Secretion of chitinase by *Aphanocladium album*, a hyperparasite of wheat rust[1]

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Summary. Aphanocladium album, a hyperparasite of wheat rust, *Puccinia graminis* var. *tritici*, secretes chitinase when grown on a chitin-containing medium. This enzyme was purified and shown to attack the mycelium of the parasitized rust fungus.
Key words. Biocontrol; chitinase; fungal cell walls; hyperparasite; rust fungi.

The fungal hyperparasites of plant pathogenic fungi have received considerable attention as potential biocontrol agents. A prominent feature of many of these interactions is the lysis of the cell walls of the parasitized fungus upon attack of the hyperparasites[4-8]. While this lysis has been well characterized on a morphological level[4-8], little is known about the biochemical processes responsible for it.

We have chosen the interaction of the hyperparasite, *Aphanocladium album*, with its host, *Puccinia graminis* var. *tritici*, to investigate biochemical aspects of host lysis. In this system, it had previously been shown by an ultrastructural study that the hyperparasite penetrates the uredospores through the germ pores after lysis of the germ tubes[8]. Here, we report our studies on the involvement of chitinase, secreted by the hyperparasite, in the lytic process.

Materials and methods. The hyperparasite, *Aphanocladium album* (ETH No. M 483) was maintained on malt agar medium[9]. For our experiments, six pieces (8 mm diameter) from the agar culture were inoculated into 200 ml of a synthetic liquid medium in 500-ml flasks and shaken at 140 strokes per min. The medium contained the following per l of distilled water: Chitin powder from crab shells (Fluka, Buchs, Switzerland), 10 g; glucose, 1 g; NH_4NO_3, 5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 2.5 g; $FeCl_3 \cdot 6H_2O$, 0.06 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot 4H_2O$, 2.1 g. After an appropriate period of growth, the medium was filtered, centrifuged at 3000 × g for 20 min, decanted and lyophilized. The lyophilized medium was taken up in 10 mM sodium acetate buffer, pH 5 ($1/25$ of the original volume), centrifuged at 20,000 × g for 10 min, adjusted to pH 8.4 with 1 M NaOH, and centrifuged again at 20,000 × g for 10 min to remove precipitated salts.

Chitinase was measured according to published procedures[10,11] using insoluble regenerated [^{3}H]chitin as a substrate. The amount of soluble radioactivity liberated within 30 min at 37 °C in 20 mM sodium acetate buffer, pH 5, was determined for at least three different dilutions of each enzyme preparation, using enzyme concentrations within the linear range of the standard curve (see fig. 2). 1 nkat chitinase was defined as that amount which liberated 1 nmole of GlcNAc (N-acetylglucosamine) equivalents per s.

Chitinase was purified by a one-step purification, using affinity chromatography on a column of regenerated chitin (8.3 × 3.0 cm) as described[10,11]. Protein was determined according to Bradford[12].

The host of *Aphanocladium album*, *Puccinia graminis* var. *tritici*, was grown axenically on a semi-defined agar medium[13], containing terramycin (50 μg ml^{-1}) to prevent bacterial growth, in a controlled chamber having 25 °C day temperature, 18 °C night temperature, and a relative humidity of 70%. After an appropriate growth period, the mycelium was scraped from the agar medium, boiled 30 min in distilled water, and collected by centrifugation (5 min, 2000 × g). The process of boiling and centrifugation was repeated three times with fresh water. The residue was lyophilized. Portions (1 mg) of the lyophilized mycelium were suspended in 0.2 ml 100 mM sodium acetate buffer (pH 5) and incubated with dialyzed crude or purified enzyme preparations in a final volume of 0.6 ml for 15 to 120 min. Controls received boiled enzyme preparations. At the end of the incubation period, the reaction was stopped by centrifugation (5 min at 1000 × g). The supernatant was further digested with snail gut enzyme to hydrolyze chitin oligomers[11], and the resulting GlcNAc was determined as described[11].

Results. Aphanocladium album grew well on a medium containing 0.1% glucose as an initial carbon source and 1% chitin powder. A strong activity of chitinase (ca. 4 nkat ml^{-1}) was present in the culture filtrate after 14–21 d of growth. Chitinase was purified with 69% yield by a one-step affinity technique from the culture filtrate (fig. 1). The culture filtrate was adjusted to pH 8.4 and put on a column of regenerated chitin. Most proteins eluted from the column, whereas chitinase was adsorbed. The column was washed with buffers at pH 8.4 and pH

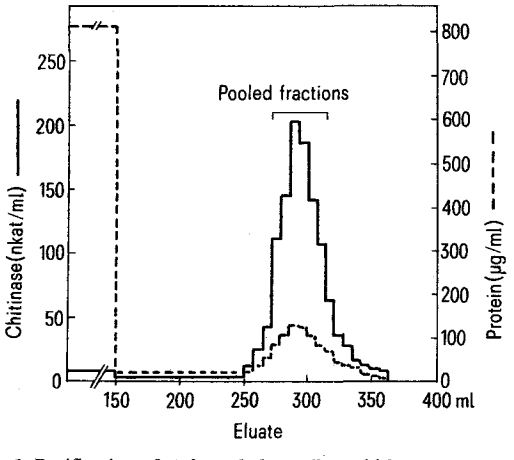

Figure 1. Purification of *Aphanocladium album* chitinase on a column of regenerated chitin. Concentrated culture medium (76 ml, containing, in total, 131 mg protein and 9150 nkat chitinase) was loaded on a column (8.3 × 3.0 cm) of regenerated chitin equilibrated with 20 mM $NaHCO_3$, pH 8.4, and washed successively with 150 ml 20 mM $NaHCO_3$ (pH 8.4), 100 ml 20 mM sodium acetate buffer (pH 5.5) and 120 ml 20 mM acetic acid (pH 3.2)[10,11]. Fractions (7 ml) of the acetic acid eluate were collected; the six fractions with the highest chitinase activity were pooled and readjusted to pH 5. The pooled fractions (42 ml) contained, in total, 4.4 mg protein and 6350 nkat chitinase.

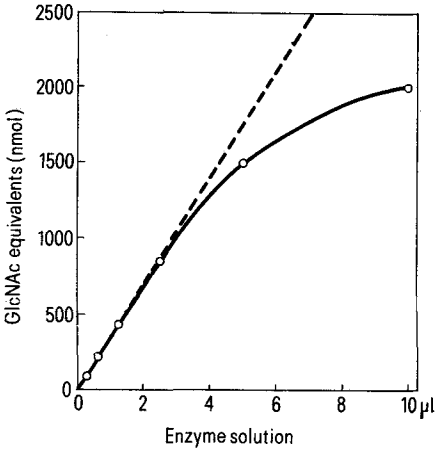

Figure 2. Product formation in the enzyme assay as a function of the concentration of *Aphanocladium album* chitinase.

5, and then eluted with acetic acid (20 mM). This liberated chitinase in a single peak with constant specific activity (fig. 1). The specific activity of purified chitinase was about 20-fold higher than in the crude culture filtrate, indicating that about 5% of the total secreted protein of *Aphanocladium album* is chitinase.

Purified chitinase of *Aphanocladium album* exhibited a broad pH optimum of pH 5 with 50% of the activity remaining at pH 3 and pH 7 (data not shown). A standard curve relating the amount of product formed in 30 min to the amount of enzyme was established (fig. 2). It was found that this standard curve was linear up to a production of 1 µmole GlcNAc equivalents from 1.2 mg substrate, corresponding to the hydrolysis of almost 20% of the substrate.

Washed, boiled mycelium of wheat rust, *Puccinia graminis* var. *tritici*, was incubated with dialyzed culture filtrate of *Aphanocladium album* containing 60 nkat chitinase or with 60 nkat purified chitinase from *Aphanocladium album*. Both preparations liberated soluble chitin fragments from the rust cell walls. Interestingly, the crude dialyzed culture filtrate was about twice as efficient as was purified chitinase. This indicates that other, unspecified enzymes of the culture filtrate enhance the potential of chitinase for attacking the mycelium of *Puccinia graminis* var. *tritici*.

Discussion. Ultrastructural studies have provided evidence that the attack of many hyperparasitic fungi involves lysis of the mycelia of their hosts[4-8]. As discussed by Tsuneda and Hiratsuka[6], this might result from autolytic processes induced in the host or from the activity of cell-wall degrading enzymes of the mycoparasites. In the present work, we have asked the question whether a mycoparasite, *Aphanocladium album*, has the enzymatic equipment to lyse the cell walls of its host, *Puccinia graminis* var. *tritici*. Rust fungi have a chitin-containing cell wall[14,15]; we therefore concentrated on chitinase. Our data show that *Aphanocladium album* secretes large amounts of chitinase on a chitin-containing synthetic medium. A distinguishing feature of this chitinase is its standard curve (fig. 2): The relation of product formation to enzyme concentration is almost linear until 20% of the substrate is hydrolyzed. This is in contrast to most other chitinases examined[10,11], which lose efficiency much sooner, when only 1–2% of the substrate is consumed, as shown by the much more curved standard curves. A chitinase resembling that of *Aphanocladium album* has previously been purified from *Serratia marcescens*[16].

We have purified *Aphanocladium album* chitinase and shown that it efficiently hydrolyzes chitin from the cell walls of its host. Interestingly, the culture medium contains other, unspecified enzymes that render the attack of chitinase yet more efficient. In this context, we found that crude culture filtrate completely dissolved the germ tubes of UV-killed *Puccinia graminis* while purified chitinase lysed the germ tubes only partially (data not shown). We conclude that chitinase is one important element in the attack of *Aphanocladium album* on *Puccinia graminis* var. *tritici* but is not in itself sufficient to bring about effective lysis of its host.

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4 Acha, G.I., Leal, J.A., and Villanueva, J.R., Phytopathology *55* (1965) 40.
5 Carling, D.E., Brown, M.F., and Millikan, D.F., Phytopathology *66* (1976) 419.
6 Tsuneda, A., and Hiratsuka, Y., Phytopathology *70* (1980) 1101.
7 Spencer, D.M., and Atkey, P.T., Trans. Br. Mycol. Soc. *77* (1981) 535.
8 Koç, N.K., Forrer, H.R., and Kern, H., Phytopath. Z. *101* (1981) 131.
9 Forrer, H.R., Phytopath. Z. *88* (1977) 306.
10 Molano, J., Polacheck, I., Duran, A., and Cabib, E., J. biol. Chem. *254* (1979) 4901.
11 Boller, T., Gehri, A., Mauch, F., and Vögeli, U., Planta *157* (1983) 22.
12 Bradford, M.M., Analyt. Biochem. *72* (1976) 248.
13 Williams, P.G., Scott, K.J., and Maclean, D.J., Phytopathology *57* (1967) 326.
14 Bartnicki-Garcia, S., A. Rev. Microbiol. *22* (1968) 87.
15 Wolf, G., in: The rust fungi, p. 152. Eds K.J. Scott and A.K. Chakravorty. Academic Press, New York 1982.
16 Roberts, R.L., and Cabib, E., Analyt. Biochem. *127* (1982) 402.

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Two mechanisms in the biological clock of *Pieris brassicae* L.: an oscillator for diapause induction; an hour-glass for diapause termination

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Summary. Resonance experiments for photoperiodic termination of pupal diapause demonstrated that *Pieris brassicae* uses a night-measuring hour-glass mechanism. In previous work the same resonance technique for diapause induction revealed that photoperiodic time-measurement is a function of the circadian system. For the first time in a living organism it has been shown that the biological clock operates by means of an oscillator for photoperiodic onset of a phenomenon and according to an hour-glass system for photoperiodic termination.
Key words. Clock; circadian oscillator; hour-glass; diapause; *Pieris*.

The biological clock, measuring time with regard to photoperiodic phenomena in living organisms, is assumed to function according to two basic principles: either a circadian oscillator[1] or an hour-glass (or a combination of the two). Resonance experiments, in which one phase, either day or night, is kept constant while the complementary phase is varied so as to obtain cycles with a period (T) of 18 to 72 h, and experiments in which an extented night is systematically interrupted by a light pulse, have proved to be most effective in revealing the existence of a circadian component. The interpretation of resonance experiments is that if the photoperiodic clock incorporates a circadian oscillation (i.e., with an endogenous periodicity, τ, close to 24 h), the product of induction is observed to be high when T is close to τ or modulo τ (i.e. the two oscillating systems resonate) or low when T is not close to modulo τ (i.e. the two oscillating systems do not resonate). In night interruption experiments, alternate peaks and troughs of a photoperiodic effect, approximately 24 h apart, are interpreted as manifestations of an underlying circadian rhythmicity (of period τ). A circadian system has been shown to exist in the majority of insects[2-6], vertebrate[7-18], and plant[19] species studied. Similar experiments have failed to reveal obvious circadian periodicity in a smaller number of species (insects[2,3,20], a lizard[21] and plants[19]), and in some cases the hour-glass interpretation is considered more appropriate. Bünning[22] examined the effects of 30-min pulses in the nights of 24-h, 36-h and 48-h cycles on larvae of the cabbage white butterfly *Pieris*