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An endo-beta-D-glycosidase from salivary glands of *Macrotermes subhyalinus* little soldier with a dual activity against carboxymethylcellulose and xylan

Fagbohoun Jean Bedel, Ahi Amedée Pascal, Karamoko Yahaya, Dabonné Soumaila *, Kouadio Eugène Jean Parfait and Kouamé Lucien Patrice

Laboratoire de Biocatalyse et des Bioprocédés de l'Université d'Abobo-Adjamé (Abidjan, Côte d'Ivoire), 02 BP 801 Abidjan 02, Côte d'Ivoire

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Abstract

This study highlights an endo-beta-D-glycosidase from little soldier of *Macrotermes subhyalinus* purified by anion exchange, cation exchange and hydrophobic interaction chromatography. The only substrates that were hydrolyzed by the purified enzyme were xylans and carboxyméthylcellulose. The enzyme showed a single protein band and its relative molecular weight was estimated to be 215.45±5.63 kDa. The specific activities towards carboxymethylcellulose and xylan from Birchwood were respectively 9.32±3.78 and 8.59±2.54 U/ mg of protein. The purified enzyme showed an optimum pH of 4.6 for cellulase activity an 5.0 for xylanase activity in acetate buffer. The optimum temperature of the enzyme with CMC and xylan from Birchwood hydrolysis were found to be 60 and 55 °C respectively.

*Corresponding Author: Soumaila Dabonné ✉ sdabonne@yahoo.fr

Introduction

Termites live in large, socially structured colonies containing workers, soldiers and reproductive members numbered in millions (Zimmerman *et al.,* 1982). Termites play a major role in the recycling of photosynthetically fixed carbon. Although, most animals cannot utilize lignocellulose as a nutrient, termites are known as significant pests in wood-based architecture all over the world. Indeed, lignocellulose is the major component of biomass found in nature, derived from agricultural residues, woods and municipal solid wastes (Pérez *et al.,* 2002). The structure of lignocellulose is comprised of tightly associated cellulose (40%), hemicelluloses (20–30%) and lignin polymers (20–30%) (Tuomela *et al.,* 2000 ; Juhász *et al.,* 2005).

Cellulases are responsible for the hydrolysis of the β-1,4-glucosidic bonds in cellulose. According to their mechanism of cellulose degradation, they are subdivided into either non-processive cellulases (endocellulases) or processive cellulases (including different exocellulases and some new processive endocellulases) (Barr *et al.*, 1996 ; Reverbel-Leroy, 1997).

Lignocellulose enzymes degrading are widely distributed in nature predominantly in microorganisms (Lama *et al.,* 2004 ; Ninawe *et al.,* 2008; Gaffney *et al.,* 2009) and insects (Séa *et al.,* 2006 ; Binaté *et al.,* 2008 ; Arakawa *et al.,* 2009). Then, cellulases and xylanases from termite workers have been characterized extensively (Rouland *et al.*, 1988a, b; Veivers *et al.,* 1991; Kouamé *et al.,* 2005; Faulet *et al.,* 2006). Furthermore, several studies on cellulases and xylanases from termites have shown that their salivary glands play a significant role in the cellulose and xylan digestion (Watanabe *et al.,* 1997, 1998 ; Tokuda *et al.*, 1999; Bléi *et al.*, 2010). In this respect, Nakashima *et al.* (2002) have found that salivary glands contribute significantly to hemicelluloses degradation in workers of the termite *Coptotermes formosanus*. However, few studies have been devoted to enzymes from termite soldiers. There are no reports concerning the purification of cellulases and hemicellulases from little soldiers of the termite *Macrotermes subhyalinus*. This paper describes the purification and characterization of an endo-beta-D-glycosidase, exhibiting cellulase and xylanase activities, from the little soldier salivary glands in order to elucidate its role in the digestive tract.

Material and methods

Enzymatic source and preparation of crude extract

Little soldiers of the termite *Macrotermes subhyalinus* were from the savannah of Lamto (Abidjan, Côte d'Ivoire). They were collected directly from their nests and then stored frozen at -20°C. Salivary glands (10 g) were dissected and homogenized with 20 mL 0.9 % NaCl (w/v) solution using a blender (Ultra-Turrax) and then sonicated as previously described by Rouland *et al.* (1988a). The homogenate was centrifuged at 20,000 x g for 15 min. The collected supernatant constituted the crude extract. After freezing at -180°C in liquid nitrogen, the crude extract was stored at -20°C (Kouamé *et al.,* 2005).

Chemicals

Polysaccharides, oligosaccharides and *p*-nitrophenylglycopyranosides were purchased from Sigma Aldrich. ANX-Sepharose 4 Fast-Flow, CM-Sepharose CL-6B and Phenyl Sepharose CL-4B gels were obtained from Pharmacia-LKB Biotech. Bovine serum albumin (BSA) and the chemicals used for polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. All other chemicals and reagents were of analytical grade.

Enzyme assays

Under the standard test conditions, xylanase or cellulase activity was assayed spectrophotometrically by measuring the release of reducing sugars from Birchwood xylan or carboxyméthylcellulose (CMC). The reaction mixture (0.38 ml) contained 0.2 ml of

0.5% xylan or CMC (w/v) dissolved in 20 mM acetate buffer (pH 5.0) and 0.1 ml enzyme solution. After 30 min of incubation at 45°C, the reaction was terminated by adding 0.3 ml of dinitrosalicylic acid solution followed by 5 min incubation in a boiling water bath. The product was analysed by measuring the optical density at 540 nm.

One unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing one µmol of reducing sugar per min under the defined reaction conditions. Specific activity was expressed as units per mg of protein (U/mg of protein). Protein concentrations were determined spectrophotometrically at 660 nm by method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Zymogram

The cellulose and xylan zymogram analyses were performed by using a 0.1% CMC or xylan (w/v) incorporated into the polyacrylamide. CMC or xylan was mixed with polyacrylamide during gel preparation. The gel for activity staining was incubated in 0.2 M acetate buffer, pH 5.0 at 45°C. The clearing zones that corresponded to enzyme activities were visualized using 0.5% (w/v) Congo red. The gel was de-stained with 1 M NaCl to visualize the clearing zone of hydrolysis. The gel was further exposed to 5% acetic acid to increase the colour contrast between the hydrolysis zone and the remaining portion of the gel (Chadha *et al*., 1999).

Purification strategy

All the purification procedure was carried out in the cold room (4°C). The crude extract of the termite salivary glands was loaded onto an anion-exchange chromatography using a DEAE-Sepharose Fast Flow column (2.5 cm x 4.5 cm), equilibrated with 20 mM sodium acetate buffer (pH 5.0). The column was washed at a flow rate of 90 mL/h with two bed volumes of equilibration buffer to remove unbound proteins. Bound proteins were then eluted with a stepwise salt gradient (0.1, 0.2, 0.4 and 2 M) of NaCl in 20 mM sodium acetate buffer (pH 5.0), and fractions of 2 mL were collected. One peak of enzyme possessing endo-xylanase/endo-cellulase activity was obtained and the active fractions were pooled. The pooled active fractions were loaded onto a cation-exchange chromatography using a CM-Sepharose CL-6B column (2.6 cm x 4.0 cm), equilibrated with 20 mM sodium acetate buffer (pH 5.0). The column was washed with the same buffer at a flow rate of 90 mL/h. endo-xylanase/endo-cellulase activity was eluted with a stepwise salt gradient (0.1, 0.2, 0.4 and 2 M) NaCl in 20 mM sodium acetate buffer (pH 5.0). Fractions of 2 mL were collected and, to the pooled active fractions, solid sodium thiosulphate was slowly added to give a final concentration of 1.7 M and the resulting enzyme solution was subsequently applied on a Phenyl Sepharose 6 Fast Flow column (1.5 cm x 3.2 cm) previously equilibrated with 20 mM sodium acetate buffer (pH 5.0) containing 1.7 M of sodium thiosulphate salt. The column was washed with a reverse stepwise gradient of sodium thiosulphate concentrations (from 1.7-0 M) dissolved in the same sodium acetate buffer at a flow rate of 78 mL/h and fractions of 1 mL were collected. The pooled active fractions were dialyzed overnight against 20 mM sodium acetate buffer (pH 5.0) and constituted the purified enzyme solution.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was carried out by using the Laemmli (1970) method on 10% (w/v) acrylamide gels under denaturing and non-denaturing conditions. Under denaturing conditions, samples were incubated for 5 min at 100°C with SDS-PAGE sample-buffer containing 2-mercaptoethanol. Under non-denaturing conditions, samples were mixed just before running in sample-buffer without 2-mercaptoethanol and SDS. Silver staining was used to localize protein bands (Blum *et al.,* 1987). The standard molecular weights (Bio-Rad) comprising myosin (209 kDa), β-galactosidase (124 kDa), carbonic anhydrase (34.8

kDa), BSA (80 kDa) and inhibiteur trypsique de soja (45.0 kDa) were used.

Native molecular weight determination

The purified enzyme was applied to gel filtration on a Sephacryl S-200 HR column (0.8 cm x 35 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0) to estimate the native molecular weight. Elution was done at a flow rate of 0.2 mL/min and fractions of 0.5 mL were collected. Standard molecular weights (SIGMA) used for calibration were beta-amylase (206 kDa), cellulase (26 kDa), bovine serum albumin (66.2 kDa), ovalbumine (45 kDa) and amyloglucosidase (63 kDa).

Effect of pH

The effect of pH on the activity of the purified enzyme was determined by performing the hydrolysis of xylan or carboxymethylcellulose in a serie of buffers (100 mM) at various pH values (3.6–10.0). Buffers used were sodium acetate (pH 3.6–5.6), phosphate citrate (2.6-7.0), glycin- NaOH (8.0 and 10.0) and sodium phosphate (5.6-8.0). pH values of each buffer were determined at 25°C.

Effect of temperature

The effect of temperature on purified enzyme activity was performed in 100 mM sodium acetate buffer (pH 5.0) over a temperature range of 30 to 80°C by using xylan or carboxymethylcellulose (0,5%) as substrate under the enzyme assay conditions.

Results

Identification of β-D-endoglycosidase

Zymogram analysis of the enzyme activity showed a single band of protein coincident with a positive stain for xylanase and cellulase activities .

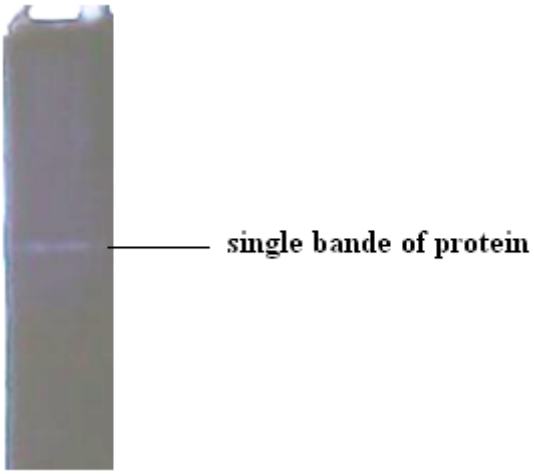

Fig 1. Zymogram of the active fraction using carboxyméthylcellulose as substrate Polyacrylamide gel electrophoresis in native

Enzyme purification

A single enzyme was purified from crude salivary gland extracts of the termite *Macrotermes subhyalinus* little soldier. The purification procedure involved three steps including anion-exchange and hydrophobic interaction chromatographiées as summarized in table 1. A single peak of activity was resolved on the anion exchange (DEAE-ANX-Sepharose4 Fast flow) chromatography used as the first step of purification (Fig. 2A). Active proteins showing xylanase and cellulase activities after this step were subjected to a cation exchange chromatography on a CM-Sepharose CL-6B column. A single peak showing xylanase and cellulase eluted with 0 M of NaCl (Fig. 2B). The enzyme was further purified by using an ultimate hydrophobic chromatography on a Phenyl-Sepharose CL-4B column. The active proteins showing xylanase and cellulase activities were eluted with 0.25 M of sodium thiosulfate (Fig. 2C). After purification, the specific activities towards carboxyméthylcellulose and xylan from Birchwood were 9.32±3.78 and 8.59±2.54 U/ mg of protein, respectly (Table 1).

Table 1. Purification procedure of a carboxymethylcellulase and xylanase from little soldier salivary glands of the termite *Macrotermes subhyalinus*.

Purification steps	Total protein (mg)	Total activity [a](Units)	Specific activity (Units/ mg)	Yield (%)	Purification Fold
Crude extract					
Carboxymethylcellulase	72.16±9.51	18.43±8.85	0.26±0.04	100	1
Xylanase	72.16±9.51	22.17±6.24	0.31±0.06	100	1
ANX-Sepharose 4 Fast-flow					
Carboxymethylcellulase	18.74±7.77	12.22±5.25	0.65±0.08	66.30±4.57	2.50±0.86
Xylanase	18.74±7.77	13.83±5.90	0.74±0.17	62.38±3.36	2.38±0.99
CM-Sepharose CL-6B					
Carboxymethylcellulase	1.02±2.53	6.44±6.84	6.31±1.81	34.94±2.55	24.26±1.60
Xylanase	1.02±2.53	7.32±4.21	6.42±2.07	33.02±1.87	20.71±1.45
Phenyl-Sepharose CL-4B					
Carboxymethylcellulase	0.34±0.06	3.17±1.26	9.32±3.78	17.20±3.45	35.85±2.67
Xylanase	0.34±0.06	2.92±1.49	8.59±2.54	13.17±2.62	27.70±1.75

a : One unit equals 1 µmol of reductor sugar release per min.

The enzyme showed a single protein band of silver staining polyacrylamide gel electrophoresis in native (Fig. 3A) and denaturing condition (Fig. 3B).

Molecular weight

After SDS-PAGE analysis under reducing conditions, the enzyme showed a single protein band and its relative molecular weight was estimated to be 215.45±5.63 kDa (Table 2). The relative molecular weight of the native glycosidase, as determined by gel filtration Sephacryl S-200 HR column, was approximately 223.56±4.10 kDa (Table 2).

pH and Temperature optima

The enzyme showed an optimum pH of 4.6 for cellulase activity an 5.0 for xylanase activity in acetate buffer (Figure 4A, B). The optimum temperature of the enzyme with CMC and xylan from Birchwood hydrolysis were found to be 60 and 55 °C respectively (figure 5). The temperature coefficient (Q_{10}) for cellulase activity as calculated between 50 and 60°C was around 1.41±0,05 from the Arrhenius plot, the activation energy was found to be 53.12±3.61 kJ/ mol (Table 2). Concerning xylanase activity, the value of temperature coefficient (Q_{10}) calculated between 45 and 55°C was 1.26±0.02 and the activation was found to be 22.12±1.74kJ/ mol (Table 2).

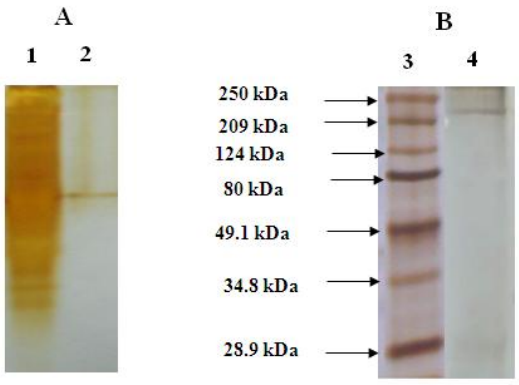

Fig 3. Polyacrylamide gel electrophoresis in native (A) and denaturing (B) conditions of the carboxyméthylcellulase and xylanase from the little soldier salivary glands of the termite *Macrotermes*

subhyalinus. Lanes 1 crude extract lane 2 and 4 purified enzyme, lane 3 molecular weight markers.

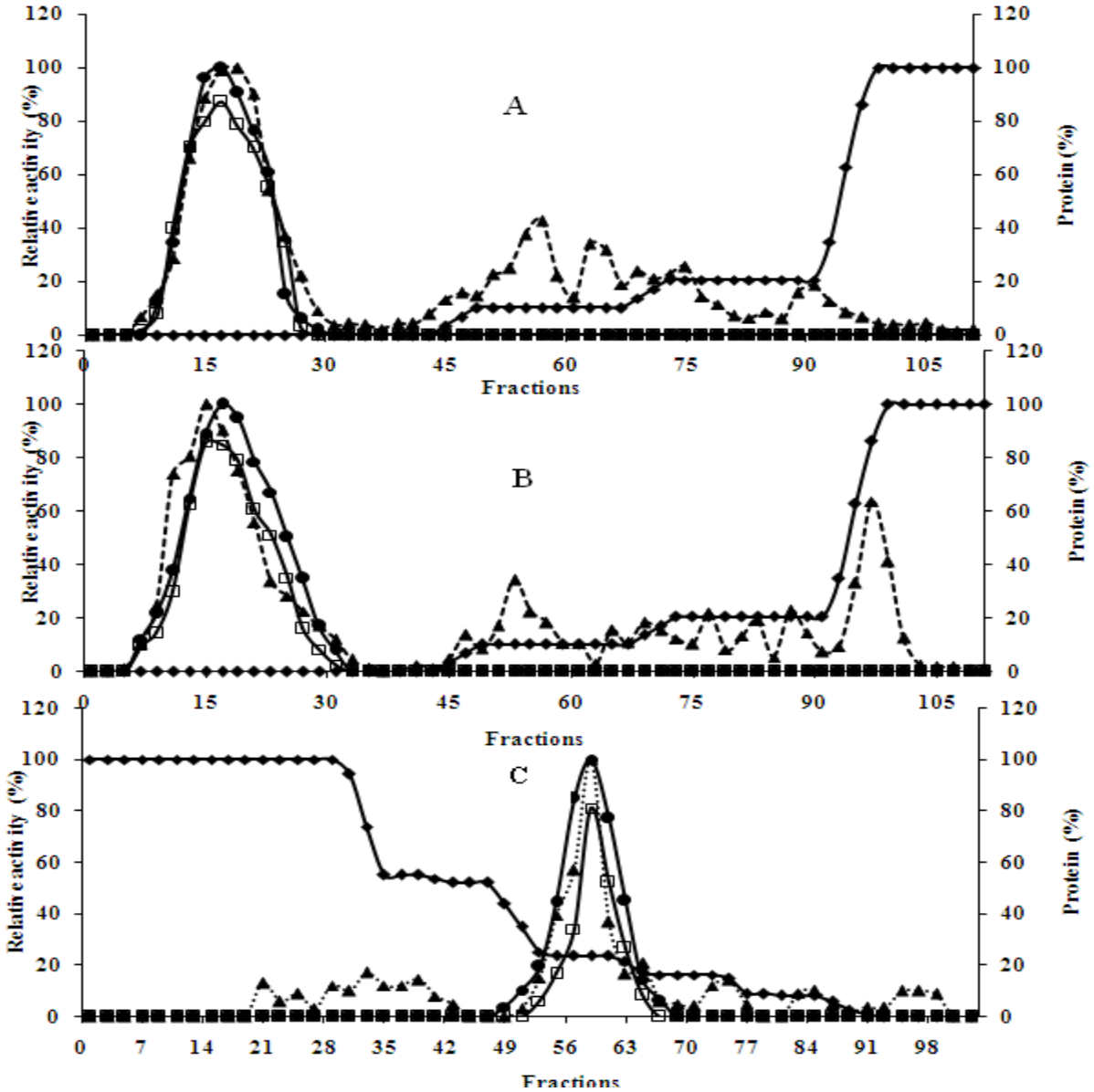

Fig 2. Purification profile of little soldier salivary glands of the termite *Macrotermes subhyalinus* carboxyméthylcellulase and xylanase. (A) Anion exchange chromatography on ANX-Sepharose 4 Fast Flow; (B) Cation exchange chromatography on CM-Sepharose CL-6B; (C) Gel hydrophobic chromatography on Phenyl-Sepharose CL-4B. Carboxymethylcellulase activity (■), xylanase activity (○), chloride sodium or sodium thiosulfate (♦), protein (▲)

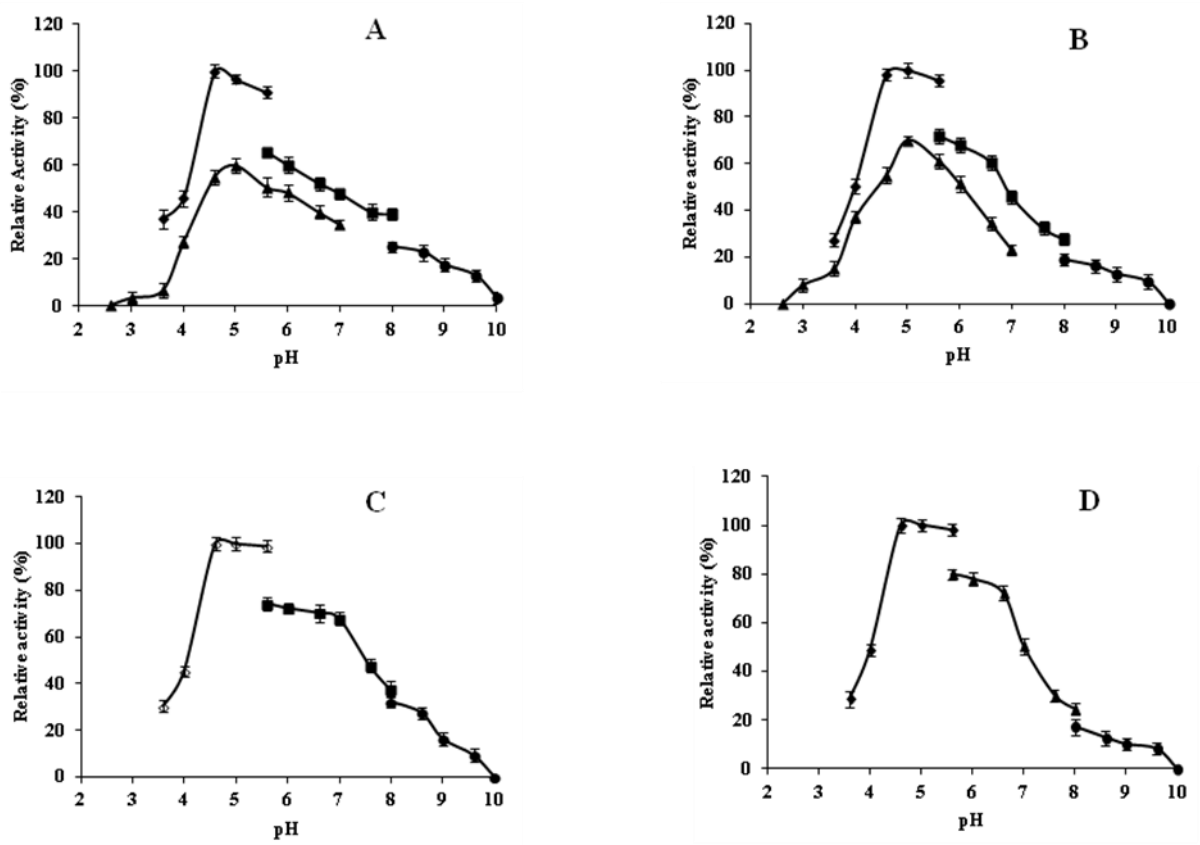

Fig 4. Effect of pH on the carboxymethylcellulase and xylanase from the little soldier salivary glands of the termite *Macrotermes subhyalinus*. (A) Optimum pH of carboxymethylcellulase activity; (B) Optimum pH of xylanase activity; (C); pH stability range. of carboxymethylcellulase activity; (D) pH stability range. of xylanase activity. Acetate buffer (♦), phosphate buffer (■), citrate-phosphate buffer (▲), glycin-NaOH buffer (●).

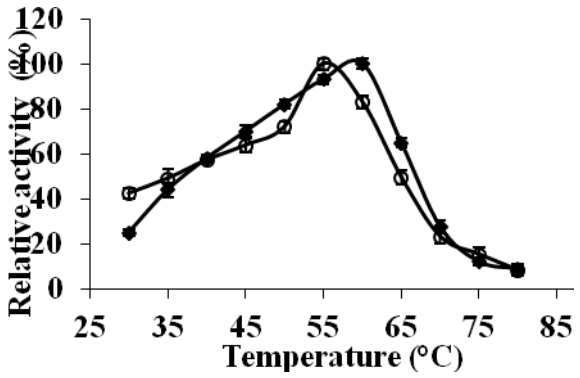

Fig 5: effect of temperature on the carboxymethylcellulase and xylanase from little soldier salivary glands of the termite *Macrotermes subhyalinus*. Carboxymethylcellulase activity (♦), Xylanase activity (○).

Discussion

Glycosidase from termite were largely studied with regard to their hydrolytic and transglycosylation activities in order to understand their important role in the breakdown of cellulose and hemicellulose in natural environments. However, no work concerning little soldier of termites *Macrotermes subhyalinus* was so far reported. A new enzyme with dual activity (carboxyméthylcellulase and xylanase) from little soldier salivary glands of the termite *M. Subhyalinus* was purified to homogeneity. The specific activities towards Beechwood xylan and carboxymethylcellulose of the purified enzyme are similar to those obtained for the three xylanases (Faulet *et al.,* 2006) and the two cellulases (Séa *et al.,* 2006) purified previously from the worker of the same termite.

The relative molecular weight of the purified enzyme was estimated to be 215.45 kDa and 223.56 kDa by SDS-PAGE and gel filtration on Sephacryl S-200 HR, respectively. This result, similar to beta-glucosidase of *Macrotermes bellicosus* (Binaté *et al*., 2008) suggests that the purified enzyme is a monomeric protein. A native PAGE analysis of the purified glycosidase sample showed a single band of protein correspond with zymogram analysis of the enzyme with dual activity.

Table 2. Some physicochemical characteristics of the carboxymethylcellulase and xylanase from little soldier salivary glands of the termite *Macrotermes subhyalinus*.

Physicochical properties	Carboxymethylcellulase activity	Xylanase activiy
Optimum pH	4.6	5.0
pH stability range	4.6 – 5.6	4.6 – 5.6
Optimum temperature (°C)	60	55
Activation energy (kJ / mol)	53.12±3.61	22.12±1.74
Temperature coefficient (Q10)	1.41±0.05	1.26±0.02.
Molecular weight (kDa)		
Mobility in SDS-PAGE[a]	215.45±5.63	
Gel filtration	223.56±4.10	

The different temperature and pH activity profiles determined for the purified enzyme with the substrates carboxyméthylcellulose and xylan from Birchwood suggest that this protein could have two active sites : one for each activity. This patterm seems to reflect the activity of the bifunctional polysaccharidases from *Cellulomonas flavigena* (Pérez-Avalos *et al.,* 2008) and *Macrotermes subhyalinus* (Blei *et al.,* 2010). The apparent bi-functional protein is significantly different from the termite *M. Subhyalinus* worker xylanases (Faulet *et al.,* 2006 a) and cellulases (Séa *et al*., 2006).

Conclusion

The present study finally showed that the purified enzyme form little soldier salivary glands of the termite *M. Subhyalinus* was a bifunctional polysaccharidase which was active in acid condition and possessing an endohydrolytic mode of action. It is a very stable enzyme mésophile in the plug acetate 20 mM with 37°C. The role of this enzyme in the digestive tract could be the hydrolysis of xylan (hemicellulose) and potentially cellulose. The weak hydrolytic activities of this enzyme could be due to the presence of defensive substances in salivary glands of the termite.

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