# AUTOLYSIS IN THE ANAEROBIC FUNGUS *Piromyces communis* OTS1 : PRESENCE OF CHITINASE AND β-1, 3-GLUCANASE ACTIVITIES

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## Summary

The degree of autolysis and presence of cell-wall degrading enzymes in an anaerobic ruminal fungus, *Piromyces communis* OTS1, grown in liquid medium, was monitored to evaluate the effect of self-digestion on fungal biomass. After a 30 days incubation period fungal dry weight decreased by 45% and the cell wall component chitin decreased by 22%. Chitinase activity detected in the supernatant was mainly of the endotype and peaked at day 6 of the incubation. β-1, 3-glucanase was detected from day 4 and increased throughout the incubation period. Autolysis was a slow process, and under natural conditions it is unlikely that it plays a significant role in the degradation of the spent fungal vegetative stage in the rumen.

(**Key Words** : Anaerobic Fungus, Autolysis, Chitinase Activity, β-1, 3-Glucanase Activity)

## Introduction

Cell walls protect against osmotic and mechanical stresses coming from the environment, and maintain the morphology and cytokinesis of the fungal cells. Polysaccharides and protein-polysaccharide complexes are the main components of fungal cell walls (Farkas, 1990). The anaerobic fungi that inhabit the gastrointestinal tract of ruminants and other herbivores possess the polysaccharide chitin as a common component in their cell walls (Orpin, 1977). Chitin is a (1-4)-β-linked homopolymer of *N*-acetyl-D-glucosamine resistant to degradation by microorganisms. It has been postulated that chitin may protect ruminal fungi from bacterial and protozoal attack in the rumen and thus fungal protein, which has a high digestibility and biological value, will be available to the host animal in the duodenum (Gulati et al., 1989, Kemp et al., 1985). However, due to the particular biology of their life-cycle, fungi are selectively retained in the rumen, where protozoa have the ability to degrade the fungal cell walls and expose intracellular contents, like proteins, to enzymatic digestion (Morgavi et al., 1993, 1994).

Chitin-containing organisms, on the other hand, produce chitinolytic enzymes which play a morphogenetic and autolytic role (Gooday, 1990). The anaerobic fungi are not an exception and chitinase activity from *Piromyces communis* OTS1 has been reported (Sakurada et al., 1995). However, the extent to which these enzymes affect chitin degradation during autolysis is not yet known.

This work was undertaken to investigate how the spent vegetative growth (with special emphasis on chitin) in the anaerobic ruminal fungus *Piromyces communis* OTS1 is affected by autodegradative enzymes.

## Materials and Methods

### Microorganism and culture conditions

The fungus was isolated by the roll-tube method (Joblin, 1981) from the rumen of a ruminally fistulated goat (Japanese native breed, 35kg) fed twice a day, at 09:00 and 17:00 h, a ration consisting of 300 g alfalfa (*Medicago sativa*) hay cubes and 100 g concentrated feed (17% protein, 72% total digestible nutrients, α-Dairy Mix, Chubu-Shiryo, Chita, Japan) and was maintained in a modified medium 10 of Caldwell and Bryant (1966, Morgavi et al., 1993). Experimental cultures (9.5 ml) were started by transferring 0.5 ml of a 2-day-old fungal culture. Incubations were carried out under anaerobic conditions at 39℃ for 30 days.

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Received May 30, 1995
Accepted January 5, 1996

### Sampling

Samples were taken periodically in triplicate. The pH of the medium was measured immediately after the tubes were opened. The fungus was collected by centrifugation (1,500 × g, 20 min, room temperature), washed with distilled water and dried as described (Morgavi et al., 1994) for determination of dry weight. The culture supernatant was kept at −80℃ until analysis. Chitin was determined in triplicate in samples taken at 2, 6, 15 and 30 days of incubation.

### Analysis

Chitinolytic activities were assayed as before by estimating the liberation of a fluorogenic substrate (Morgavi et al., 1994). 4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide (4-MU-GlcNAc) for the determination of *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.52) activity and 4-methylumbelliferyl *N*, *N'*-diacetylchitobiose (4-MU-$(GlcNAc)_2$) and 4-methylumbelliferyl *N*, *N'*, *N''*-triacetylchitotriose (4-MU-$(GlcNAc)_3$) for chitinase (EC 3.2.1.14) activity were used as substrates. The reaction mixture contained 0.05 M citrate phosphate buffer (pH 5.5) and was incubated at 39℃ for 20 min. The amount of 4-methylumbelliferyl was measured with a Shimadzu RF-5000 spectrofluorometer (360 excitation and 450 nm emission).

β-1, 3-Glucanase (EC 3.2.1.39) was assayed using laminarin as substrate. Reducing substances liberated by the hydrolysis of 0.4% laminarin in 0.05 M citrate phosphate buffer (pH 5.5) were measured by the neocuproine method (Dygert et al., 1965). Incubations were carried out at 39℃ for 5 h. Reducing sugars present in the incubation medium were measured at the same time and subtracted. One microunit (μU) was defined as the amount of enzyme which liberates 1 nmol glucose equivalent $min^{-1}$.

Total chitin was determined by a chemical quantitative colorimetric method (Chen and Johnson, 1983). Staining with a chitin-binding dye was carried out to examine the distribution of chitin in the cell. Fungal cells taken at different incubation periods were stained with 0.01% calcofluor white M2R in 0.5 M Tris-HCl (pH 8.8) for 5 min, washed with water, and examined by fluorescence microscopy using a Olympus BH microscope (Olympus Optic1 Co., Ltd., Japan).

Protein was determined according to Bradford (Bradford, 1976), using bovine serum albumin as standard.

## Results

*P. communis* OTS1 showed a normal growth in the medium used with an exponential growth stage until day 4 of the incubation (figure 1). This was accompanied by a concomitant decrease in the culture pH from an initial 6.5 at day 0 to around 6.0 at day 6 of the incubation and the depletion of the carbon source (not shown). Fungal dry weight was also maximum at day 4 of the incubation. The fungal dry weight after reaching its peak decreased steadily until stabilizing about two weeks from the onset of the incubation. *P. communis* OTS1 under the conditions studied had a 45% loss in dry weight during autolysis after a 30 days incubation period. Total fungal chitin also peaked during the first week of the incubation (229 μg/tube at day 6 of the incubation) and decreased with longer incubation times, but this decrement was not so accentuated with a 22% loss detected at the end of the incubation (179 μg/tube at day 30 of the incubation).

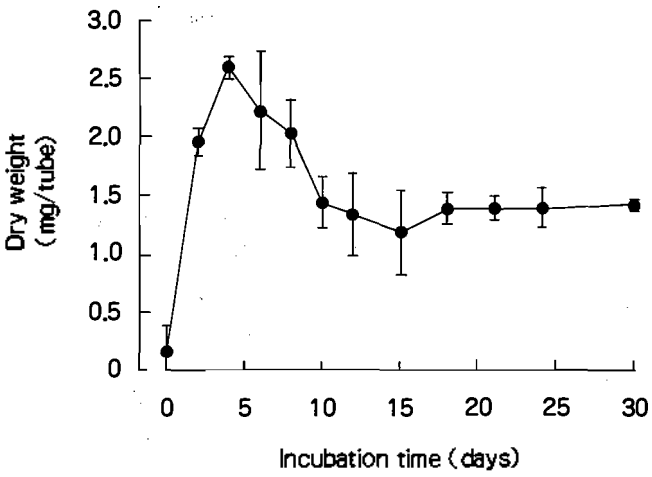


Figure 1. Changes in *Piromyces communis* OTS1 dry weight during a 30 days incubation period as described in Materials and Methods. Values are the mean ± S.D. of three tubes.

Chitin detected by fluorescence microscopy was distributed all over the fungal cell walls, but some cells showed more intensely stained regions in the rhizoid and/or the sporangium (figure 2). As the culture aged, the intensity of the fluorescence decreased with time; zoosporangia, particularly, were hardly stained after 15 days of incubation, indicating chitin depletion. No differences were observed in the intensity of the staining in cells from cultures 15 or 30 days old.

Chitinolytic activity detected in the culture medium was highest at day 6 of the incubation when the fungal dry weight already began to decrease (figure 3). The chitinase activity was mainly 'endo'-type, and fluorescence

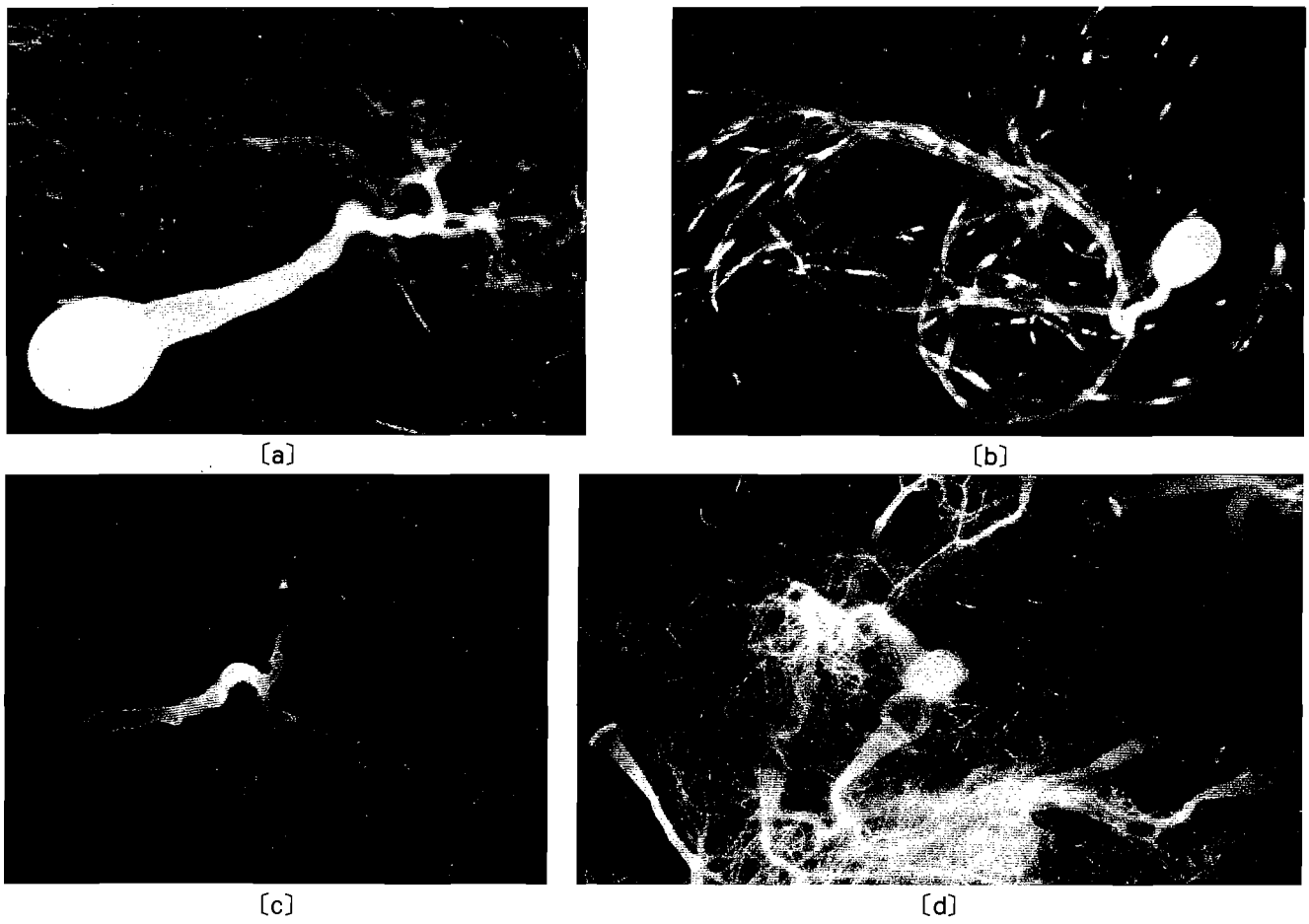


Figure 2. Staining of *Piromyces communis* OTS1 with calcofluor white. The fungus was grown *in vitro* for 4 days (a, b) and 30 days (c, d). Note that some regions of the rhizoid are more intensely stained (b) and light staining in old cultures (c, d).

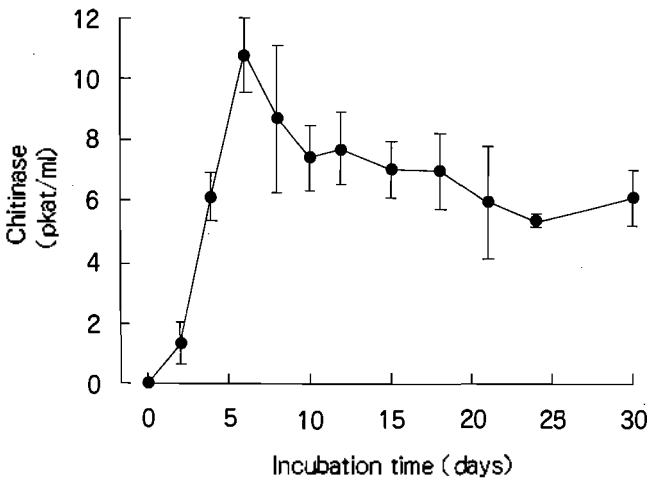


Figure 3. Chitinase activity released into the incubation medium by *Piromyces communis* OTS1. Activity was assayed with 4-MU-$(GlcNAc)_3$ as substrate. Values are the mean ± S.D. of three tubes.

liberated from 4-MU-$(GlcNAc)_3$ was 5 to 6 times higher than the fluorescence liberated from 4-MU-$(GlcNAc)_2$ throughout the incubation period, except at day 2 where the ratio was smaller (1.7 times). On the other hand, activity of the closely related enzyme *N*-Acetyl-$\beta$-glucosaminidase was not detected.

$\beta$-1, 3-Glucanase activity was also detected in the culture medium. The activity appeared at day 4 and incresed progressively with the incubation time (figure 4). This pattern is different from that of chitinase where the activity peaked at the beginning of the autolysis and decreased thereafter. Protein excretion into the medium was evident from days 10 to 15 of the incubation, part of these proteins were degraded with time (data not shown).

## Discussion

The fungus stopped its growth when the available carbon source was exhausted and fermentation products probably accumulated. Under these conditions no living

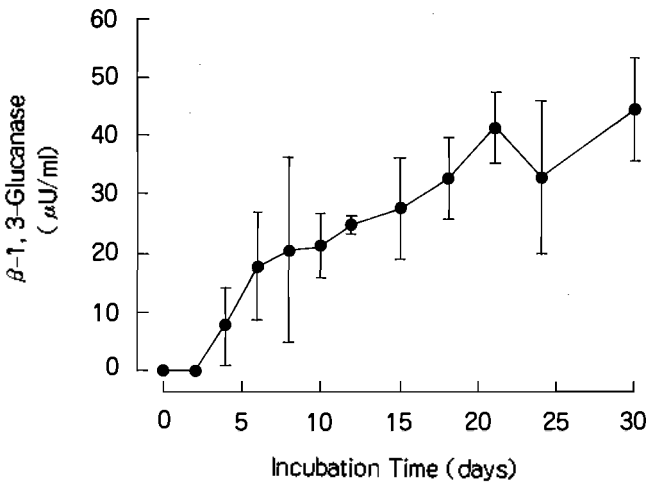


Figure 4. β-1,3-Glucanase activity released into the incubation medium by *Piromyces communis* OTS1 using laminarin as substrate. Values are the mean ± S.D. of three tubes.

living cells are found in 8 to 9-day-old cultures.

Calcofluor white used to localize chitin in fungal cells have the disadvantage that is also absorbed onto a variety of polysaccharides containing contiguous β-1, 4-linked D-glucopyranosyl units (Moore, 1990). Combination of calcofluor white and fluorescein isothiocyanate-conjugated wheat germ agglutinin is considered more accurate (Lukěs et al., 1993), but the presence of chitin in the cell wall is not a matter of discussion in the present study and the objective was to check for any localized changes in chitin during autolysis.

*P. communis* OTS1 has a predominantly endo-type chitinase activity as many other fungal species (Sahai and Manocha, 1993, Sakurada et al., 1995). The presence of *N*-acetyl-β-glucosaminidase activity was not detected, although it is thought that the coexistence of chitinases and *N*-acetyl-β-glucosaminidases is essential in any fungal chitinolytic system (Rast et al., 1991). This enzyme was also absent in different cell fractions of *P. communis* OTS1 (Sakurada et al., 1995). The apparent lack of this enzymatic activity may be the reason of the relatively low degree of chitin degraded, less than 25% after 30 days of incubation. The comparative resistance of chitin to degradation is indicated by the fact that this compound accounted for 10.3% of the fungal dry weight at day 6 of the incubation and its percentage increased to 12.5 at the end of the incubation.

1,3-β-glucanase released into the culture medium has been associated with the autolytic process in other fungi (Nuero et al., 1993. Perez-Leblic et al., 1985). Although the exact chemical composition of the anaerobic fungi cell wall have not been reported, the finding of 1,3-β-glucanase activity in the culture medium suggest that 1,3-β-linked glucans are normal constituents of *P. communis* OTS1 cell wall.

Cell wall degrading enzymes appeared in the cultures before the maximum dry weight was achieved. Due to the biology of the anaerobic fungi life cycle, dying cells can be found in the culture medium at ealry stages of the incubation, coexisting with actively growing cells which makes difficult to define clearly the onset of the autolysis as occurred in other fungal species (Nuero et al., 1993, Perez-Leblic et al., 1985, Reyes et al., 1988). In aging cultures cell wall polysaccharides and also cytoplasmic components are degraded, like reserve carbohydrates, which contributes to the fungal dry weight loss (Phillips and Gordon, 1989). Fungal proteases (Wallace and Joblin, 1985) may also have a role during autolysis.

The degradation of chitin during the autolysis of the ruminal anaerobic fungus *P. communis* OTS1 began in the zoosporangium; the main rhizoid was the most resistant part and remained intact even after long incubation periods. Chitin seems to be one of the compounds more resistant to the action of autodegradative enzymes. Autolysis of *P. communis* OTS1 was a slow process under the conditions studied. Although the ruminal fungi attach to plant fibres which have a long retention time in the rumen (2 to 3 days) (Lechner-Doll et al., 1991), it is unlikely that the autolytic process plays a significant role in the degradation of the spent fungal vegetative stage in the rumen.

## Acknowledgments

We thank Drs. K. Ashizawa and H. Numaguchi, Miyazaki University, for allowing the use of some of the equipment utilized in this work. DPM is the recipient of a Japan Society for the Promotion of Science (JSPS) fellowship in 1995.

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