Chitinolytic Activity in Viable Spores of Encephalitozoon Species

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By employing 4-methylumbelliferyl-beta-D-NN',N"-triacetylchitotriose substrate in a semi quantitative assay, chitinolytic activity in viable spores of Encephalitozoon cuniculi *and* E. intestinalis *was detected and dependence on reaction time, spore concentration, concentration of substrate and temperature were demonstrated. It was possible to block the chitinolytic activity by chitin hydrolysate. By incubation at 80°C for 10 min or at 55°C for 20 min the spores were loosing the chitinolytic activity. Incubation of the spores in trypsin reduced the chitinolytic activity. Cellulase activity could not be detected.*

Key words: microspora - cellulase - chitinase activity - inhibition

Microsporidia are spore-forming, obligate intracellular living protozoa belonging to the phylum microspora, Battaglia 1884 (Wittner & Weiss 1999). These unicellular parasites have a wide host range in the invertebrate and vertebrate phyla, including humans (Sprague et al. 1992, Weber & Bryan 1994, Silveira & Canning 1995, Hollister et al. 1996, Weiss & Voss Brinck 1998) and recently became very important as opportunist organism promoting infections in immunocompromissed host (Weber & Brian 1994, Wittner & Weiss 1999). Vavra and Cha1upsky (1982) were the first to describe chitin as a component of microsporidia spore walls. We have only a little knowledge of the mechanisms of polar tube extrusion through the chitinous spore wall. In *Plasmodium gallinaceum* motile ookinetes are reported to produce chitinase to penetrate the chitinous peritrophic membrane which surrounds the blood meal (Huber et al. 1991). Chitin is a component of the cyst wall of *Entamoeba invadens* (Arrayo-Begovich & Carbez-Trejo 1982) and the chitinase inhibitor allosamidin (Sakuda et al. 1987) inhibits cyst formation in this species (Vega et al. 1997). Chitinase is secreted by *Leishmania* in culture medium as well as in the sand fly vector (Schlein et al. 1991) and cellulase activity has also been found associated with these parasites (Jacobson & Schlein 1997). Therefore we have looked for chitinase and cellulase activity in the spores of *Encephalitozoon cuniculi* and *E. intestinalis*.

MATERIALS AND METHODS

Parasites - The strain of *E. cuniculi* CDC:V 290 was isolated and characterized by De Groote et al. (1995) and that of *E. intestinalis* CDC:V308 was isolated and characterized by Visvesvara et al. (1995). These isolates were a gift from Prof. Dr NJ Pieniazek, Center for Disease Control and Prevention, Atlanta, GA, USA.

Culture - Both strains were maintained in tissue culture in Vero E6 cells (Kock 1998); in brief, the parasites were cultured in Vero E6 cells at 37°C using M-199 medium with Hank's salts and L-glutamine (Gibco BRL, Germany) supplemented with 2% to 5% heat-inactivated fetal bovine serum (Gibco, BRL, Germany), nonessential amino acid solution (Sigma, Deisenhofen, Germany), and penicillin/streptomycin (Gibco BRL, Germany). Spores were harvested by centrifugation (1500 x g/10 min) of the cell culture supernatant, washed three times in PBS (20 mM, pH 7.2) and stored at 4°C. Additionally to these tests the tissue culture cells were tested for chitinolytic activity but the result was negative. In the same way, the supernatant of the tissue culture was tested also with a negative result. These tests were not described in the paper.

Enzyme assays - As substrate to measure chitinolytic activity, 4-methylumbelliferyl-beta-N,N',N"-triacetylchitotriose (4-UM $(GlcNAc)_3$) (Sigma, Deisenhofen, Germany) was used. The [4-UM-$(GlcNAc)_3$] stock solution was prepared by

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dissolving 1 mM in 6.25 ml DMSO Merck, Darmstadt Germany). To 312.5 µl of this solution 1.6875 ml DMSO was added and then 60O µl of this mix were added to 9.4 ml PBS (pH 7.2), called solution 3 (sol.3). Aliquots of 1 ml of sol. 3 containing 7.5 µg of 4-MU $(GlcNAc)_3$ were stored at -20°C. All solutions were stable for several months (O'Brien & Colwell 1987).

Measurement of cellulase activity - As substrate to measure cellulase activity, 4-methylenbelliferyl-beta-D-cellobiose stock solution was prepared by dissolving 1 mM in 10 ml DMSO (Merck, Darmstadt, Germany). To 500 µl of this solution 1.5 ml DMSO was added and the 600 µl of this mix were given to 9.4 ml PBS (pH 7.2). Samples of 1 ml of this substrate solution containing 7.5 µg of 4-UM-cellobiose were stored at -20°C.

Measurement of chitinolytic activity - Free 4-methylumbellifere (4MU) is the fluorescent agent in the performed assays (McCreath & Gooday 1992), which were carried out in Quartz cuvettes and measured in a SFM 25 Kontron Spectralfluorimeter, wavelength range 200 nm-800 nm (Kontron AG Zürich Switzerland), exitation 355 nm, emission 460 nm and high voltage 340.

Standard curve - All tests were carried out at pH 7.2, the condition of the tissue culture for microsporidia. A standard curve was set up at this pH to show the relationship between the measured fluorescence units (fu) and the increasing amounts of free 4-MU (O'Brien & Colwell 1987). For this purpose 999 µl up to 990 µl buffer citrate acid-Na_2 HPO_4 -McIlvaine buffer solution (Dawson et al. 1993) were stepwise supplemented with 1µl up to 10 µl 4-MU and the fu determined under the conditions described above.

Determination of chitinolytic activity of the spores -The spores were harvested from tissue culture (Kock 1998) washed three times in cold PBS and counted. The first test (constant spore concentration, variable substrate volume (sol. 3) was carried out with 7 x 10^7 spores per 2.3 ml reaction mix 5 ml spore-containing buffer plus 1.8 ml (sol. 3), at second 1.4 ml spore-containing buffer plus 0.9 ml sol. 3 and at third 1.85 ml spore-containing buffer plus 0.45 ml sol. 3 were mixed. The second test was carried out with constant concentration of substrate (sol. 3) but the amounts of spores were varying from 1.1 x 10^8 through 5.5. x 10^7 down to 2.75 x 10^7 spores per 2.3 ml reaction mix composed of 500 µl spore-containing buffer plus 1.8 ml sol. 3. All tests were performed on ice. Before incubation, at 37°C in water bath, the tests were measured and set to nil. In 15 min intervals the fluorescence was measured up to 120 min.

Determination of cellulase activity of the spores - For this test only the spores of *E. cuniculi* were harvested and counted as mentioned; 1.1 x 10^8 spores were taken off in 500 µl buffer and mixed with 1. 8 ml sol. 3. Before incubation, at 37°C waterbath, the test was measured as described above for the chitinolytic activity and set to nil. In 15 min intervals, the fluorescence was measured up to 120 min.

Inhibition of chitinolytic enzyme activity by chitin hydrolysate - Allosamidin was introduced as inhibitor for chitinase (Sakuda et al. 1987, Nishimoto et al. 1991, McNab & Glover 1991, Vega et al. 1997) but it is not commercially available. Therefore we have tested the possibility of blocking the chitinolytic activity of the spores by chitin hydrolysate (Vector Lab., Burlington, USA; Dunn Comp., Asbach, Germany). To control the inhibition a defined amount of chitinase (1 x 10^5 U/ml PBS; Sigma, Deisenhofen, Germany) in 250 µl buffer and 250 µl chitin hydrolysate stock solution were mixed with 1.8 ml sol. 3. In a second test the 250 µl chitin hydrolysate stock solution were mixed with 1. 8 ml sol. 3. Furthermore, spores (1.1 x 10^8) of *E. cuniculi* and spores (7 x 10^7) of *E. intestinalis* in 250 ml buffer were incubated with 250 µl chitin hydrolysate plus 1.8 ml sol. 3. Additionally, the same amount of spores were taken off in 500 µl buffer plus l.8 ml sol. 3. The tests were prepared on ice. Before incubation, at 37°C in waterbath, the tests were measured and set at nil. In 15 min intervals the fluorescence was measured up to 120 min.

Influence of the chitinolytic, activity of the spores by trypsin - To test the influence of trypsin on the chitinolytic activity of the spore only spores of *E. cuniculi* (1.1 x 10^8) were taken off in 500 µl buffer plus 1 mg trypsin (T-8642 Sigma, Deisenhofen, Germany) and 1.8 ml sol. 3. For control, the same test was carried out without trypsin. The performance of both tests were carried out as described above.

Influence of incubation temperature on the chitinolytic activity of the spores - 1.1x 10^8 spores of *E. cuniculi* and 7 x 10^7 spores of *E. intestinalis* in 500 µl buffer plus 1. 8 ml sol. 3 were incubated for 120 min at 4 °C, 37°C and 55°C. Additionally, the same amount of spores were incubated for 10 min at 80°C and afterwards at 37°C as well. The tests were carried out as described above.

RESULTS

All tests showed similar results whether spores of *E. cuniculi* or *E. intestinalis* were used. Therefore only the results of *E. cuniculi* were presented here: (1) the tests show a chitinolytic activity of viable spores depending on the concentration of

substrate (Fig. 1) and on the amount of spores (Fig. 2); (2) it is possible to block this activity by chitin hydrolysate (Fig. 3) and by treatment with trypsin (Fig. 3); (3) an activity of cellulase could not be detected (Fig. 3); (4) in comparison to an incubation temperature of the spores at 37°C the chitinolytic activity of them decreased at 55°C (Fig. 4) because the spores were loosing their ability to lysate the substrate of 4-UM-(GleNAc)3. Only a weak chitinolytic activity was measured at 4°C but this ability was completely lost after incubation of the spores for 10 min at 80°C (Fig. 4).

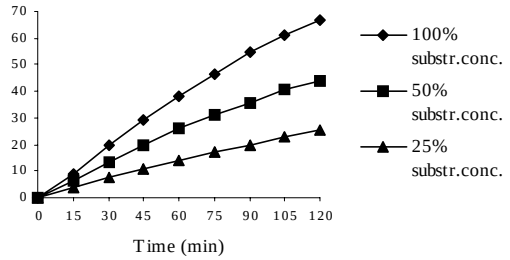

Fig. 1: measured fluorescence shows the kinetics of chitinolytic activity of spores of *Encephalitozoon cuniculi* with constant concentration of spores and available substrate volume.

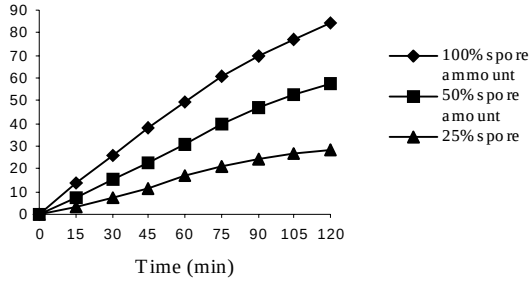

Fig. 2: measured fluorescence shows the kinetics of chitinolytic activity of spores of *Encephalitozoon cuniculi* with constant substrate volume and variable amount of spores.

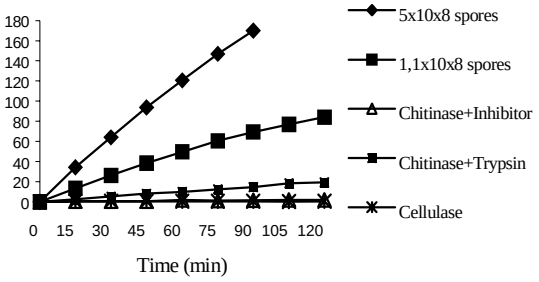

Fig. 3: measured fluorescence shows chitinolytic activity of *Encephalitozoon cuniculi* spores and its inhibition by chitin hydrolysat and trypsin as well as cellulase activity.

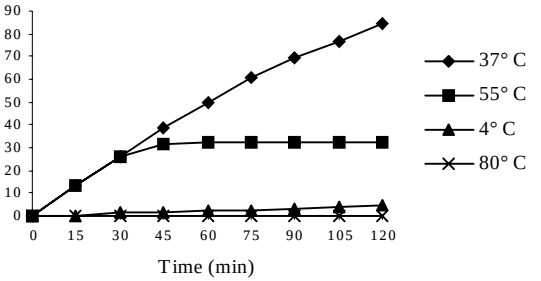

Fig.4: measured fluorescence shows the variability of chitinolytic activity of *Encephalitozoon cuniculi* spores on different temperatures. Incubation temperatures: 4°C; 37°C; 55°C; preincubation at 80°C for 10 min followed by incubation at 37°C.

DISCUSSION

This is the first report of the presence of chitinolytic activity in the spores of the human pathogenic microsporidia *E. cuniculi* and *E. intestinalis* and the absence of cellulase activity. Cellulase activity was described for the phytopathogenic Protozoa *Phytomonas* sp. (Sanchez-Moreno et al. 1992). With this enzyme this pathogen is well adapted to its life in plants (Dollet 1984). Whether this enzyme is integrated in the pathogenesis of plant diseases caused by these flagellates is unknown. This cellulose degrading enzyme was also detected in *Leishmania*, *Sauroleishmania*, *Leptomonas*, *Crithidia*, *Herpetomonas* and *Trypanosoma* (Jacobson & Schlein 1997). The authors could demonstrate that *Leishmania* can be cultivated in the presence of cellulose. The hydrolysis of cellulose by the mentioned flagellates may be a source of their nutrients.

In contrast to cellulase, chitinase is involved in the life cycle of several parasites. By the evidence of chitinolytic, activity of not destructed, viable *Encephalitozoon* spores, which follows the rules of enzymatic kinetics, we presume the existence of chitinase or chitinases in this organism. An evidence of the existence of this enzyme in these parasites is the inhibition of the chitinolytic activity by chitin hydrolysate and few influence by trypsin.

The chitinolytic activity could be stopped by incubation of the viable spores at 55°C, and could be completely eliminated by incubation of the spores for 10 min at 80°C.

Chitin and chitinases are widespread in nature (Flach et al. 1992). Chitinase was found in vertebrates, in human, and in guinea pig serum (Overdijk & Van Steijn 1994, Overdijk et al. 1996). Chitin is a component in the sheat of *Brugia malayi* (Fuhrman & Piessens 1985), *Onchocerca volvu-*

lus and *B. gibsoni* (Brydon et al. 1987) or in the egg-shell of *Ascaris suum* (Ward & Fairbairn 1972, Dubinsky et al. 1986a, b). The presence of chitin in nematodes is supplemented by the detection of chitinolytic enzyme activities in *B. malayi*, *B. phalange* and in *B. gibsoni* (Gooday et al. 1988). Genes for chitinases are expressed in the infective larvae of *O. volvolus* and *A. vitae* (Wu et al. 1996). Chitin is an essential component of the peritrophic membrane in insects. Schlein (1993) found that the chitinolytic activity of *Leishmania* promastigotes in the vector is necessary to lyse the chitin framework of the peritrophic membrane which surrounds the bloodmeal with the flagellate inside so that they become free to continue their development in the intestine. Shahabuddin et al. (1993) found that the inhibition of the chitinolytic activity of the ookinets of *P. gallinaceum* by allosamidin completely inhibits the development of oocysts because the ookinets were no longer able to pass through the chitin containing peritrophic membrane which surrounds the bloodmeal in the mosquito. In *Trichomonas vaginalis* and *T. fetus* chitin is probably integrated in the adherence mechanism within the urogenital tract. Chitin is also a structural component of *Giardia* cysts (Ward et al. 1985) as well as in the cysts of *E. histolytica*, *E. dispar* and *E. invadens* (Vega et al. 1997). This cyst formation can be blocked by allosamidin. *Entamoeba* chitinases accumulate to maximal levels in the process of excysting (Vega et al. 1997). Ward and Fairbairn (1972) described that chitinase is needed for the hatching of infective eggs of *A. suum*. Gooday et al. (1988) agree that filarial chitinases from females of *B. gibsoni* are not only integrated in the formation of the egg-shells but also in the hatching.

The chitinolytic activity in the microsporidia spores is perhaps also part of the extrusion mechanism by which the polar tube process through the chitinous spore wall and integrated in the maturation process of the spore wall.

In microsporidia spores chitin is normally described as part of the endospore (Vavra 1967, 1976, Erickson & Blanquet 1969, Canning & Hollister 1991). Bigliardi et al. (1996) demonstrated that chitin is also integrated in the exospore. This result is underlined by the finding of Kock (1998) that the lectin of *Triticum vulgaris* conjugated with gold reacts to a high degree with the exospore. This exospore-chitin could be the ligand for the chitin binding receptor on human macrophages (Renkema et al. 1998) because macrophages can serve as host cells for microsporidia (Couzinet et al. 1997) so that macrophages could play a part in the dissemination of these spores in a host. The dissemination of these parasites in a host is an unsolved problem. A monoclonal antibody against the chitinase of *B. malayi* can block the transmission of the microfilaria (Fuhrman & Piessens 1985). The importance of the chitinolytic activity for the infectivity of the spores of human pathogenic microsporidia and the importance of the exospore-chitin for the dissemination of the spores need further studies.

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