KINETIC PARAMETERS OF N-ACETYLGLUCOSAMINIDASE IN ADULT FEMALE *NIPPOSTRONGYLUS BRASILIENSIS* BY A QUANTITATIVE COLORIMETRIC MICROMETHOD

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Summary :

Chitin, the 1,4- β -linked homopolymer of N-acetylglucosamine, is a component of Nematode eggshell. Chitin is hydrolyzed to its constituent monomer by the binary chitinase enzyme system. Chitinase (EC 3.2.1.14) hydrolyses first the polymer to N,N'-diacetylchitobiose which is then split to N-acetylglucosamine by N-acetylglucosaminidase (EC 3.2.1.30). N-acetylglucosaminidase is also able to release N-acetylglucosamine monomers directly from chitin. This study reports the identification of

N-acetylglucosaminidase in adult female *Nippostrongylus brasiliensis*. A quantitative colorimetric micromethod has been developed to detect N-acetylglucosaminidase activity from *N. brasiliensis* using p-nitrophenyl-N-acetylglucosaminide as substrate. The assay conditions were optimized as follows: 50 μ l of protein extract were incubated at 37 °C for 2 h in the presence of 50 μ l of 19 mM substrate solution and 50 μ l of 0.2 M potassium phosphate buffer pH 6.0. The reaction was stopped by the addition of 50 μ l 0.1 N NaOH and the absorbance was measured on a Multiskan MS spectrophotometer at 405 nm within 30 min. after the reaction was stopped. The optimal pH was 6.0. Specific activity of protein extract was found at 94.4 ± 5.6 pmol/min/mg protein ($n = 4$). V_{max} app. and K_m app. for p-nitrophenyl-N-acetylglucosaminide were respectively 280 ± 11 pmol/min/mg protein ($n = 3$) and 1.8 ± 0.2 mM ($n = 3$). This test can now be proposed as a tool for screening anthelmintic compounds in the field of new inhibitors of chitin metabolism.

KEY WORDS : *Nippostrongylus brasiliensis*, intestinal Nematode, N-acetylglucosaminidase, chitin metabolism.

Résumé : PARAMÈTRES CINÉTIQUES DE LA N-ACÉTYLGLUCOSAMINIDASE CHEZ LE VER FEMELLE *NIPPOSTRONGYLUS BRASILIENSIS* PAR UNE MICROMÉTHODE COLORIMÉTRIQUE QUANTITATIVE

La chitine, un homopolymère de N-acétylglucosamine lié par des liaisons β -1,4, est un composant de la paroi des oeufs de Nématodes. Les chitinases (EC 3.2.1.14) constituent un système qui permet l'hydrolyse enzymatique de la chitine en N,N'-diacétylchitobiose. La N-acétylglucosaminidase (EC 3.2.1.30) est l'enzyme qui hydrolyse le N,N'-diacétylchitobiose en monomères de N-acétylglucosamine et qui libère aussi des monomères à partir de la chaîne de chitine. Cette étude décrit l'identification d'une activité N-acétylglucosaminidase chez les femelles adultes de *Nippostrongylus brasiliensis*. Une microméthode colorimétrique a donc été mise au point pour quantifier l'activité de la N-acétylglucosaminidase chez *N. brasiliensis* en utilisant le p-nitrophényl-N-acétylglucosaminide comme substrat. Les conditions du dosage ont été optimisées de la manière suivante : 50 μ l d'extrait protéique ont été incubés à 37 °C pendant 2 h en présence de 50 μ l de solution de substrat à 19 mM et de 50 μ l de tampon phosphate potassique 0,2 M pH 6. La réaction a été arrêtée par l'addition de 50 μ l de NaOH 0,1 N et la densité optique a été mesurée sur un spectrophotomètre Multiskan MS à 405 nm dans la demi-heure qui suit l'arrêt de la réaction. Le pH optimal est de 6,0. L'activité spécifique de l'extrait protéique est de $94,4 \pm 5,6$ pmol/min/mg protéines ($n = 4$). Les calculs du V_{max} apparent et du K_m apparent pour le p-nitrophényl-N-acétylglucosaminide donnent respectivement les valeurs de 280 ± 11 pmol/min/mg protéines ($n = 3$) et $1,8 \pm 0,2$ mM ($n = 3$). Ce test peut maintenant être proposé pour le criblage de composés anthelmintiques dans le domaine des inhibiteurs du métabolisme de la chitine.

MOTS CLÉS : *Nippostrongylus brasiliensis*, nématode intestinal, N-acétylglucosaminidase, métabolisme de la chitine.

INTRODUCTION

Chitin is the carbohydrate with the second largest biomass and is only surpassed by cellulose. It is composed of unbranched, helicoidal microfibrils of β (1-4)-linked aminosugars. Chitin is most often called a homopolymer of N-acetyl- β -D-glucosamine, which may not be completely true since glucosamine is also found to various degrees in chitin (Spindler *et al.*, 1990).

Chitin is an important component of the eggshell in Nematodes (Wharton, 1980; Brydon *et al.*, 1987) and in eggshell-derived structures such as filarial sheath in *Brugia malayi* (Fuhrman & Piessens, 1985). It is not synthesized by vertebrates. It therefore represents a useful target for drugs against Nematodes (Spindler *et al.*, 1990). In Nematodes, the chitin layer, probably α -chitin, is laid down by the fertilised egg, not by the uterus of the female. Nematode egg chitin shows minor differences in optical properties and higher flexibility with typical arthropod chitin (Barrett, 1981). Chitin is specifically degraded by the action in concert of chitinase (EC 3.2.1.14) and β -N-acetylglucosaminidase (EC 3.2.1.30) (Arnold *et al.*, 1993). The latter (NAGase) hydrolyses the

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non-reducing ends of the chitin chains producing monomers of N-acetylglucosamine. These enzymes have been characterized in *Onchocerca gibsoni* (Gooday *et al.*, 1988), *Brugia malayi* (Fuhrman *et al.*, 1992) and *Heligmosomoides polygyrus* (Arnold *et al.*, 1993). This paper describes the determination of NAGase activity and some properties of NAGase from an intestinal rat nematode, *Nippostrongylus brasiliensis* female adult worm by a quantitative colorimetric micromethod. This assay may be used for the research of Nematode NAGase specific inhibitors.

MATERIALS AND METHODS

PARASITE AND LABORATORY CYCLE

A strain of *Nippostrongylus brasiliensis* was maintained in female white rat (Wistar, Charles River, Cléon, France). Faeces from infected rats were used to make faecal cultures in Petri dishes which were incubated in a humidified room at 25 °C. Five days later, third stage infective larvae (L3) migrated and remained attached to the edge of the filter paper. Because of their positive thermal tropism, larvae could be harvested by filling the Petri dish with pre-warmed (37 °C) distilled water. Then, they were washed several times and injected into female rats subcutaneously in a minimal volume (0.4 ml of 0.15 M NaCl containing about 2,500 L3 per 250 g female rat). After migration to the lungs, the third moult occurred within 24-32 h. Then L4 migrated to the small intestine via the trachea and oesophagus and molted into adults (Kassai, 1982). Under our experimental conditions, eggs started to appear in faeces on the 5th day (D5) post-infection. All worms were spontaneously expelled owing to the immunological reaction of the host two weeks post-infection (Kassai, 1982).

PREPARATION OF WORMS

The day of infection was termed day 1. Worms were collected at Day 8. Parasite-infected rats were killed by CO₂ inhalation and the small intestine was removed from the duodenum to jejunum. It was then filled with physiological saline (0.15 M NaCl) at 37 °C and opened longitudinally. The mucosa was excised and placed in a gauze bag which was then placed in a beaker containing physiological saline at 37 °C for 1 h. Worms settled at the bottom of the beaker. They were collected from the beaker and screened according to sex and counted within 3 h using a binocular microscope. They were washed four times in pre-warmed (37 °C) saline to avoid contamination. Samples of worms containing about 500 females, collected from the same rat, were weighed and frozen at -70 °C. Each experiment was performed in triplicate using worms from different rats.

CHEMICALS AND REAGENTS

p-nitrophenyl-N-acetyl- β -D-glucosaminide and all other reagents were analytical grade and were purchased from Sigma Chemical Co.

ENZYME EXTRACTION

Batches of about 500 females were separately crushed in an ice bath. Each batch was homogenized in 4 ml of 0.2 M potassium phosphate buffer pH 6.0. The homogenate was centrifuged at 20,000 g for 20 min at 4 °C. The supernatant fraction was assayed for NAGase activity.

ENZYME ASSAY

NAGase activity was assayed by a colorimetric measurement of *p*-nitrophenol (PNP) liberated from *p*-nitrophenyl-N-acetyl- β -D-glucosaminide as described by Mega *et al.*, 1970, with slight modifications. The assay was performed in Nunclon® 96-well plates as follows: 50 μ l of supernatant fraction were incubated for 2 h at 37 °C in the dark with 50 μ l of 0.2 M potassium phosphate pH 6.0 and 50 μ l of a *p*-nitrophenyl-N-acetyl- β -D-glucosaminide solution to obtain a substrate concentration in final volume at 7 mM. The reaction was stopped by the addition of 50 μ l of 0.1 N NaOH. The absorbance was measured at 405 nm on a Multiskan MS Spectrophotometer. Several controls including wells with no substrate or no protein extract were used in the enzyme assay.

One unit of NAGase is defined as the amount of enzyme that generates 1.0 μ mol PNP in 1 h under the standard assay conditions.

PH OPTIMUM DETERMINATION

The true pH of the reaction mixture was measured during the determination of the pH optimum on the supernatant fraction. Several buffers were used to cover the required pH range: 0.2 M glycine-HCl (pH range, 2.2-3.6), 0.2 M sodium acetate (pH range, 3.6-5.6), 0.2 M potassium phosphate (pH range, 5.6-8.0).

PROTEIN DETERMINATION

Protein concentrations were determined by the method of Bradford, 1976.

K_m VALUE FOR *p*-NITROPHENYL- β -D-N-ACETYLGLUCOSAMINIDE

Apparent K_m value was calculated for *p*-nitrophenyl- β -D-N-acetylglucosaminide from Lineweaver-Burk (1934) reciprocal plots fitted by a weighted regression.

RESULTS

NAGase activity was always detected in adult female *N. brasiliensis* batches. The enzyme assay was optimized for the highest activity. The colorimetric measurement of PNP liberated from *p*-nitrophenyl- β -D-N-acetylglucosaminide was carried out in the thirty minutes after the reaction was stopped with 0.1 N NaOH. When the measurement was carried out 24 h after the reaction was stopped, chemical hydrolysis of the substrate occurred amplifying the coloration of the medium. The activity of NAGase

increased more than twice from 27 °C to 37 °C (Fig. 1). The enzyme reaction was a linear function of time up to a 4 h incubation period (Fig. 2). Female adult *N. brasiliensis* NAGase had a pH optimum of 6.0 (Fig. 3).

The apparent Michaelis constant (K_m value) was determined at 1.8 ± 0.2 mM ($n = 3$) for the substrate *p*-nitrophenyl- β -D-N-acetylglucosaminide and the linear correlation coefficient was above 0.99 (Fig. 4). Specific activity of the protein extract was at 94.4 ± 5.6 pmol/min/mg protein ($n = 4$). V_{max} was found at 280 ± 11 pmol/min/mg protein ($n = 3$).

Fig. 1. – Effect of temperature on the NAGase activity after a 2-h incubation period. The substrate concentrations are given in final volume.

Fig. 3. – The pH curve for *N. brasiliensis* adult female NAGase. The assay conditions were as described in the Materials and Methods section. The activity determined at each pH value was the mean of three measurements at 37 °C.

Fig. 2. – Linearity of the enzyme reaction of adult female *N. brasiliensis* NAGase as a function of incubation time at 37 °C. The substrate concentration in the reaction mixture was 7 mM.

Fig. 4. – Lineweaver-Burk plot of *N. brasiliensis* adult female NAGase. The enzyme fraction was incubated with various concentrations of the substrate, *p*-nitrophenyl-N-acetylglucosaminide. Velocity (V) is expressed in U/ml.

DISCUSSION

The cuticle of Nematodes does not contain chitin but its main components are collagen-like proteins whereas *Pentastomida* for example have a chitin containing cuticle (Mehlhorn *et al.*, 1991). Nevertheless, chitinase activity was found in *Onchocerca gibsoni* adult female and the authors postulated a role for this enzyme in embryogenesis (Gooday *et al.*, 1988). Since the chitinase assays were performed on extracts of the adult worm, the activity could not be localized as maternal or embryonic, leaving its biological role obscure. Moreover, chitinase and NAGase activities have been described in both sexes of adult *Heligmosomoides polygyrus* (Arnold *et al.*, 1993). The low levels of chitinase activity observed in males were unexpected; they were associated primarily with the mixed membrane fraction. In contrast, enzyme activities in females and eggs were predominantly in the soluble fraction.

This study pointed out the presence of NAGase in adult *N. brasiliensis*. The NAGase activity found in this Nematode is not surprising because of the ubiquity of the enzyme. Since NAGase hydrolyses chitin to N-acetylglucosamine, its inhibition could be of interest to block chitinase systems. Only adult females were considered for their NAGase activity since the strategy of inhibition requires studies on homogeneous enzyme batch *N. brasiliensis*.

The effect of incubation time on the degree of hydrolysis is shown in Fig. 2. NAGase activity increased linearly with time up to 180 min. On the basis of these findings, a 2 h incubation time was selected for subsequent experiments. In our conditions, a 2 h incubation time gave an absorbance value of 0.5 for NAGase activity. Such a value is suitable in order to evaluate the effect of potential NAGase inhibitors in further experiments.

The apparent K_m value determined at 1.8 ± 0.2 mM showed that substrate had not a high affinity for the enzyme. The substrate concentration used for NAGase assay was chosen at 7 mM; this value corresponds to about 4 fold the K_m app. value. In these conditions of substrate saturation, the reaction velocity was maximum.

Further NAGase purification is needed to determine isoforms. The characteristics of the purified enzyme is necessary to appreciate its interest as a biochemical target for chemotherapy.

This quantitative colorimetric micromethod using supernatant fraction from crude extract can now be proposed as a tool to screen specific inhibitors.

Further studies will be based on the research of polyvalent chitinase-NAGase inhibitors because it is

expected that high chitobiose concentrations could inhibit chitinase system.

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