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A novel polysaccharidase with endo-beta-D-xylanase and endo-beta-D-glucanase activities in the gut of the major soldier of the termite *Macrotermes subhyalinus*.

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1 SUMMARY

A novel polysaccharidase from major soldier of the termite *Macrotermes subhyalinus* was purified to homogeneity by a three-step procedure consisting of ion-exchange, size-exclusion and hydrophobic interaction chromatographies in order to elucidate its contribution to the degradation of plant material. The only substrates that were hydrolyzed by the purified enzyme were xylans and carboxymethylcellulose. The specific activities towards carboxymethylcellulose and xylan from Birchwood were respectively 2.00 and 2.75 U/mg of protein. The molecular weight was measured to be 78.90 kDa by gel filtration and 76.95 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that the enzyme behaved as a monomer. The optimum temperatures of the enzyme were found to be 45°C using carboxymethylcellulose and 60°C using xylan from Birchwood as substrates which pointed out that this enzyme had separate sites for each activity. The pH-activity optimum was pH 5.0 for both substrates. The enzyme was capable of hydrolyzing both beta-1, 4-glucosidic and beta-1, 4-xylosidic bonds in cellulose and xylan respectively. Based on thin-layer chromatographic analysis of the degradation products, the cellulase activity produced cellobiose and cellodextrins from carboxymethylcellulose as the substrate. When xylan from Birchwood was used, end products were xylose, xylobiose and xylodextrins. The catalytic efficiency values for carboxymethylcellulose and xylan from Birchwood were respectively 13.60 and 31.74 U.ml/mg^2.
The polysaccharidase purified is an endo-beta-xylanase with endo-beta-glucanase activity. The enzyme catalyzed both hydrolysis and transglycosylation. It appears to be distinct from the other termite and *Termitomyces* sp cellulases and xylanases so far reported in terms of substrate specificity and low activity values against carboxymethylcellulose and xylans. The role of the purified enzyme in the digestive tract is the hydrolysis of amorphous cellulose and xylans from the plant material.

2 INTRODUCTION

Cellulose and hemicelluloses are the most abundant polysaccharides in nature. They build structural components of plant cell walls and are associated with lignin and other

polysaccharides (Hongpattarakere, 2002). Xylan is the main carbohydrate found in hemicelluloses. Its complete degradation requires actions of several types of endo-and exo-acting xylanases. These include beta-1, 4-endoxylanase (xylanases; EC 3.2.1.8), beta-1, 4-exoxylanase; alpha-L-arabinofuranosidase (EC 3.2.1.55), alpha-glucuronidase (EC 3.2.1), O-acetyl xylan esterase (EC 3.1.1.6), beta-1, 4-xylosidase (EC 3.2.1.37) (Wong *et al.*, 1988) and ferulic/*p*-coumaric acid esterases (Borneman *et al.*, 1991). In general, enzymic hydrolysis of cellulose requires the co-operative action of different enzymes, including endoglucanases (1, 4-beta-D-glucan glucanohydrolase, EC 3.2.1.4), which form free reducing chain ends and short-chain oligosaccharides by random cleavage of the beta (1-4)-bonds of cellulose, and exoglucanases(1,4-beta-D-glucan cellobiohydrolase, EC3.2.1.91). This form mainly cellobiose by endwise hydrolysis of the free reducing end of cellulose or cello-oligosaccharides. Another major component of activity is beta-glucosidase (beta-glucoside glucohydrolase, EC 3.2.1.21), which forms glucose by endwise cleavage of cello-oligosaccharides (Romaniec *et al.*, 1992).

Termites have developed cellulose and xylan digestion capabilities that allow them to obtain energy and nutrition from nutritionally poor food sources, such as plant material and residues derived from it (e.g., wood and humus) (Zhou *et al.*, 2007). The ability of the worker termites to hydrolyze these compounds has been the subject of various studies (Rouland *et al.*, 1988a, 1988b; Veivers *et al.*, 1991; Watanabe *et al.*, 1997; Kouamé *et al.*, 2005a, 2005b; Faulet *et al.*, 2006a, 2006b). However, little attention has been paid to these enzymes from soldier termites. Recent works reported the purification and characterization of a bifunctional endo-beta-D-glucanase/endo-beta-D-xylanase from the major soldier salivary glands of the termite *Macrotermes subhyalinus*. This enzyme is not able to degrade crystalline cellulose (Bléi, 2009, unpublished data). Does this pattern imply that the major soldier of this termite does not contain enzymes exhibiting exoglucanase and exoxylanase activities?

This question prompted this study to purify and investigate the characteristics of the enzymes exhibiting cellulase and xylanase activities found in the digestive tract from major soldiers of the termite *Macrotermes subhyalinus* in order to elucidate their contribution to the degradation of plant material and to understand more accurately the cellulose and xylan digestive mechanisms.

3 MATERIALS AND METHODS

3.1 Chemicals: Polysaccharides, oligosaccharides and *p*-nitrophenyl-glycopyranosides were purchased from Sigma Aldrich. ANX-Sepharose 4 Fast-Flow, Sephacryl S100 HR and Phenyl Sepharose CL-4B gels were obtained from Pharmacia-LKB Biotech. The chemicals used for polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. All other chemicals and reagents were of analytical grade.

3.2 Enzymatic source and preparation of crude extract: The major soldiers of the termite *Macrotermes subhyalinus* originated from the savannah of Lamto (Abidjan, Côte d'Ivoire). They were collected directly from their nests and then stored frozen at -20°C. These termites (10 g) were homogenized with 20 ml 0.9 % NaCl (w/v) solution in an Ultra-Turrax and then sonicated as previously described by Rouland *et al.* (1988a). The homogenate was centrifuged at 20000 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.*, 2005a).

3.3 Enzyme and protein assays: Under the standard test conditions, xylanase or cellulose activity was assayed spectrophotometrically by measuring the release of reducing sugars from Birchwood xylan or carboxymethylcellulose (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. Determination of other polysaccharidase activities was carried out under the same experimental conditions. The reference cell contained all reactants except the enzyme. After 30 min of

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incubation at 45°C, the reaction was terminated by adding 0.3 ml of dinitrosalicylic acid solution (Bernfeld, 1955) followed by 5 min incubation in a boiling water bath. The tubes were cooled to room temperature (25°C) for 10 min and 2 ml of distilled water was added. The product was analyzed by measuring the optical density at 540 nm.

The oligo-saccharidase activity was determined by measuring the amount of glucose or xylose liberated from oligosaccharide by incubation at 45°C for 30 min in a 20 mM acetate (pH 5.0), containing 10 mM oligosaccharide. The reference cell contained all reactants except the enzyme. The amount of glucose was determined by the glucose oxidase-peroxidase method (Kunst *et al*., 1984) after heating the reaction mixture at 100°C for 5 min. The hydrolysis of xylobiose was assayed by withdrawing aliquots (100 µl) which were heated at 100°C for 5 min. After filtration through a 0.45 µm hydrophilic Durapore membrane (millipore), the reaction mixture (20 µl) was analyzed quantitatively by HPLC at room temperature. Chromatographic separation of sugars (xylobiose and xylose) were performed on a Supelcosyl LC-NH_2 (5 µm) column (0.46 x 25 cm) from Supelco using acetonitrile/water (75: 25; v/v) as the eluent, and monitored by refractometric detection. The flow rate was maintained at 0.75 ml min^{-1} (Kouamé *et al*., 2001).

Enzymatic activity against the *p*-nitrophenyl-glycopyranoside was measured by the release of *p*-nitrophenol. An assay mixture (0.25 ml) consisting of a 20 mM acetate buffer (pH 5.0), 1.5 mM *p*-nitrophenyl-glycopyranoside and enzyme solution was incubated at 45°C for 10 min. The reference cell contained all reactants except the enzyme. The reaction was stopped by the addition of sodium carbonate (2 ml) at a concentration of 2 % (w/v) and absorbance of the reaction mixture was measured at 410 nm (Kouamé *et al*., 2005a; Yapi *et al*., 2007).

Protein concentrations were determined spectrophotometrically at 660 nm by method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

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).

3.4 Purification procedures: Fifteen (15) ml of crude extract was loaded onto a ANX-Sepharose 4 Fast-Flow (2.2 x 7.3 cm) that had been equilibrated previously with 20 mM acetate buffer pH 5.0. The unbound proteins were removed from the column by washing with 60 ml of the same buffer pH 5.0. The retained proteins were eluted with a gradient of NaCl (0-2 M). Fractions (2 ml each) were collected at a flow rate of 90 ml/h and assayed for enzyme activity. The fractions (retained proteins) possessing the highest endo-xylanase/endo-cellulase activity were pooled and saturated overnight by 80 % ammonium sulfate in a cold room. The precipitated pellet was then separated by centrifugation at 20000 x g for 30 min and dissolved in 1 ml of 20 mM acetate buffer pH 5.0. The enzyme solution was loaded directly into a Sephacryl S-100 HR (1.6 cm x 65 cm) which was pre-equilibrated with the same buffer pH 5.0. Proteins were eluted at a flow rate of 18 ml/h using 20 mM acetate buffer pH 5.0. Fractions of 1 ml were collected and active (endo-xylanase/endo-cellulase activity) fractions were pooled. The pooled fraction from the previous step was saturated to a final concentration of 1.7 M sodium thiosulfate and applied on a Phenyl-Sepharose CL-4B column (1.6 x 2.7 cm) previously equilibrated with 20 mM acetate buffer pH 5.0 containing 1.7 M sodium thiosulfate. The column was washed with equilibration buffer and the retained proteins were then eluted using a gradient with sodium thiosulfate (1.7-0 M). Fractions of 0.5 ml were collected at a flow rate of 78 ml/h and active fractions (endo-xylanase/endo-cellulase activity) were pooled. The pooled fraction was dialysed against 20 mM acetate buffer pH 5.0 overnight in a cold room.

3.5 Electrophoretic methods: To check purity and determine molecular weight, the purified enzyme was analyzed in native and denaturing conditions using polyacrylamide gel electrophoresis on a 10% separating gel and a 4% stacking gel (Hoefer mini-gel system; Hoefer Pharmacia Biotech, San Francisco, USA), according to the procedure of Laemmli (1970) at 10°C and constant current 20 mM. Proteins were stained with silver nitrate according to Blum *et al*. (1987). In denaturing conditions, the sample was denatured by a 5 min treatment at 100°C. Electrophoretic buffers were contained sodium dodecyl sulfate (SDS) and beta-mercaptoethanol. The molecular weight (M_w) of the purified enzyme was determined using the plot of log M_w of standard protein markers versus their relative mobility. In native conditions, the sample was not denatured by a 5 min treatment at 100°C,

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sodium dodecyl sulfate and beta-mercaptoethanol were not introduced in electrophoretic buffers.

3.6 Native molecular weight determination: The native molecular weight of the purified enzyme was determined using gel filtration on Sephacryl S 200 HR. The column Sephacryl S 100 HR (1.2 cm × 48 cm) equilibrated and eluted in 20 mM acetate buffer (pH 5.0) was calibrated with beta-amylase (206 kDa), cellulase (26 kDa), bovine serum albumin (66.2 kDa), ovalbumine (45 kDa) and amyloglucosidase (63 kDa). Fractions of 0.5 ml were collected at a flow rate of 10 ml/h. The M_w of the purified enzyme was determined using the plot of log M_w of standard protein markers versus their elution volume.

3.7 Temperature and pH optima: The purified enzyme activity, as a function of pH, was determined under standard conditions using various buffers in the pH buffering range 3.0-8.0: acetate (20 mM) buffer for pH range 3.6-5.6; citrate-phosphate (20 mM) buffer for pH range 3.0-7.0; phosphate (20 mM) buffer for pH range 5.6-8.0. The optimum pH for this enzyme was obtained using two substrates: carboxymethylcellulose and xylan from Birchwood (0.5%, w/v). The pH value corresponding to the highest enzyme activity was taken as the optimum pH. Optimum temperature was estimated under standard conditions using the cellulase or xylanase activity assay at temperatures between 30°C and 80°C. The temperature value corresponding to the highest enzyme activity was taken as the optimum temperature.

3.8 pH and temperature stabilities: The stability of the purified enzyme was followed over the pH range of 3.0 to 8.0 in 20 mM buffers. The buffers were the same as in the study of the pH and temperature optima (above). After 2 h incubation at 25°C, aliquots were taken and immediately assayed for residual xylanase or cellulase activity. The thermal stability of the purified enzyme was determined at 45 and 65°C after exposure to each temperature for a period from 30 to 360 min. The enzyme was incubated in 20 mM acetate buffer pH 5.0. Aliquots were drawn at intervals and immediately cooled in ice-cold water. Residual activities, determined in both cases at 45°C under the standard test conditions, are expressed as percentage activity of zero-time control of untreated enzyme.

3.9 Kinetic data analysis and substrate specificity: The specificity of the purified enzyme was determined by using a variety of commercial grade substrates (*p*-nitrophenyl-glycopyranoside, avicel, sigmacel 50-cellulose, carboxymethylcellulose, xylans, cellobiose, sucrose, xylobiose, inulin and starch) under standard conditions. The kinetic parameters (K_M, V_{max} and V_{max}/K_M) were determined in 20 mM acetate buffer (pH 5.0) at 45°C. Hydrolysis of xylans (Birchwood and Beechwood) or carboxymethylcellulose was quantified on the basis of released reducing sugars similarly as in the standard enzyme assay. K_M and V_{max} were determined from Lineweaver-Burk plot using different concentrations of xylan (2.0-10.50 mg/ml, w/v) and carboxymethylcellulose (2.0-10.50 mg/ml, w/v).

3.10 Thin-layer chromatography analysis of hydrolysate: The reaction mixture consisting of 0.1 ml of carboxymethylcellulose or xylan from Birchwood (0.5%, w/v) in 20 mM acetate buffer (pH 5.0) and 0.1 ml of enzyme was incubated at 45ºC. At definite intervals (30 min, 3 h, 6 h, 12 h, 24h), 0.05 ml aliquots were taken and the reaction was terminated by heating at 100°C for 5 min. Monosaccharides and oligosaccharides were analyzed by thin-layer chromatography on silica Gel G-60, using butanol/ethanol/water (3:5:2, v/v/v) as the mobile phase system. The bands were visualised with 3% (w/v) phenol in sulphuric acid/ ethanol (5-95, v/v).

3.11 Effect of chemical agents: The enzyme was incubated with 1 mM or 1% (w/v) of different chemical agents for 2 h at 25ºC (various cations in the form of chlorides). After incubation, the residual activity was determined by the standard enzyme assay using xylan from Birchwood or carboxymethylcellulose as a substrate. The activity of enzyme assayed in the absence of the chemical agents was taken as 100%.

4 RESULTS

4.1 Purification of enzyme: Xylan from Birchwood and carboxymethylcellulose were used as the substrates to monitor the enzymatic activity. The crude extract subjected to ANX-Sepharose 4 Fast-Flow chromatography showed two peaks (GX_1 and GX_2) of xylanase/cellulase activity (Figure 1A).

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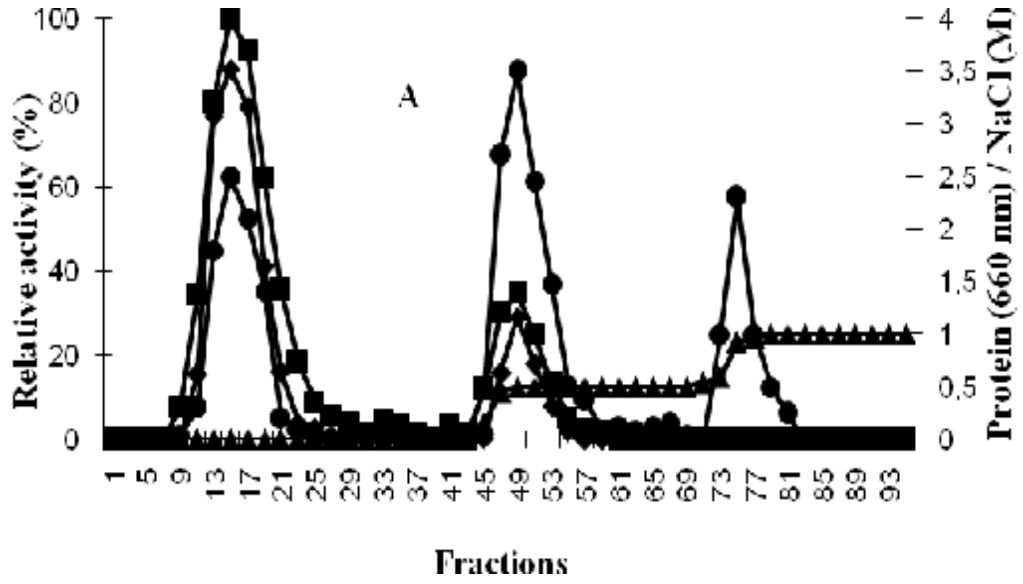

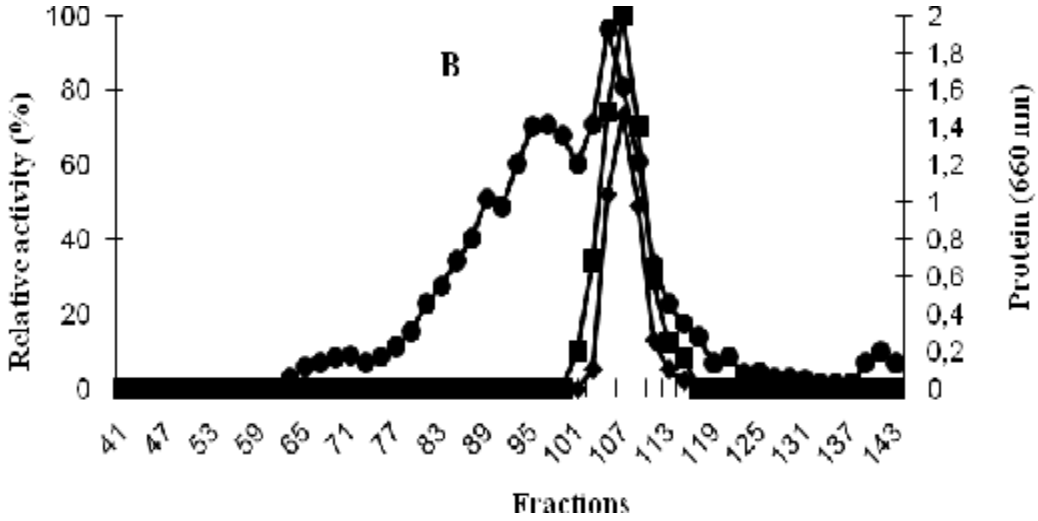

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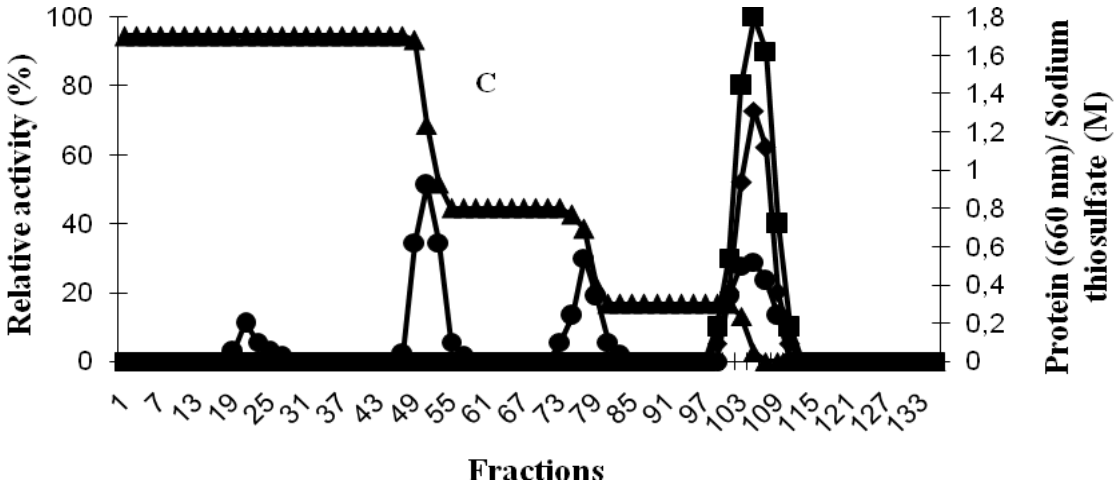

Figure 1: Purification profile of the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus*.
(A) Ion exchange chromatography (ANX-Sepharose 4 Fast Flow);
(B) Gel filtration chromatography (Sephacryl S-100 HR);
(C) Gel hydrophobic chromatography (Phenyl-Sepharose CL-4B).
Xylanase activity (■), cellulase activity (♦), chloride sodium or sodium thiosulfate (▲) and protein contents (●).

GX_1 was eluted in the pre-gradient fractions, while GX_2 was adsorbed to the ion exchange resin (Figure 1A). It was eluted at 0.5 M NaCl. Purification of GX_2 (pooled fractions 47 to 51) on Sephacryl S100 HR column showed one peak of protein containing xylanase/cellulase activity (Figure 1B). Pooled fractions (107 to 111) were further purified in a final step using hydrophobic interaction on Phenyl Sepharose CL-4B (Figure 1C). The enzyme (pooled fractions 103 to 107) was eluted at 0.2 M sodium thiosulfate. Table 1 indicates the degree of purification and yield for each step. The enzyme showed a single protein band by polyacrylamide gel electrophoresis in native and denaturing conditions (Figure 2).

Table 1: Purification of the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus*

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract					
Carboxymethylcellulase	340.0	15.42	0.05	100	1
Xylanase	340.0	38.03	0.11	100	1
ANX-Sepharose 4 Fast-Flow					
Carboxymethylcellulase	8.57	2.17	0.25	14.07	5.55
Xylanase	8.57	3.75	0.44	9.86	4.00
Sephacryl S-100 HR					
Carboxymethylcellulase	0.71	0.70	1.0	4.54	22
Xylanase	0.71	0.98	1.38	2.58	12.55
Phenyl-Sepharose CL-4B					
Carboxymethylcellulase	0.20	0.40	2.00	2.59	44.44
Xylanase	0.20	0.55	2.75	1.45	25.00

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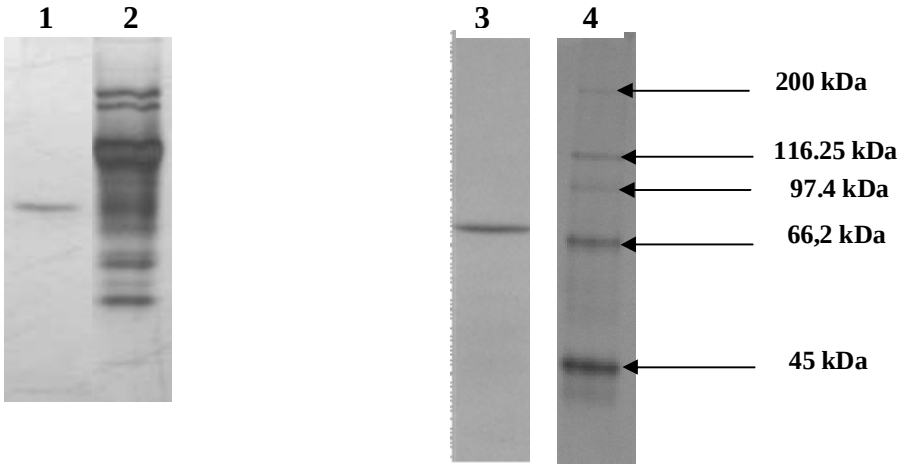

Figure 2: Polyacrylamide gel electrophoresis in native (A) and denaturing (B) conditions of the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus*. Lanes 1 and 3, purified enzyme; lane 2; crude extract; lane 4, molecular weight markers.

4.2 Molecular weight: From the migration pattern of the standard, the molecular weight was calculated to be 76.95 kDa. The molecular weight determined by gel filtration chromatography was 78.90 kDa (Table 2).

4.3 pH and temperature optima: The optimum pH value, for xylan from Birchwood and carboxymethylcellulose hydrolysis was 5.0 (Table 2).

Table 2: Some physicochemical characteristics of the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus*

Physicochemical properties	**Carboxymethylcellulase activity**	**Xylanase activity**
Optimum pH	5.0	5.0
pH stability range	4.6-6.0	4.6-5.6
Optimum temperature (°C)	45°C	60°C
Activation energy (kJ/mol/K)	49.82	26.97
Temperature coefficient (Q10)	1.91	1.41
Half life		
at 45°C (min)	180	-
at 60°C (min)	25	90
Michaelis Menten equation	obeyed	obeyed
Presence of transglycosylation activity	yes	yes
Mode of action	endo	endo
Molecular weight		
Mobility in SDS-PAGE[a]	76.95	
Gel filtration	78.90	

a = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The purified enzyme retained more than 60 % of its cellulase activity in the range pH 4.6 to 6.6 (Figure 3A). Concerning xylanase activity, the enzyme retained more than 60 % of its activity in the range pH 4.6 to 6.0 (Figure 3B).

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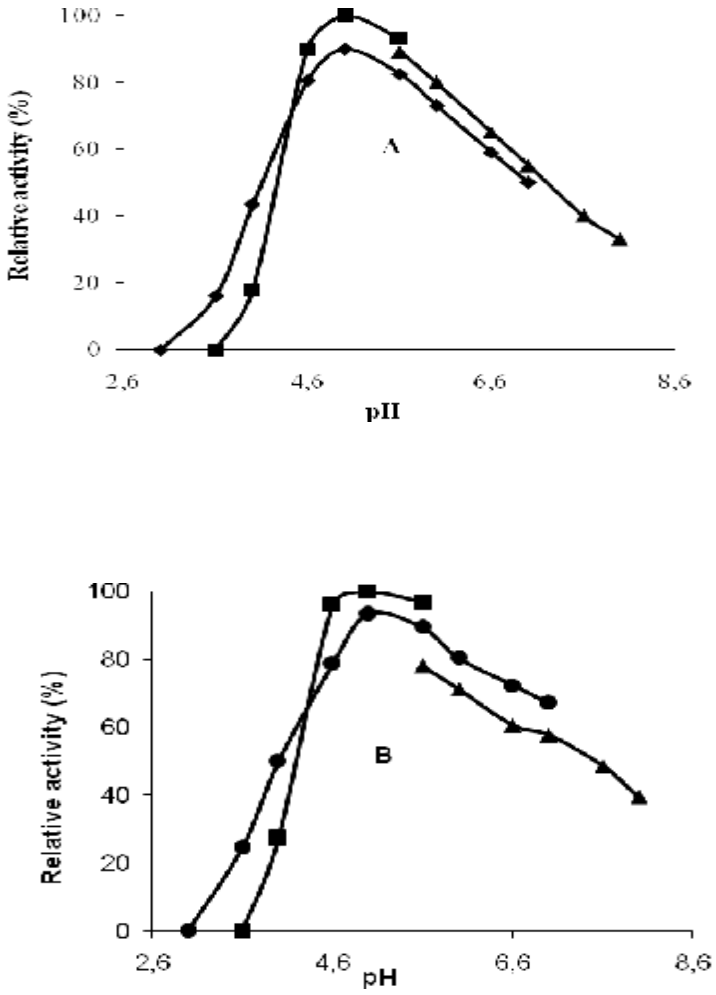

Figure 3: Effect of pH on the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus.*
Cellulase activity (A), xylanase activity (B), acetate buffer (■), citrate-phosphate buffer (●) and phosphate buffer (▲).

The optimum temperatures of the enzyme with xylan from Birchwood and carboxymethylcellulose hydrolysis were found to be respectively 60 and 45°C (Table 2). The enzyme retained more than 70 % of its cellulase activity in the range 40°C to 50°C (Figure 4). The value of the temperature coefficient (Q_{10}) calculated between 30°C and 40°C was 1.91 (Table 2). From the Arrhenius plot, the activation energy was found to be 49.82 KJ/mol (Table 2). Concerning xylanase activity, the enzyme retained more than 70 % of its activity in the range 50 to 65°C (Figure 4). The value of the temperature coefficient (Q_{10}) calculated between 45°C and 55°C was 1.41 and the activation energy was found to be 26.97 KJ/mol (Table 2).

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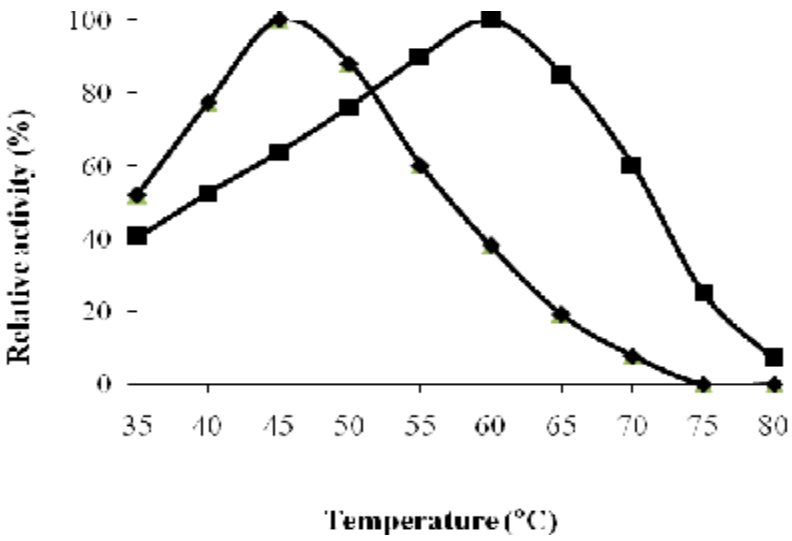

Figure 4: Effect of temperature on the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus*. Xylanase activity (■) and cellulase activity (♦)

4.4 pH and temperature stabilities: At 25°C, the carboxymethylcellulase activity of the enzyme was stable over a wide pH range of 4.6-6.0 for 120 min (Table 2). The same activity was fully stable for 30 min at 45°C in 20 mM acetate buffer pH 5.0. At 60°C, the half life of the carboxymethylcellulase activity was 25 min (Figure 5). Concerning xylanase activity, the enzyme was stable over a wide pH range of 4.6-5.6 for 120 min (Table 2). At 60°C, this activity was fully stable for 30 min in 20 mM acetate buffer pH 5.0, but at 45°C, it was stable for 360 min (Figure 5). At 60°C, the half life of the xylanase activity was 90 min (Table 2).

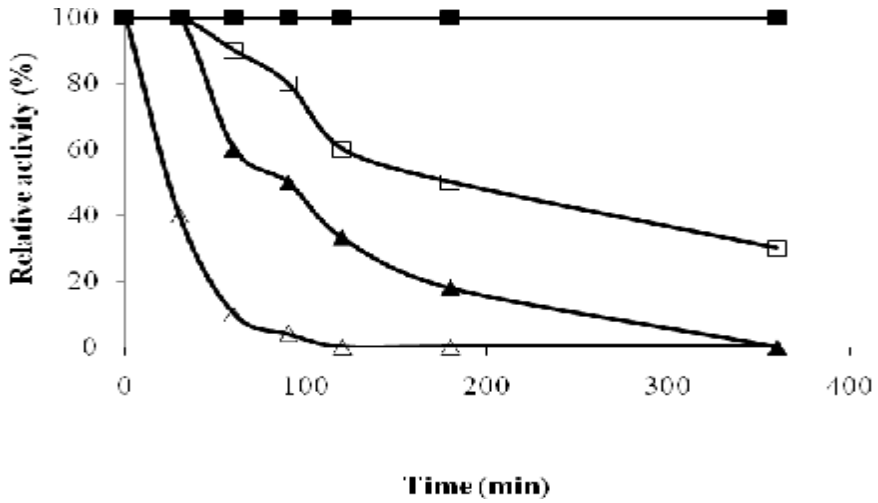

Figure 5: Thermal stability of the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus*. Xylanase activity 45°C (■), cellulase activity at 45°C (□), xylanase activity at 60°C (▲), cellulase activity at 60°C (Δ), xylanase activity at 65°C (●) and cellulase activity at 65°C (○).

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4.5 Substrate specificity and kinetic parameters: The enzyme purified did not attack *p*-nitrophenyl glycosides, cellobiose, sucrose, xylobiose, avicel, sigmacel 50-cellulose, inulin and starch (Table 3). Although, the enzyme degraded carboxymethylcellulose and xylans (Beechwood and Birchwood) (Table 3). The greatest activity was detected with xylans followed by carboxymethylcellulose. The effect of substrate concentration on enzymatic activity was studied with carboxymethylcellulose and xylans (Beechwood and Birchwood). With these substrates, the enzyme obeyed the Michaelis-Menten equation (Table 2). The apparent K_M and V_{max} values for carboxymethyl cellulase and xylanase activities were 0.52 and 7.04 mg/ml and 0.54 and 17.12 IU/mg respectively, according to Lineweaver–Burk plots (Table 4). The catalytic efficiency of the enzyme, given by the V_{max}/K_M ratio is much higher for the xylans than the carboxymethylcellulose (Table 4).

Table 3: Activities of the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus* on synthetic chromogenic, oligosaccharide and polysaccharide substrates.

Substrate	**Concentration in assay**	**Relative rate of hydrolysis (%)**
Carboxymethylcellulose	2.6 mg/ml	100
Xylan (Birchwood xylan)	2.6 mg/ml	137.50
Xylan (Beechwood xylan)	2.6 mg/ml	118.37
Avicel	2.6 mg/ml	0
Sigmacel 50-cellulose	2.6 mg/ml	0
Inulin	2.6 mg/ml	0
Starch	2.6 mg/ml	0
Sucrose	10 mM	0
Cellobiose	10 mM	0
Xylobiose	10 mM	0
p-Nitrophenyl-glycopyranoside	1.5 mM	0

Table 4: Kinetic parameters of the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus* towards carboxymethylcellulose, xylan from Birchwood and xylan from Beechwood

Substrate	**K_M (mg/ml)**	**V_{max} (U/mg)**	**V_{max}/K_M (Uxml/mg^2)**
Carboxymethylcellulose	0.52	7.04	13.60
Xylan from Birchwood	0.54	17.12	31.74
Xylan from Beechwood	0.45	13.30	29.56

4.6 Thin-layer chromatography analysis of hydrolysate: Within the first 12 h of the reaction, the enzyme hydrolyzed carboxymethylcellulose to cellobiose and cellodextrins and subsequently degraded xylan from Birchwood to accumulate xylose, xylobiose and xylodextrins. After 12 h of incubation, the hydrolysis products of carboxymethylcellulose such as cellobiose disappeared in the reaction (Figure 6.

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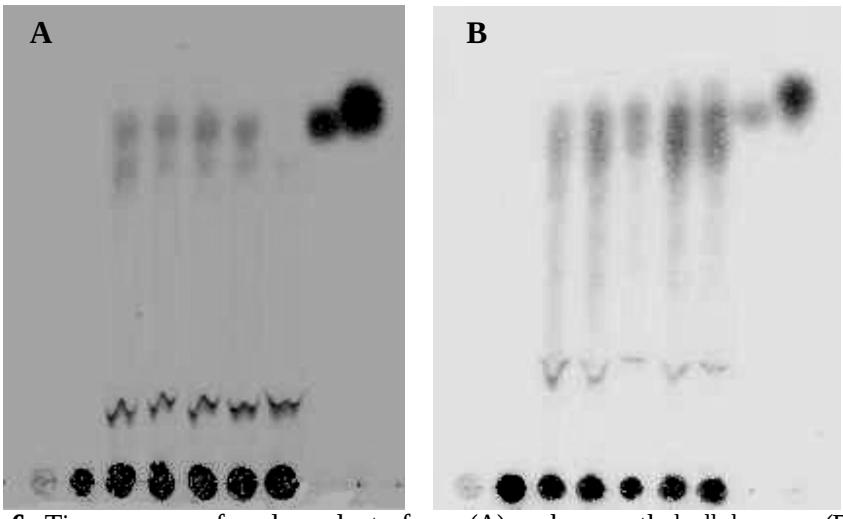

Figure 6: Time course of end products from (A) carboxymethylcellulose or (B) xylan from Birchwood hydrolysis for the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus.* G_1= glucose, G_2= cellobiose, X_1= xylose, X_2 = xylobiose, 0_1= enzyme, $0_{2=}$ substrate (xylan from Birchwood or carboxymethylcellulose)

4.7 Effect of chemical agents on enzyme activity: SDS showed an inhibitory effect on the purified enzyme. NaCl, KCl, EDTA, *p*CMB and DTNB had no effect on the same enzyme activity (Table 5). However, it was activated by $FeCl_2$, $CuCl_2$, $CaCl_2$ and $BaCl_2$ (Table 5).

Table 5: Effect of chemical agents on the 76.95 kDa polysaccharidase from the major soldier of the termite *Macrotermes subhyalinus*

Chemical agents	Concentration in assay	Relative activity (%)	
		Carboxymethyl cellulase activity	Xylanase activity
Control	0	100	100
$ZnCl_2$	1	100	73.9
$MgCl_2$	1	147.37	100
$BaCl_2$	1	147.63	167.39
$CaCl_2$	1	100	173.91
$CuCl_2$	1	178.20	182.61
$FeCl_2$	1	121.05	113.04
KCl	1	100	100
NaCl	1	100	100
EDTA[b] (%, w/v)	1	100	100
DTNB[c] (%, w/v)	1	100	82.40
*p*CMB[d] (%, w/v)	1	100	100
SDS[e] (%, w/v)	1	0	0

b = sodium ethylendiamintetraacetate, **c** = 5,5-dithio-bis(2-nitrobenzoate), **d** = *p*-chloromercuribenzoate, **e** = sodium dodecyl sulfate

5 DISCUSSION

The whole body extract from a major soldier of the termite *Macrotermes subhyalinus* subjected to ANX-Sepharose 4 Fast-Flow chromatography showed two peaks (GX_1 and GX_2) of xylanase/ cellulase activity. GX_1 purified to homogeneity had been characterized. Its biochemical and catalytic

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properties are similar to those of a bifunctional endo-beta-glucanase/endo-beta-xylanase from the salivary glands of the same termite (Bléi, 2009, unpublished data). GX_2, the novel polysaccharidase, was purified by a three-step procedure consisting of ion-exchange, size-exclusion and hydrophobic interaction chromatographies. The preparation showed only one band in sodium dodecyl sulfate polyacrylamide gel electrophoresis and in polyacrylamide gel electrophoresis. The activity of the purified enzyme markedly decreased in either acidic or alkaline pH ranges. The unfolding of the enzyme molecules might occur at extreme acidic or alkaline pH owing to the decrease in electrostatic bonds leading to the loss in its activity. The result was in accordance with Zverlov *et al*. (1998) who reported that the optimum pH of carboxymethylcellulose hydrolysis from the extreme thermophile *Anaerocellum thermophilum* was 5.0. The optimum temperatures of the enzyme were found to be 45°C using carboxymethylcellulose and 60°C using xylan from Birchwood as substrates. This phenomenon may be attributed to the presence of two separate catalytic domains with presumably different properties. This is in line with the bifunctional polysaccharidases from *Ruminococcus flavefaciens* (Flint *et al*., 1993) and *Cellulomonas flavigena* (Pe´rez-Avalos *et al*., 2008).

The only substrates that were hydrolyzed by the polysaccharidase were xylans (Birchwood and Beechwood) and carboxymethylcellulose. This result suggests that the enzyme is a polysaccharidase with a high specificity for the beta-anomeric configuration of the glucosidic and xylosidic linkages. This pattern seems to reflect the bifunctional polysaccharidases from *Ruminococcus flavefaciens* (Flint *et al*., 1993), *Cellulomonas flavigena* (Pe´rez-Avalos *et al*., 2008) and the symbiotic fungus *Termitomyces sp* of the termite *Macrotermes subhyalinus* worker (Faulet *et al*., 2006b). The polysaccharidase is significantly different from the *Trinervitermes trinervoides* (Potts and Hewitt, 1974), *Reticulitermes sperutus* (Watanabe *et al*., 1997), *Coptotermes fornosunus* (Nakashima *et al*., 2002), *Odontotermes formosanus* (Yang *et al*., 2004) and *Macrotermes subhyalinus* (Faulet *et al*., 2006b) workers xylanases and cellulases. The resultant findings confirm the previously held view that xylanases can either show narrow substrate specificities (Gilbert *et al*., 1988) or are capable of hydrolyzing cellulosic substrates in addition to xylan.

The purified enzyme was not able to attack the microcrystalline cellulose substrate. This observation is in close agreement to reports of Munoz *et al*. (2001) who reported that endoglucanases are more active against amorphous cellulose while exoglucanases are more active against crystalline cellulose. Similarly, Wood (1989) had reported that it is generally agreed that synergism between endoglucanases and exoglucanases during cellulose hydrolysis is apparent when crystalline cellulose is the substrate, but is less significant when the enzymes are cleaving amorphous or soluble forms of the polysaccharide. This studies observation is in contrast to Cohen *et al*. (2005), who reported that the brown rot basidiomycete *Gloeophyllum trabeum* produced an endoglucanase Cel5A capable of yielding cellobiose from crystalline cellulose (Avicel).

The catalytic efficiency of the enzyme, given by the V_{max}/K_M ratio is much higher for the xylans than the carboxymethylcellulose. The polysaccharidase purified is an endo-beta-xylanase with endo-beta-D-glucanase activity. The physiological role of the purified enzyme is the digestion of amorphous cellulose and xylans from the plant material. This pattern seems to imply that the gut from major soldier of the termite *Macrotermes subhyalinus* does not contain enzymes exhibiting exoglucanase and exoxylanase activities. The absence of exoglucanase in termites might be explained by the presence of mandibles which physically crush wood into small particles ranging from 20 to 50 μm (Yoshimura *et al*., 1996) or to less than 50 μm (Itakura *et al*., 1995) in size. This could effectively allow the cellulolytic enzymes easy access to substrates due to the increase in accessible surface area. This could therefore compensate for the absence of the crystalline cellulose-attacking ability of cellobiohydrolase (exoglucanase) (Watanabe and Tokuda, 2001). Within the first 12 h of the reaction, the hydrolysis of the two substrates carboxymethyl cellulose and xylan (Birchwood) produced the expected oligosaccharides, disaccharides and monosaccharides from them. These results indicate that the purified enzyme randomly cleaved internal beta-1,4-glucosidic and beta-1,4-xylosidic bonds in these substrates respectively as a bifunctional polysaccharidase possessing endoglucanase and endoxylanase activities.

After 12 h of incubation, the hydrolysis products of carboxymethylcellulose such as glucose and cellobiose disappeared in the reaction. It seems that

the transglycosylation reaction occurred with the hydrolysis carboxymethylcellulose, judging from the fact that the polysaccharidase can transfer part of the monosaccharide-based carboxymethylcellulose to monosaccharide-based oligosaccharides. The rate of transglycosylation product formation was largely favored relative to the rate of hydrolysis.

There are some polysaccharidases known to have dual transglycosylation and hydrolysis activities. The XTH enzyme, which cuts the glucan backbone of xyloglucan and either attaches the newly created reducing end to xyloglucan derived oligosaccharides (xyloglucan endotransglycosylase activity; XET), or to water (xyloglucan endohydrolase; XEH) (Rose *et al*., 2002).

The specific activities towards carboxymethylcellulose, Birchwood xylan and Beechwood xylan are considerably lower than those obtained for the three xylanases (Faulet *et al*., 2006a, 2006b) and the two cellulases (Séa *et al*., 2006) purified previously from worker of the same termite. These observations give an indication of how trophallaxis can be efficiently maintained within a termite colony. The termite *Macrotermes subhyalinus* worker could regurgitate its own salivary glands secretions to supplement the digestive needs of the soldier that live in close association with the worker, receiving these liquids by trophallaxis. It is possible that this natural phenomenon is done essentially to complete the soldier enzymatic activities in its digestive tract.

The relative molecular weight of the native enzyme was calculated to be approximately equal to 78.90 kDa when estimated by means of Sephacryl S200 HR. On sodium dodecyl sulfate polyacrylamide gel electrophoresis, under completely denaturing conditions, the purified enzyme features a single band corresponding to a molecular weight of 76.95 kDa. This result would suggest that, in contrast to the cellulases and xylanases obtained from workers (Matoub and Rouland, 1995; Séa *et al*., 2006; Faulet *et al*., 2006a, 2006b) of the termites *Macrotermes subhyalinus*, the purified enzyme is a monomeric protein.

6 CONCLUSION

It can be concluded from the present study that the major soldier of the termite *Macrotermes subhyalinus* apparently produce an endo-beta-xylanase with dual activity against carboxymethyl cellulose. The apparent role of this enzyme in the digestive tract is the hydrolysis of xylan (hemicellulose) and amorphous cellulose. This protein possesses two distinct catalytic sites and low enzymatic activities. The enzyme catalyzed both hydrolysis and transglycosylation. This protein is significantly different from the worker of the termite *Macrotermes subhyalinus* xylanases and cellulases. The major soldier of this termite does not contain enzymes exhibiting exoglucanase and exoxylanase activities. This study hypothesizes that the low activity is one of the causes of the trophallaxis phenomenon.

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