Full Length Research Paper

Purification and characterization of an endo-beta-D-xylanase from major soldier salivary glands of the termite *Macrotermes subhyalinus* with dual activity against carboxymethylcellulose

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An enzyme with apparent dual functions as a xylanase and carboxymethylcellulase was purified from the major soldier salivary glands of the termite *Macrotermes subhyalinus*. The preparation was found homogeneous by gel electrophoresis after successive chromatography on anion-exchange, cation-exchange and hydrophobic interaction columns. The specific activities towards carboxymethylcellulose and xylan were respectively 3.59 and 5.68 U/mg of protein. The molecular weight was measured to be 57.12 kDa by gel filtration and 14.47 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme showed a pH optimum of 5.6 for carboxymethylcellulase activity and 5.0 for xylanase activity. The optimum temperature for carboxymethylcellulase and xylanase activities was respectively 60 and 65°C. The enzyme was capable of hydrolyzing both beta-1,4-glycosidic and beta-1,4-xylosidic bonds in carboxymethylcellulose and xylans, respectively. Based on thin-layer chromatographic analysis of the degradation products, the carboxymethylcellulase activity produced glucose, cellobiose and cellodextrins from carboxymethylcellulose as the substrate. When xylan from Birchwood was used, end products were xylobiose and xylodextrins. The salivary glands of *M. subhyalinus* soldier apparently produce an endo-beta-xylanase with dual activity against carboxymethylcellulose. The apparent role of this enzyme in the digestive tract is the hydrolysis of xylan and potentially cellulose.

Key words: Endo-beta-D-glucanase, endo-beta-D-xylanase, major soldier, physiological role, salivary glands, termite *Macrotermes subhyalinus*.

INTRODUCTION

Lignocellulose is the most abundant organic material on earth, representing 50% of the total plant biomass and presenting an estimated annual production of 5×10^{10} tons (Rajarathnam and Bano, 1989). It consists of three types of polymers, cellulose, hemicellulose and lignin that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross linkages (Pérez et al., 2002).

The lignin is rather difficult to biodegrade and reduces the availability of the other polymers by means of a physical restriction (Ladisch et al., 1983). Hemicelluloses are biodegraded to monomeric sugars and acetic acid. Xylan is the main carbohydrate found in hemicellulose. Its complete degradation requires orchestrated actions of various enzymes including endo-xylanase, beta-D-xylosidase, alpha-glucuronidase, acetyl esterase and alpha-L-arabinofuranosidase (Beg et al., 2001). Endo-xylanase randomly hydrolyzes the xylan backbone, while beta-D-xylosidase converts xylobiose and xylo-oligosac-charide into xylose (Polizeli et al., 2005). In addition, alpha-L-arabinofuranosidase, alpha-D-glucuronidase, acetyl

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xylan esterases, ferulic-acid esterases, and *p*-coumaric-acid esterases are required for the removal of the side chains (Subramaniyan and Prema, 2002). Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific (Béguin and Aubert, 1994). These include several endo-1-4-beta-glucanase, exo-1-4-beta-glucanase and beta-glucosidase. The former two enzymes can degrade native cellulose synergistically to generate cellobiose which is a product inhibitor for these enzymes (Bhat and Bhat, 1997). Beta-Glucosidase plays an important role of scavenging the end product cellobiose by cleaving the beta (1 - 4) linkage to generate D-glucose and also in the regulation of exo and endo-cellulases synthesis. Furthermore, when a beta-glucosidase preparation is added to lignocellulosic materials, it plays a major role in release of phenolic compounds, suggesting that cellulose degrading enzymes may also be involved to facilitate the breakdown of polymeric phenolic matrices (Zheng and Shetty, 2000). These enzymes are widely spread in nature, predominantly being produced by micro organisms such as molds, fungi, bacteria (Bayer et al., 1998) and insects (Martin and Martin, 1978, 1979). Cellulases and xylanases from termite workers have been characterized extensively (Rouland et al., 1988a, b; Veivers et al., 1991; Kouamé et al., 2005a, b; Faulet et al., 2006a, b). However, little attention has been paid to enzymes from termite soldiers. Therefore, no data have been published on purification and characterization of termite major soldier enzymes. Here we report for the first time, the purification and characterization of an endo-beta-D-xylanase from major soldier salivary glands of the termite *Macrotermes subhyalinus* with dual activity against carboxy-methylcellulose. This was done in order to elucidate it role in the digestive tract and increase our knowledge on trophallaxis phenomenon.

MATERIALS AND METHODS

Chemicals

Polysaccharides, oligosaccharides and *p*-nitrophenyl-glyco-pyranosides 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. The chemicals used for polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. All other chemicals and reagents were of analytical grade.

Enzymatic source and preparation of crude extract

The major soldier of the termite *M. subhyalinus* originated from the savanna of Lamto (Abidjan, Côte d'Ivoire). They were collected directly from their nests and then stored frozen at -20°C. Salivary glands (9 g) were dissected and 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).

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 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 for 10 min and 2 ml of distilled water was added. The product was analysed by measuring the optical density at 540 nm.

The disaccharidase activity was determined by measuring the amount of glucose or xylose liberated from disaccharide by incubation at 45°C for 30 min in a 20 mM acetate (pH 5.0), containing 10 mM disaccharide. 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 analysed 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).

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.

Purification procedures

Fifteen (15) ml of crude extract was loaded onto an 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 two column volumes 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 containing the highest xylanase and cellulase activities were pooled and submitted to cation-exchange chromatography in a CM-Sepharose CL-6B column (1.6 x 4.0 cm) equilibrated with 20 mM acetate buffer pH 5.0 at a flow rate of 72

ml/h. The column was washed with the same buffer and eluted with a gradient of NaCl (0 - 2 M). Fractions of 0.5 ml were collected and active fractions were pooled together. 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 M). Fractions of 0.5 ml were collected at a flow rate of 78 ml/h and active fractions (cellulase and xylanase activities) were pooled. The pooled fraction was dialysed against 20 mM acetate buffer pH 5.0 overnight in a cold room.

Electrophoretic methods

To check purity and determine molecular weight, the purified enzyme was analysed 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 sodium dodecyl sulfate and beta-mercaptoethanol were not used. The sample was not heated.

Native molecular weight determination

The native molecular weight of the enzyme was determined using gel filtration on Sephacryl S200 HR. The column Sephacryl S200 HR (1.2 × 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.

Temperature and pH optima

Optimum pH was estimated using the cellulase (CMC) or xylanase activity (Birchwood xylan) assay over a pH range between 3.0 and 8.0: acetate (20 mM) buffer for pH range 3.6 to 5.6; citrate-phosphate (20 mM) buffer for pH range 3.0 to 7.0; phosphate (20 mM) buffer for pH range 5.6 - 8.0. Optimum temperature was estimated using the cellulase or xylanase activity assay at temperatures between 30 and 80°C.

pH and temperature stabilities

The stability of the 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 enzyme was determined at 45, 60 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.

Determination of kinetic parameters

The kinetic parameters (K_M, V_{max} and k_{cat}/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 to 10.50 mg/ml, w/v).

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 and 24 h), 0.05 ml aliquots were taken and the reaction was terminated by heating at 100°C for 5 min. Mono and oligosaccharides were analyzed by thin-layer chromatography on silica Gel G-60, using butanol/ethanol/water (3,5,2 v) as the mobile phase system. The bands were visualised with 3% (w/v) phenol in sulphuric acid/ ethanol (5 - 95, v).

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 CMC as a substrate. The activity of enzyme assayed in the absence of the chemical agents was taken as 100%.

RESULTS

Purification of enzyme

A single enzyme was purified from crude salivary gland extracts prepared from major soldier of the termite *M. subhyalinus*. Xylan from Birchwood and CMC were used as the major substrates to monitor enzymatic activity. The purification protocol involves three steps of column chromatography: anion-exchange, cation-exchange and hydrophobic interaction. A single peak of activity was eluted from ANX-Sepharose 4 Fast-Flow (Figure 1A). Pooled fractions (13 to 18) showing xylanase and cellulase activities after this first step were subjected to a cation-exchange chromatography on a CM-Sepharose CL-6B. One peak showing xylanase and cellulase activities were resolved in this step (Figure 1B). The enzyme (pooled fractions 16 to 22) was further purified in a final step using hydrophobic interaction on Phenyl Sepharose CL-4B (Figure 1C). After purification, the
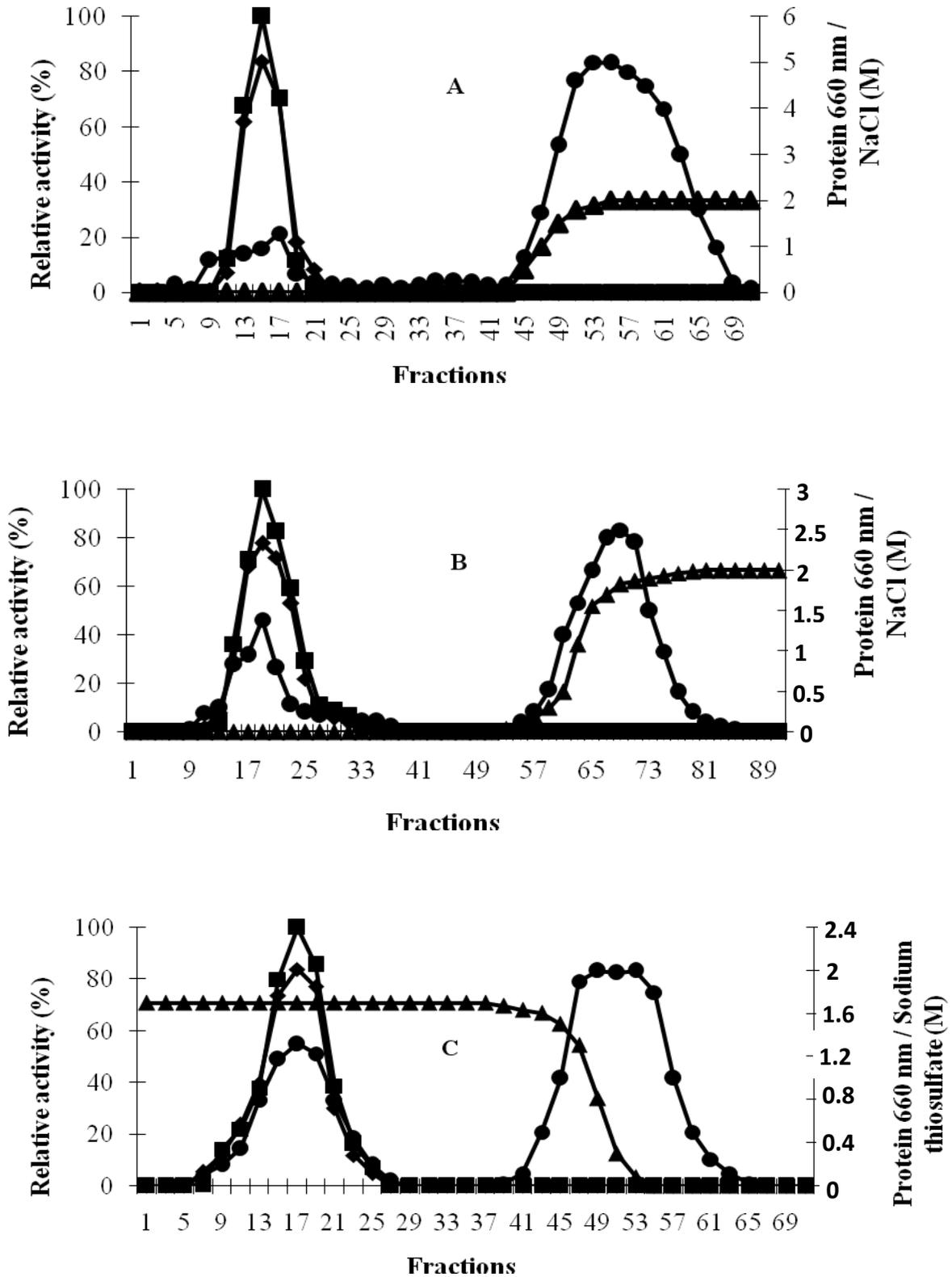
Figure 1. Purification profile of an endo-beta-D-xylanase from major soldier salivary glands of the termite *M. subhyalinus* with dual activity against carboxymethylcellulose. (A) Ion exchange chromatography (ANX-Sepharose 4 Fast Flow); (B) Ion exchange chromatography (CM-Sepharose CL 6B); (C) Gel hydrophobic chromatography (Phenyl-Sepharose CL-4B). Xylanase activity (■), cellulase activity (♦), chloride sodium or sodium thiosulfate (▲) and protein contents (●).
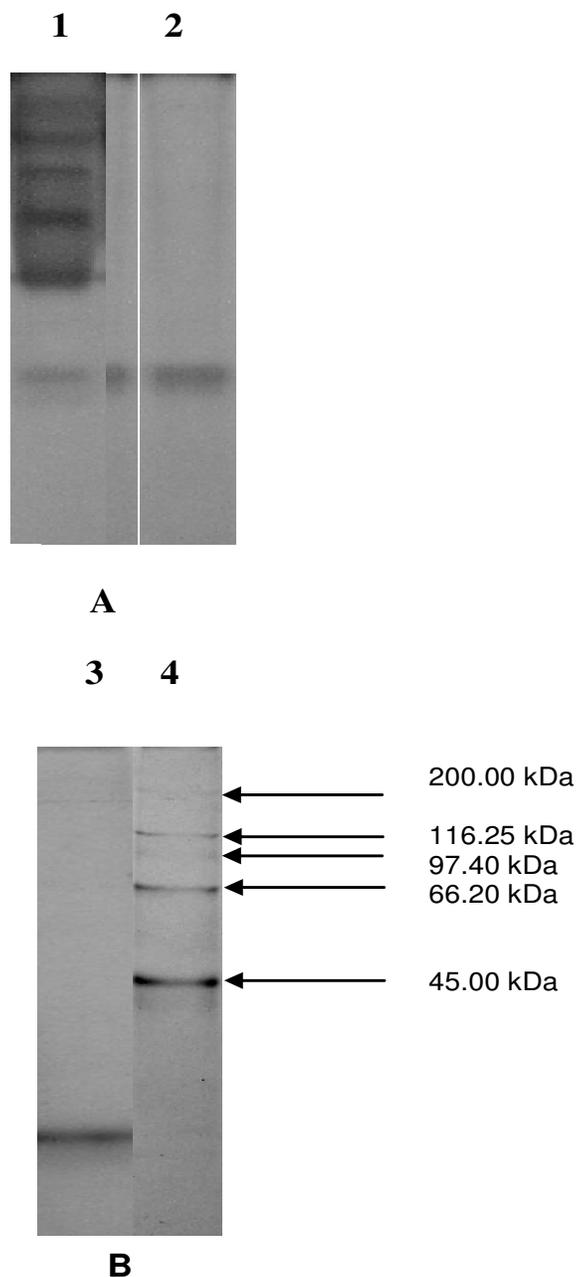
Figure 2. Polyacrylamide gel electrophoresis in native (A) and denaturing (B) conditions of an endo-beta-D-xylanase from major soldier salivary glands of the termite *M. subhyalinus* with dual activity against carboxymethyl-cellulose. Lanes 1 and 3, purified enzyme; lane 2; crude extract; lane 4, molecular weight markers.

specific activities towards carboxymethylcellulose and xylan from Birchwood (pooled fractions 14 to 18) were respectively 3.50 and 5.35 U/mg of protein (Table 1). The enzyme showed a single protein band by polyacrylamide gel electrophoresis in native (Figure 2A) and denaturing conditions (Figure 2B).

Molecular weight

From the migration pattern of the standard, the molecular weight was calculated to be 14.47 kDa. The molecular weight determined by gel filtration chromatography was57.12 kDa (Table 2).

Table 1. Purification of an endo-beta-D-xylanase from major soldier salivary glands of the termite *Macrotermes subhyalinus* with dual activity against carboxymethylcellulose.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract					
Carboxymethylcellulase	109.41	2.73	0.03	100	1
Xylanase	109.41	4.70	0.04	100	1
ANX-Sepharose 4 Fast Flow					
Carboxymethylcellulase	6.29	1.77	0.28	64.84	9.38
Xylanase	6.29	2.67	0.43	56.86	10.33
CM-Sepharose CL-6B					
Carboxymethylcellulase	0.67	0.59	0.80	21.61	29.33
Xylanase	0.67	0.89	1.33	32.60	33.25
Phenyl-Sepharose CL-4B					
Carboxymethylcellulase	0.08	0.28	3.50	10.25	116.67
Xylanase	0.08	0.43	5.35	9.15	133.75

Table 2. Some physicochemical characteristics of an endo-beta-D-xylanase from major soldier salivary glands of the termite *M. subhyalinus* with dual activity against carboxymethylcellulose.

Physicochemical properties	Carboxymethylcellulase activity	Xylanase activity
Optimum pH	5.6	5.0
pH stability range	4.6-6.0	4.6-5.6
Optimum temperature	60	65°C
Activation energy (KJ/mol)	48.18	26.87
Temperature coefficient (Q10)	1.74	1.35
Half life		
at 60°C (min)	100	120
at 65°C (min)	30	90
Michaelis Menten equation	Obeyed	Obeyed
Presence of transglycosylation activity	Yes	Yes
Mode of action	Endo	Endo
Molecular weight		
Mobility in SDS-PAGE[a]	14.47	
Gel filtration	57.12	

a = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

pH and temperature optima

The enzyme showed an optimum pH of 5.6 for cellulase activity and 5.0 for xylanase activity in acetate buffer (Table 2). It retained more than 60% of its cellulase activity in the range pH 5.0 to 6.0 (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).The optimum température of the enzyme with xylan from Birchwood and CMC hydrolysis were found to be respectively 65 and 60°C (Table 2). The enzyme retained more than 70% of its cellulase activity in the range 55 to65°C (Figure 4). The value of the temperature coefficient (Q_{10}) calculated between 45 and 55°C was
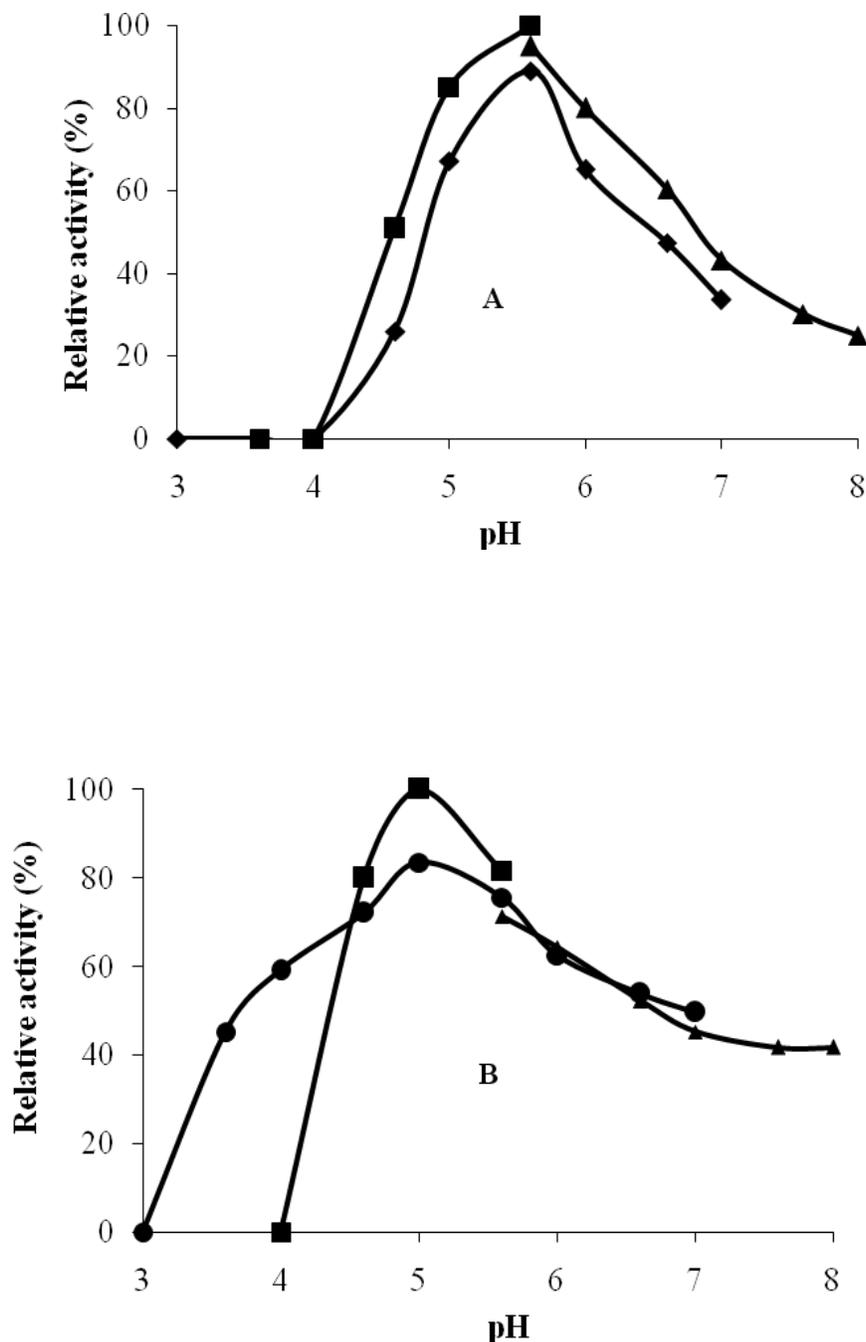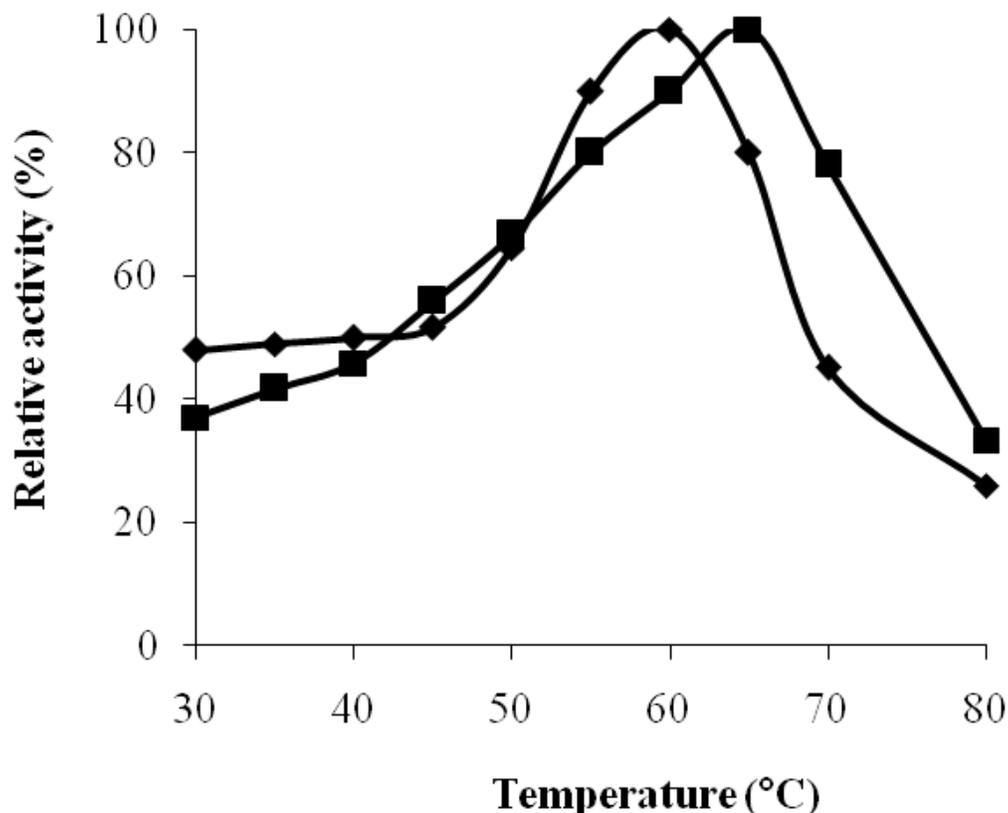
Figure 3. Effect of pH on an endo-beta-D-xylanase from major soldier salivary glands of the termite *M. subhyalinus* with dual activity against carboxymethylcellulose. Cellulase activity (A), xylanase activity (B), Acetate (■); citrate-phosphate (●); phosphate.

1.74 (Table 2). From the Arrhenius plot, the activation energy was found to be 22.58 KJ/mol (Table 2). Concerning xylanaseactivity, the enzyme retained more than 70% of its activity in the range 55 to 70°C (Figure 4). The value of the temperature coefficient (Q_{10}) calculated between 50 and 60°C was 1.35 and the activation energy

was found to be 23.52 KJ/mol (Table 2).

pH and temperature stabilities

At 25°C, the cellulase activity of the purified enzyme was

Figure 4. Effect of temperature on an endo-beta-D-xylanase from major soldier salivary glands of the termite *M. subhyalinus* with dual activity against carboxymethylcellulose. Xylanase activity (■), cellulase activity (♦).

stable over a wide pH range of 4.6 to 6.0 for 120 min (Table 2). The same activity was fully stable for 30 min at 60°C, but at 45°C, it was stable for 360 min in 20 mM acetate buffer pH 5.0. At 60°C, the half life of the cellulase activity was 100 min (Figure 5). Concerning xylanase activity, the purified enzyme was stable over a wide pH range of 4.6 TO 5.6 for 120 min (Table 2). At 60 and 65°C, this activity was unstable in 20 mM acetate buffer pH 5.0, but at 45°C, it was stable for 360 min (Figure 5). At 65°C, the half life of the xylanase activity was 90 min (Table 2).

Substrate specificity and kinetic parameters

The purified enzyme did not attack *p*-nitrophenyl glycopyranosides, cellobiose, sucrose, lactose, xylobiose, maltose, inulin, avicel, *sigmacel* 50-cellulose and starch (Table 3). Although, the enzyme degraded carboxymethylcellulose and xylans (Beechwood and Birchwood) (Table 3). The effect of substrate concentration on enzymatic activity was studied with carboxymethylcellulose and xylans. With these

substrates, the enzyme obeyed the Michaelis-Menten equation (Table 2). The K_M, V_{max} and V_{max}/K_M values are reported in Table 4. The catalytic efficiency of the enzyme, given by the V_{max}/K_M ratio is much higher for the carboxymethylcellulose than the xylans (Table 4).

Thin-layer chromatography analysis of hydrolysate

Within the first 12 h of the reaction, the hydrolysis of carboxymethylcellulose produced the expected oligosaccharides, disaccharides and monosaccharides. From xylan (Birchwood), the hydrolysate contained oligosaccharides and disaccharides. After 12 h of incubation, the hydrolysis products such as monosaccharides and disaccharides disappeared in the reaction (Figure 6).

Effect of chemical agents on enzyme activity

Chemical agents KCl, FeCl$_2$, SDS, DTNB and *p*CMB showed an inhibitory effect on cellulase activity of the
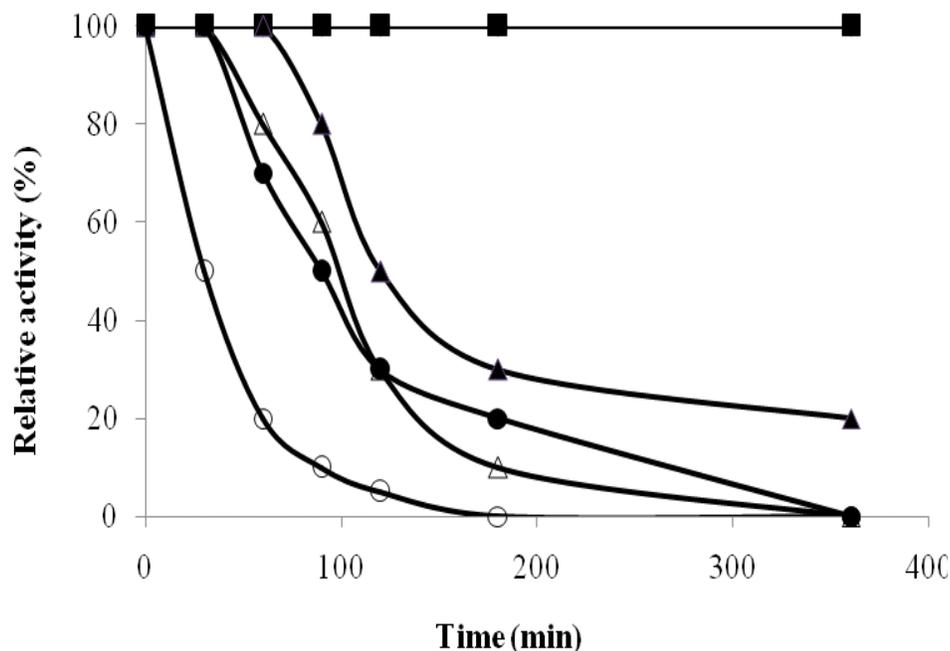
Figure 5. Thermal stability of an endo-beta-D-xylanase from major soldier salivary glands of the termite *M. subhyalinus* with dual activity against carboxymethylcellulose. Xylanase activity 45°C (■), cellulase activity 45°C (□), xylanase activity 60°C (▲), cellulase activity 60°C (△), xylanase activity 65°C (●), cellulase activity 65°C (○).

Table 3. Activities of an endo-beta-D-xylanase from major soldier salivary glands of the termite *M. subhyalinus* on synthetic chromogenic, disaccharide and polysaccharide substrates.

Substrate	Concentration in assay	Relative rate of hydrolysis (%)
Carboxymethylcellulose	2.6 mg/ml	100.00
Xylan (Birchwood xylan)	2.6 mg/ml	160.80
Xylan (Beechwood xylan)	2.6 mg/ml	116.80
Avicel	2.6 mg/ml	0.00
Sigmacel 50-cellulose	2.6 mg/ml	0.00
Inulin	2.6 mg/ml	0.00
Starch	2.6 mg/ml	0.00
Maltose	10.0 mM	0.00
Sucrose	10.0 mM	0.00
Lactose	10.0 mM	0.00
Cellobiose	10.0 mM	0.00
Xylobiose	10.0 mM	
p-Nitrophenyl-glycopyranoside	1.5 mM	0.00

purified enzyme. However, $BaCl_2$, $CuCl_2$, EDTA and $CaCl_2$ had no effect on the same enzyme activity (Table 2). Concerning xylanase activity, the enzyme was inhibited by $ZnCl_2$ and SDS. However, it was activated by $MgCl_2$, $BaCl_2$ and $CaCl_2$. EDTA, DTNB, *p*CMB, $CuCl_2$, $FeCl_2$, KCl and NaCl had no effect on the same enzyme activity (Table 5).

DISCUSSION

A single enzyme with dual activity (carboxymethyl cellulase and xylanase) was purified from the major soldier salivary glands of the termite *Macrotermes subhyalinus*. The preparation showed only one band in sodium dodecyl sulfate polyacrylamide gel electrophoresis

Table 4. Kinetic parameters of an endo-beta-D-xylanase from major soldier salivary glands of the termite *M. subhyalinus* towards carboxymethylcellulose, xylan from Birchwood and xylan from Beechwood.
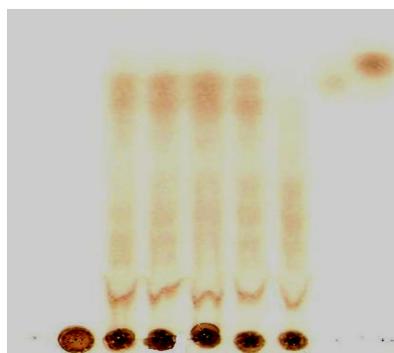
Substrate	K_M (mg/ml)	V_{max} (U/mg)	V_{max}/K_M (Uxml/mg^2)
Carboxymethylcellulose	0.46	9.92	21.56
Xylan from Birchwood	1.10	15.61	14.19
Xylan from Beechwood	1.21	11.74	9.70

A
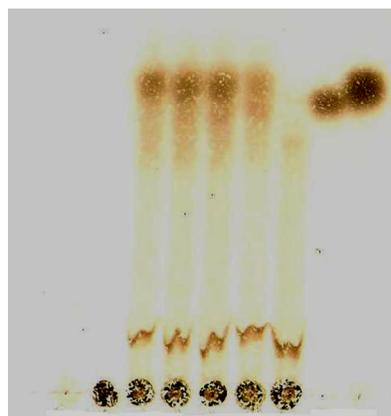
0_1 0_2 1/2 3 6 12 24 G_2 G_1

Time (h)

B

Time (h)

0_1 0_2 1/2 3   6   12 24 X_2 X_1

Figure 6. Time course of end products from (A) carboxymethylcellulose or (B) xylan from Birchwood hydrolysis for an endo-beta-D-xylanase from major soldier salivary glands of the termite *M. subhyalinus* with dual activity against carboxymethylcellulose. G_1= glucose, G_2= cellobiose, X_1= xylose, X_2 = xylobiose, 0_1= enzyme, 0_2= substrate (xylan Birchwood or carboxymethylcellulose).

Table 5. Effect of chemical agents on an endo-beta-D-xylanase from major soldier salivary glands of the termite *M. subhyalinus* with dual activity against carboxymethylcellulose.

Chemical agents	Concentration in assay	Relative activity (%)	
		Carboxymethylcellulase activity	Xylanase activity
Control	0	100	100
$ZnCl_2$	1	127.20	88.13
$MgCl_2$	1	135.96	156.15
$BaCl_2$	1	97.36	163.04
$CaCl_2$	1	100	169.56
$CuCl_2$	1	100	100
$FeCl_2$	1	61.27	100
KCl	1	80.70	95.24
NaCl	1	100	100
EDTA[b] (%, w/v)	1	100	100
DTNB[c] (%, w/v)	1	47.14	100
pCMB[d] (%, w/v)	1	79.11	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.

and in polyacrylamide gel electrophoresis. The purified enzyme had no contaminating glycosidase activities such as glucosidase, fucosidase, mannosidase, arabinosidase and xylosidase. The only substrates that were hydrolyzed by the enzyme were xylan and carboxymethylcellulose. These results are in agreement with those for the bifunctional polysaccharidase from the symbiotic fungus *Termitomyces* sp of the termite *M. subhyalinus* (Faulet et al., 2006b). But, they differ to cellulolytic and xylanolytic enzymes from the termite *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 *M. subhyalinus* workers (Faulet et al., 2006b).

Within the first 12 h of the reaction, the hydrolysis of the two substrates carboxymethylcellulose and xylan from 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 polysaccharidase possessing endo-glucanase and endo-xylanase activities. Carboxymethylcellulose, which measures endo-beta-1,4-glucanase activity, is one of the most popular artificial substrates for measuring cellulase activity because of its high solubility in water. Thus, carboxymethylcellulose has been preferentially used in most studies of cellulose digestion in termites and other insects. However, it has long been recognized that evaluation of cellulose digestibility by carboxymethyl-cellulose degradation assays is somewhat insufficient.

This observation is supported by the incapacity of the major soldier salivary glands to degrade *in vitro* crystal-line cellulose, a pure constituent of native cellulose, into which penetration by water-soluble enzymes is difficult due to tightly packed cellulose fibres joined to each other by hydrogen bonds and van der Waals forces (Gardner and Blackwell, 1974). The digestibility of native cellulose is highly dependent upon its crystallinity and its association with other structural polymers, especially lignin (Wood and Saddler, 1988). In insects, the grinding action of the mandibles and the highly alkaline conditions that prevail in the midgets of some species might also serve to reduce the crystallinity of ingested cellulose (Martin, 1991). It seems that the apparent role of this enzyme in the digestive tract is the hydrolysis of xylan (hemicellulose) and potentially cellulose.

The specific activities towards xylans from Birchwood and Beechwood are considerably lower than those obtained for the three xylanases (Faulet et al., 2006a, b) and the two cellulases (Séa et al., 2006) purified previously from the worker of the same termite. These observations suggest how trophallaxis might serve socio-nutritional needs in termite colonies. The worker of the termite *M. subhyalinus* could regurgitate its own salivary glands secretions to supplement the digestive needs of the soldier of the same insect 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 different temperature and pH activity profiles determined for the purified enzyme with the substrates

carboxymethylcellulose and xylan from Birchwood suggest that this protein has two active sites: one for each activity. This pattern seems to reflect the activity of the bifunctional polysaccharidases from *Ruminococcus flavefaciens* (Flint et al., 1993) and *Cellulomonas flavigena* (Pe´rez-Avalos et al., 2008). The apparent bi-functional protein is significantly different from the termite *M. subhyalinus* worker xylanases (Faulet et al., 2006b) and cellulases (Séa et al., 2006). This is a strong indication that the catalytic domains of these enzymes (from termite *M. subhyalinus*) can be grouped into well defined families, indicating that their genes evolved divergently from relatively few ancestral sequences. This observation is in close agreement to reports of Han (1987), who reported that in the course of *M. subhyalinus* caste differentiation, the most dynamic morphogenesis occurs in the stage of moulting from minor worker to presoldier. Miura et al. (1999) recently identified a gene expressed specifically in the mandibular glands of soldiers, but not workers, and subsequent studies have found numerous transcription factor, structural and enzyme-coding genes that differ in expression between soldiers and workers (Scharf et al., 2003).

After 12 h of incubation, the carboxymethylcellulose or xylan (Beechwood) hydrolysis products such as monosaccharides and disaccharides disappeared in the reaction. It seems that the transglycosylation reaction occurred with the hydrolysis of xylan or carboxymethylcellulose, judging from the fact that the polysaccharidase can transfer part of the monosaccha-ride-based polysaccharide to monosaccha-ride-based oligosaccharides. The rate of transglyco-sylation product formation was largely favored relative to the rate of hydrolysis. These enzymatic activities are analogous to the dual activities of XTH enzyme, which cuts the glucan backbone of xyloglucan and either attaches the newly created reducing end to xyloglucan derived oligosaccharides (xyloglucan endo-transglycosylase activity; XET), or to water (xyloglucan endohydrolase; XEH) (Rose et al., 2002).

The relative molecular weight of the purified enzyme was estimated to be 14.47 and 57.12 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration on Sephacryl S 200 HR, respectively. This result would suggest that, in contrast to the cellulases (Séa et al., 2006) and xylanases (Matoub and Rouland, 1995; Faulet et al., 2006b) obtained from workers of the termites *M. subhyalinus,* and *Macrotermes bellicosus*, the purified enzyme is a homotetrameric protein.

Conclusion

The salivary glands of *M. subhyalinus* soldier apparently produce an endo-beta-xylanase with dual activity against carboxymethylcellulose. The role of this enzyme in the digestive tract is the hydrolysis of xylan (hemicellulose)

and potentially cellulose. We hypothesize that the low activity is one of the causes of the trophallaxis phenomenon. This protein is significantly different from the termite *M. subhyalinus* worker xylanases and cellulases.

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