

CHITINOLYTIC ACTIVITIES IN *TRICHOMONAS VAGINALIS*

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Summary :

Chitinolytic activities were identified in the Protozoa *Trichomonas vaginalis*. Overall chitinase activity assessed using chitine-azure as substrate was 10.93 ± 1.21 nmoles/min/mg prot. End nonreducing chitobiosidase (exochitinase) and chitotriosidase (endochitinase) activities were shown using *p*-nitrophenyl-substrates and had specific activities of 4.55 ± 0.53 and 0.47 ± 0.06 nmol/min/mg prot, $K_{m,app} = 1.32$ mM and $5 \mu\text{M}$ and pH optimum = 7.0 and 6.1 respectively. β -N-acetylhexosaminidase (NAHase) activity was also detected with a specific activity of 5.40 ± 0.65 nmol/min/mg prot., $K_{m,app} = 0.656$ mM and pH optimum at 7.0. No release of these enzymes into the culture medium was found. The possible role of chitinases in *T. vaginalis* remains to be investigated.

KEY WORDS : *Trichomonas vaginalis*, chitinolytic activity, β -N-acetylhexosaminidase, chitobiosidase, chitotriosidase.

MOTS CLÉS : *Trichomonas vaginalis*, activité chitinolytique, β -N-acetylhexosaminidase, chitobiosidase, chitotriosidase.

Résumé : ACTIVITÉS CHITINOLYTIQUES OBSERVÉES CHEZ *TRICHOMONAS VAGINALIS*

Des activités chitinolytiques ont été identifiées chez le Protozoaire *Trichomonas vaginalis*. L'activité chitinasique totale s'élève à $10,93 \pm 1,21$ nmoles/min/mg protéine en utilisant la chitine-azure comme substrat. Les activités chitobiosidase (exochitinase) et chitotriosidase (endochitinase) libérant respectivement des dimères et des trimères de β -D-N-acétylglucosamine à partir de l'extrémité non réductrice ont été montrées en utilisant des substrats *paranitrophénylés*. Les activités spécifiques chitobiosidase et chitotriosidase s'élèvent respectivement à $4,55 \pm 0,53$ et $0,47 \pm 0,06$ nmol/min/mg protéine avec des $K_{m,app}$ à $1,32$ mM et $5 \mu\text{M}$ et des pH optima à 7,0 et 6,1 pour chacun des systèmes enzymatiques. Une activité β -N-acetylhexosaminidase (NAHase) a aussi été détectée à $5,40 \pm 0,65$ nmol/min/mg prot. avec un $K_{m,app}$ à $0,656$ mM et un pH optimum à 7,0. Aucune libération de ces enzymes n'a pu être mise en évidence dans le milieu de culture. Le rôle des chitinases de *T. vaginalis* est maintenant en cours d'étude.

INTRODUCTION

Trichomonas vaginalis is a common cause of infection of the human genital tract and trichomoniasis is recognized as a major sexually transmitted disease. Metronidazole is the most commonly used antitrichomonal drug (Lloyd & Paget, 1991). However, the occurrence of 5-nitroimidazole-resistant *T. vaginalis* strains has prompted us to identify new potential targets for therapy (Meingassner & Thurner, 1979). A knowledge of parasite metabolism and its cytopathic effects are therefore of interest for the design of new drugs to control trichomoniasis. Chitinases are enzymes found in plants, insects and parasites (Flach *et al.*, 1992). These enzymes cleave β -1- \rightarrow 4 linkages between N-acetylglucosamine (GlcNAc) residues, releasing oligomers. Two classes of chiti-

nases can be distinguished: endochitinases which cleave within chitin chains and exochitinases that cleave [GlcNAc]₂ units from the non-reducing end of chitin chains. Their presence has been described in Protozoa such as *Leishmania* (Schlein *et al.*, 1991), *Plasmodium* (Huber *et al.*, 1991) and *Entamoeba* sp. (Lopez-Romero & Villagomez-Castro, 1993). They are involved in the insect-parasite interaction in *Leishmania* and *Plasmodium* and in the encystation process of *Entamoeba*. No data is available in the literature concerning the occurrence of chitinases in *T. vaginalis*, except for the presence of β -D-N-acetylhexosaminidase (NAHase) (Lockwood *et al.*, 1988). This paper reports the presence and chitinolytic properties of chitobiosidase (exochitinase) and chitotriosidase (endochitinase) in *T. vaginalis*.

MATERIALS AND METHODS

PARASITE AND CULTURE

Trichomonas vaginalis strain CMP (Châtenay-Malabry Parasitology), sensitive to metronidazole was isolated in 1987 from a woman

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suffering from trichomoniasis and stored as a stabilate in conventional medium containing 6 % dimethylsulphoxide as cryoprotector in liquid nitrogen. Trichomonads were cultured axenically *in vitro* in trypticase-yeast extract-maltose (TYM) medium (Diamond, 1957), supplemented with 10 % heat-inactivated filtered horse serum (Institut Pasteur, Cat. No. 61311, Paris, France) at 37 °C. The parasites were subcultured every two days in TYM medium. Trichomonads were in logarithmic phase of growth within 48 h of subculture.

PREPARATION OF PARASITES

The parasites were harvested and washed three times by centrifugation (3,000 g for ten min) in phosphate buffered saline (PBS) pH 7.2 at 4 °C. Parasites were counted using a haemocytometer and adjusted to a suspension of 3.5×10^7 living organisms per ml in the same buffer.

ENZYME EXTRACTION

Cell lysis was obtained by three cycles of freezing and thawing and assessed microscopically. The lysate was centrifuged at 3,000 g for ten min at 4 °C and the supernatant was used as the enzyme source. Moreover, the samples of medium from *T. vaginalis* cultures were filtered at low pressure through a nitrocellulose Millipore filter (pore size 0.45 µm) and were also assayed for chitinase activity.

CHEMICALS AND REAGENTS

Chitin azure, *p*-nitrophenyl β-D-N-acetylglucosaminide, *p*-nitrophenyl β-D-N,N'-diacetylchitobioside, *p*-nitrophenyl β-D-N,N',N''-triacetylchitotrioside, *Streptomyces griseus* chitinase, were purchased from Sigma Chemical Co. (Saint-Quentin Fallavier, France).

ENZYME ASSAYS

Overall chitinase activity was determined using chitin azure as substrate, suspended in 0.2 M sodium acetate buffer (pH 5.2). The final concentration of chitin azure particles was 10 mg/ml. Degradation of soluble azure after centrifugation (2000 g, ten min) was monitored by spectrophotometric detection at 550 nm (Hackman & Goldberg, 1964).

NAHase, chitobiosidase (exochitinase) and chitotriosidase (endochitinase) activities were determined with the *p*-nitrophenyl-substrates according to Mega *et al.*, 1970 for NAHase and Renkema *et al.*, 1995 for chitobiosidase and chitotriosidase. The assay was performed in Nunclon® 96-well plates. *p*-nitrophenyl β-N-acetylglucosaminide, *p*-nitrophenyl β-N,N'-diacetylchitobioside and *p*-nitrophenyl β-N,N',N''-triacetylchitotrioside were used at final concentrations of 6 mM, 6 mM and 270 µM respectively. NAHase was assayed in a 0.2 M

sodium phosphate buffer (pH 7.0) whereas chitobiosidase and chitotriosidase were assayed in 0.2 M sodium acetate buffer (pH 5.2). Assays (final volume, 120 µl) were stopped with 50 µl of 0.2 M glycine-NaOH buffer (pH 10.6). The *p*-nitrophenol formed was determined spectrophotometrically at 405 nm.

PROTEIN DETERMINATION

Proteins were determined by the method of Sedmak & Grossberg, 1977.

pH OPTIMUM DETERMINATION

The true pH of the reaction mixture was measured during the determination of the pH optimum on the supernatant fraction. Two buffers were used to cover the required pH range: 0.2 M sodium acetate buffer (pH range, 3.6-5.6) and 0.2 M sodium phosphate buffer (pH range, 5.6-8).

K_M VALUES FOR *p*-NITROPHENYL-β-D-OLIGOSACCHARIDES

Apparent K_m values were calculated for *p*-nitrophenyl-β-D-N-acetylglucosaminide, *p*-nitrophenyl-β-D-N,N'-diacetylchitobioside and *p*-nitrophenyl-β-D-N,N',N''-triacetylchitotrioside from Lineweaver-Burk reciprocal plots fitted by weighted regression.

THERMAL STABILITY OF NAHASE, CHITOBIOSIDASE AND CHITOTRIOSIDASE

In order to determine the heat-stability of the enzymes, cell extracts were incubated at 65 °C for various periods of time and then re-assayed. Enzyme half-life was determined as the time of incubation at 65 °C which provoked the decrease of 50 % enzyme activity.

LDH (LACTATE DEHYDROGENASE) AND MDH (MALATE DEHYDROGENASE) DETERMINATIONS

LDH was assayed in the medium in which the cells had been grown according to the method described by Marchat *et al.*, 1996. MDH was assayed following Loiseau *et al.*, 1993.

RESULTS

Three chitinolytic enzymes were identified in crude extracts of *Trichomonas vaginalis* trophozoites: NAHase, chitobiosidase and chitotriosidase, with a total activity of 10.93 nmoles/min/mg protein (Table I). NAHase activity was 5.40 nmoles/min/mg protein with an optimum pH at 7.0, similar to exochitinase. Endochitinase had maximum activity at pH 6.1. When enzyme extracts

Chitinolytic enzymes	Total activity*	NAHase**	Chitobiosidase**	Chitotriosidase**
Specific activity (nmol/min/mg prot)	10.93 ± 1.21	5.40 ± 0.65	4.55 ± 0.53	0.47 ± 0.06
pH optimum	5.2	7.0	7.0	6.1
K _m app (mM)	/	0.656	1.320	0.005
Half-life (min) at 65 °C	/	7	7	5

Assays were carried out in triplicate.

/ : not determined

* : chitin azure as substrate.

** : *p*-nitrophenyl-oligosaccharides as substrates.

Table I. — Chitinolytic activities in *Trichomonas vaginalis*.

were preincubated at 65 °C before assay, the enzymes activity was reduced to half its initial value after seven, seven and five min for NAHase, chitobiosidase and chitotriosidase respectively.

Concerning the possibility of *T. vaginalis* to secrete chitinases in the culture medium, we had to verify the presence of chitinase activity in fresh culture medium. We found that horse serum itself, a component of the medium, contains NAHase, chitobiosidase and chitotriosidase activities. Only 30 % of serum chitinase was inactivated by heating at 56 °C for 30 min. and complete inactivation could only be achieved at 65 °C. The chitinolytic activities found in fresh culture medium containing 10 % heat-inactivated horse serum, were 0.813, 0.282 and 0.400 nmol/min/mg protein for NAHase, chitobiosidase and chitotriosidase, respectively. A culture medium in which cells had been grown for 48 h gave similar results.

We also checked the influence of cell lysis in the culture by assaying for two exclusively intracellular enzymes. These were lactate dehydrogenase (LDH) which is cytosolic and malate dehydrogenase (decarboxylating) (MDH), a hydrogenosomal enzyme. LDH and MDH activities were detectable when cells were washed in PBS pH 7.2 and lysed by freezing and thawing in this buffer. Similar activities were observed when cells were lysed in fresh culture medium, indicating that medium components did not affect the assays. Nevertheless, during growth (24, 30 and 48 h), no significant activity could be detected in the medium in which the cells had been grown and then removed by centrifugation and filtration. From the limit of detection of LDH, it was calculated that less than 1 % of the cells could have been lysed after a 48 h culture.

DISCUSSION

During axenic growth *in vitro*, NAHase has been described as being released into the growth medium by *T. vaginalis*, accompanied

by other hydrolytic enzymes such as acid phosphatase and α -mannosidase (Lockwood *et al.*, 1988; North & Buchan, 1990). It has therefore been suggested that secretion of hydrolases contributes to the pathology of this parasite (Savoia & Martinotti, 1989). Nevertheless, these authors did not carry out assays on fresh culture medium containing heat-inactivated horse serum. Chitinases had been detected in mammalian serum by Lundblad *et al.*, 1974; 1979. According to our results, no chitinase activity from *T. vaginalis* was detected in the medium and the chitinase activity detected in the medium could be ascribed only to horse serum. This suggests that no chitinolytic enzymes were released in the culture medium and the chitinolytic activities detected by Lockwood *et al.*, 1988 should therefore be attributed to horse serum chitinases.

The present study demonstrates for the first time chitinolytic activity in *T. vaginalis*. No physiological function for these enzymes is as yet apparent. They may be involved in digestion of nutrients inside the cell. Alternatively, they may be required specifically for the establishment of the parasite within the host, perhaps by providing a means of countering host defence systems. Thus, the pathogenic effect of *T. vaginalis* has been reported being mediated by a surface membrane lectin specific for mannose, β -N-acetylglucosamine and β -N-acetylmannosamine (Roussel *et al.*, 1991). Intracellular chitinases could provide β -N-acetylglucosamine, which would then modulate the cytopathic effect. Moreover, the *T. vaginalis* chitinases described here may also be involved in defence against chitin-containing pathogens such as fungi. Further studies will be performed on these enzymes in order to understand their contribution to the cytopathic effect of this parasite.

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