2$. (lane 3). (b) PAS and Alcian blue stain after treatment with W-7 (10^{-5} M) for 10 h.

nematodes, and on the mucus production on the surface of adhesive knobs. W-7 inhibited trapping, and inhibition increased with the concentration of W-7 for 3 h after initiation of incubation. Further, 10 h of incubation with W-7 and W-5 also decreased the number of trapped nematodes (Fig. 6a). The trapping was significantly ($p<0.05$) decreased at 10^{-5} M and 10^{-4} M concentrations of W-7 as compared to that of W-5. This indicates the possible involvement of calmodulin in this function of nematode-trapping. However, 10^{-3} M W-7 caused cell-damage, because the treated fungus did not recover its trapping ability after incubation with DW for 5 h (Fig. 6b). Further, a Ca-binding protein was obtained from the *A. ellipsospora* (Fig. 7a). Therefore, calmodulin probably exists in this fungus. Although acid mucicpolysaccharide was still observed on the surface of trapping knob, its production was drastically decreased in the fungus treated with W-7, and the number of trapped nematodes was reduced (Fig. 7b). These results indicate that Ca is involved in calmodulin for producing mucus. However, this is not sufficient to explain why this fungus lost its ability to trap nematodes after washing with chelating agents for 2 min. Therefore, the Ca ion must have a more direct role of nematode trapping, and the non-specific sticky mucus might be supported or connected by Ca-binding substances like lectin.

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