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Purification and biochemical characteristics of a new strictly specific endoxylanase from termite *Macrotermes subhyalinus* workers

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Abstract

Termite *Macrotermes subhyalinus* (Rambur) (Isoptera Termitidae Macrotermitinae) worker produced a new strictly specific xylanase. The enzyme was purified by DEAE-Sepharose CL-6B, Sephacryl S-200 HR, CM-Sepharose CL-6B and Phenyl-Sepharose CL-4B chromatographies. It exhibited a molecular mass of 60.1-61.2 kDa. This enzyme was indicated to be an endo-xylanase, which produced monomers and oligomers of xylose from xylan. It had an optimum pH of 5.0-5.6 and optimum temperature of 60 °C. However, it had pH stability between 4.6 and 5.6. The enzyme was stable at 37 and 50 °C for more than 6 h. At pH ranging from 5.0-5.6 and 60 °C, the enzyme retained fully activity for 30 min and possessed a half life of 2 h. The xylanase activity was stimulated by Na^{+}, Mn^{2+}, Sr^{2+}, Ca^{2+}, Mg^{2+} and dithiol- reducing agents, and was sensitive to Cu^{2+}, Fe^{2+}, and detergent agents. It enzymatic activity was slightly loss in presence of urea at 1 % (w/v) concentration. The enzyme could also be used in the presence of organic solvents such as acetone and dioxane (up to 10 %, v/v) without loss of activity.

Key words: endo-xylanase, biochemical, *Macrotermes subhyalinus*, termite worker, macrotermitinae, termitidae.

Introduction

The digestion of hemicellulose by xylophagous insects has interested biologists since the beginning of the 20th century. Insects themselves produced several different hemicellulosic activities (Martin, 1991; Slaytor, 1992; Breznak and Brune, 1994). The study of hemicellulose digestion in insects is most advanced in termites (Krishna, 1969).

Termites play a major role in the recycling of photosynthetically fixed carbon. With the aid of their symbiotic intestinal flora, they are able to degrade extensively wood constituents such as cellulose and hemicellulose. Nevertheless, the microbial species involved in the degradation of hemicelluloses are poorly defined (Schafer *et al.*, 1996).

The degradation of plant material by Macrotermitinae is to a great extent due to their double symbiosis: endosymbiosis and exosymbiosis with a fungus from the genus *Termitomyces* sp. (Grasse, 1982; Rouland *et al.*, 1990; Kouamé *et al.*, 2005). Electron microscopy studies have shown that plant material was degraded to a great extent from the upper part to the base of the fungus comb, in particular the pectocellulosic membrane and the polyphenol proteins (dark pigments) (Butler and Buckerflield, 1973; Martin, 1991; Cookson, 1992). The termite workers eat the inferior part of the fungus comb that has been pre-degraded by the fungus (exosymbiosis). The degradation of this ingested vegetal material is completed in the termite digestive tract by the concomitant action of enzymes from different origins [termite, fungus (endosymbiosis) microflora] (Rouland *et al.*, 1990).

Xylan is the major structural component of plant cell wall and the most renewable hemicellulose composed of 1,4-linked β-D-xylopyranosyl residues. Among annual plants, hard-woods and softwoods contain 20-25 % and 7-12 % xylan, respectively (Whistler and Richards, 1970). Xylanases (1,4- β-D-xylan xylanohydrolase; E.C. 3.2.1.8), a repertoire of hydrolytic enzymes, facilitate the complete hydrolysis of xylan. Xylanases have been reported from bacteria (Foong and Doi, 1992; Archana and Satyanarayana, 2003), fungi (Kimura *et al.*, 2002; Devillard *et al.*, 2003), actinomycetes (Holtz *et al.*, 1991; Roberge *et al.*, 2003) yeasts (Morosoli *et al.*, 1986; Amoresano *et al.*, 2000; Fierens *et al.*, 2004).

The potential application of xylanases has received a great deal of attention in food, feed, pulp and paper industries (Wong *et al.*, 1988). The environmental and legislative pressures have forced the pulp and paper industry to modify its pulping, bleaching and effluent treatment technologies to reduce the environmental impact of mill effluents (Bajpai *et al.*, 1994). Biobleaching and bioprocessing of pulps using xylanases (Garg *et al.*, 1998) is one of the most suitable biological applications to be used in the pulp and paper industry. Other significant benefits of these enzymes include higher brightness ceilings, a reduction in the amounts of bleaching chemicals needed to achieve high brightness and reduced amounts of organochlorine compounds in the bleach plant effluents (Ragauskas *et al.*, 1994). A prerequisite in pulp and paper industry is the use of cellulose free xylanases that ensure minimal damage to the pulp fibres and also generate rayon grade or superior quality dissolving pulps (Jurasek and Paice, 1987).

In this study, the biochemical characteristics of a new strictly specific endoxylanase purified from the termite worker of *Macrotermes subhyalinus* (Rambur) (Isoptera Termitidae Macrotermitinae) is reported.

Materials and methods

Enzymatic source and preparation of crude extract

The workers of the termite *M. subhyalinus* were collected to Lamto (Côte d'Ivoire) directly from the nest, then stored at -20 °C.

After thawing, the termite workers (15 g) were washed with distilled water, then harvested by centrifugation and resuspended in 10 ml 0.9 % (w/v) NaCl solution. After disruption of termite workers in an Ultra-Turrax type T25 and then sonicated as previously described by Rouland *et al*. (1988), the solution was centrifuged at 15,000 g for 15 min at 4 °C. The obtained supernatant constituted the crude extract.

Chemicals

Substrates for glycosidases, including beechwood xylan, birchwood xylan, DA-methyl xylan, carboxymethylcellulose (CMC), polygalacturonic acid, cellulose, cellobiose, starch, inulin, xylobiose and synthetic substrates (*p*-nitrophenyl-glycosides) were purchased from Sigma-Aldrich. DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, Sephacryl S-200 HR and Phenyl-Sepharose CL-4B gels were from Pharmacia-LKB Biotech. Protein Standards for molecular mass determination and the chemicals used for polyacrylamide gel electrophoresis were obtained from Bio-Rad. All other chemicals and reagents were of analytical grade.

Purification procedure

All steps of the purification procedure were performed at 4 °C. The crude extract was loaded onto a DEAE-Sepharose CL-6B column (2.5 X 6.7 cm) that had been equilibrated with 20 mM sodium acetate buffer (pH 5.0). After washing the column with the same buffer, a 60 ml increasing discontinuous gradient (0-200 mM) of NaCl dissolved in 20 mM sodium acetate buffer (pH 5.0) was applied to the column. Proteins were eluted at a flow rate of 60 ml/h and fractions of 2 ml were collected. The fractions containing the enzyme were pooled and extensively concentrated by adding ammonium sulfate to 80 % final saturation. After centrifugation at 13.000 g for 30 min, the precipitate was dissolved in 20 mM sodium acetate buffer (pH 5.0) and the resulting solution was passed through a Sephacryl S-200 HR column (1.6 X 65 cm) that had been equilibrated with the same buffer, at a flow rate of 30 ml/h; fractions of 1 ml were collected. The active fractions were pooled and extensively adsorbed on a CM-Sepharose CL-6B column (2.5 X 5.3 cm) that had been equilibrated with 20 mM sodium acetate buffer (pH 5.0). After washing the column with the same buffer, a 40 ml increasing discontinuous gradient (0-200 mM) of NaCl dissolved in 20 mM sodium acetate buffer (pH 5.0) was applied to the column. The flow rate was 56 ml/h and fractions of 2 ml were collected. The active fractions were pooled and put in a solid sodium thiosulfate 1.7 M, and then loaded onto a Phenyl-Sepharose CL-4B column (1.6 X 4.5 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0) containing 1.7 M sodium thiosulfate. After washing the column with two bed volumes of equilibration buffer, elution (flow rate, 60 ml/h; fractions, 1 ml) was carried out with a 15 ml decreasing discontinuous gradient (1.7-0 M) of sodium acetate buffer (pH 5.0). Finally, the active fractions were pooled, extensively dialyzed against 20 mM sodium acetate buffer (pH 5.0) and stored at 4 °C.

Enzyme assays

Xylanase assays were performed by incubating 250 µl of beechwood xylan (1 %, w/v) suspension in 100 mM sodium acetate buffer (pH 5.0) with 50 µl of enzyme solution at 50 °C for 20 min. Other substrates (1 % or 5 mM) were tested under the same conditions. Reactions stopped by the addition of either 300 µl of 3,5-dinitrosalicylic acid reagent for natural substrates or 2 ml of 1 M sodium carbonate for synthetic substrates. Reducing sugars were measured at 540 nm with 3,5-dinitrosalicylic acid reagent (Bernfeld, 1955) with D-xylose as a standard. *p*-Nitrophenol released from synthetic substrates was measured at 412 nm. One unit of enzyme activity was defined as the amount of enzyme which produced reducing sugar equivalent to 1 µmol of xylose or release 1 µmol of *p*-nitrophenol per min under the conditions described above.

Estimation of protein concentration

Protein concentration was measured according to the method of Smith *et al*. (1985), utilizing bicinchoninic acid (BCA). Bovine serum albumin was used as a standard.

Determination of molecular mass

The molecular mass of the xylanase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme sample was denatured by a 5 min treatment at 100 °C in a 125 mM Tris-HCl buffer (pH 6.8) containing 4% (w/v) SDS, 1 % (v/v) β-mercaptoethanol, 20 % (v/v) glycerol and 0.025 % (w/v) bromophenol blue. Electrophoresis was performed according to Laemmli (1970) on 1.5 mm thick slab gels (7 X 8 cm) containing 10 % acrylamide, 375 mM Tris-HCl buffer (pH 8.8) and 0.1 % (w/v) SDS. Electrophoresis was carried out at 10 mA with a 25 mM Tris/192 mM glycine buffer containing 0.1 % (w/v) SDS as electrode buffer. Proteins were stained with silver nitrate according to Blum *et al*. (1987).

Electrophoresis of the native enzyme was performed using essentially the same method, but without SDS and β-mercaptoethanol in the buffers.

The molecular mass of the native xylanase was estimated by gel filtration in a HPLC system, by using a TSK (QC-PAK GFC 200) column (7.8 mm X 15 cm). The standard proteins used for calibration were β-amylase (200 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and cytochrome C (12.4 kDa).

Effect of pH on activity and stability

In these experiments, the pH values of each buffer were determined at 25 °C. Xylanase activity was measured at 50 °C under the standard test conditions. For determination of the optimum pH, the xylanase activity was measured by performing the essays at various pH values in the following buffer systems: sodium acetate buffer (100 mM) from pH 3.6 to 5.6, sodium phosphate

buffer (100 mM) from pH 5.6 to 8.0, Tris-HCl buffer (100 mM) pH 7.0 to 9.0 and citrate phosphate buffer (100 mM) pH 3.0 to 7.0. For pH-stability study, the enzyme solutions were preincubated at ambient temperature for 1 h in sodium acetate and sodium phosphate buffers at various pH values between 3.6 and 8.0. After adjusting the mixtures to pH 5.0, the residual activity was measured under the standard assay conditions.

Effect of temperature on activity and stability

For determination of the optimum temperature, the incubation was performed for 20 min in 100 mM sodium acetate buffer (pH 5.0) at temperature ranging from 35 to 80 °C. The thermal inactivation of the xylanase was studied at 37, 50, 60 and 65 °C by prewarming the enzyme solutions in 100 mM sodium acetate buffer (pH 5.0). Aliquots were removed at different times and residual activity was measured at 50 °C under standard conditions.

Effect of ions and denaturing agents

Ions were incubated with the enzyme for 30 min at room temperature, and then the xylanase activity was measured under the standard test conditions. The final concentration of ions in the reaction mixture was 1 or 5 mM. Studies with denaturing agents were performed under the same conditions except for the concentration value on the enzyme.

Substrate specificity and kinetic parameters determination

The study of substrate specificity of the xylanase was performed with a variety of natural and synthetic substrates (1 %, w/v or 5 mM) incubated at 50 °C from 20 min in 100 mM sodium acetate buffer (pH 5.0) with 50 µl of purified enzyme. The reaction was stopped and quantified under the standard test conditions.

The kinetic parameters (K_M, V_{max} and V_{max}/K_M) were determined from Lineweaver-Burk representation using different concentrations (0.12 - 1 %) of beechwood xylan or birchwood xylan. Each experimental point was determined at least in triplicate and in all cases the initial rate was used for plotting.

Enzyme activity on natural substrates was essayed in 450 µl of 100 mM sodium acetate buffer (pH 5.0) containing the tested substrate at the indicated concentration. After prewarming the mixture for 5 min at 50 °C, the reaction was initiated by 75 µl of the enzyme solution. Aliquots (300 µl) were withdrawn at different times. The reaction was stopped and quantified as in the standard enzyme essay.

Analysis of degradation products

The hydrolysis of xylobiose (5 mM) and xylan (1 %, w/v) by the termite worker xylanase have been tested with 50 µl enzyme in 100 mM sodium acetate buffer (pH 5.0) at 50 °C. Hydrolysis was stopped by heating reaction solution at 100 °C for 5 min. Aliquots were analyzed on silica gel thin-layer chromatography (TLC). A portion of each sample (3 µl) was spotted onto a TLC silica gel plate 60 F_{254} (E. Merck AG, Darmstadt, Germany) and chromatographed in a solvent system containing butanol-acetic acid-water (9: 3.75: 2.25, v/v/v) at room temperature. Sugars were visualized by naphto-resorcinol staining as described by Brückner (1955).

Results

Enzyme purification

The results of the purification of the xylanase from termite workers are summarized in table 1. The purification protocol involved four steps of chromatography for the enzyme (data not shown).

One peak of xylanase activity was resolved on the DEAE-Sepharose CL-6B column at 0.4 M NaCl concentration in 20 mM sodium acetate buffer (pH 5.0). The peak of the xylanase activity resolved on the DEAE-Sepharose CL-6B column step was applied to a Sephacryl S-200 HR gel. One peak showing xylanase activity was eluted. The active fractions were subjected to cation-exchange chromatography on CM-Sepharose CL-6B column. A single peak of xylanase activity was eluted when washing the column with 20 mM sodium acetate buffer (pH 5.0). This xylanase activity was ultimately purified using hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B gel. The xylanase was eluted at 0.2 M sodium thiosulfate and was enriched about 144-fold.

The enzyme showed a single protein band on native-polyacrylamide gel electrophoresis staining with silver nitrate (figure 1).

Molecular properties

After SDS-PAGE analysis under reducing conditions, the xylanase showed a single protein band. Its relative molecular mass was estimated to be 60.1 kDa (figure 2). The relative molecular mass of the native enzyme, as determined by gel filtration, was approximately 61.2 kDa.

Table 1. Purification of the termite *M. subhyalinus* worker xylanase. Values given are the averages of at least three experiments. (UI = 1 µmol of reducing sugars released per min).

Purification steps	Total protein (mg)	Total activity (UI)	Specific activity (UI/mg)	Yield (%)	Purification factor
Crude extract	241.5	16.9	0.07	100	1
DEAE-Sepharose CL-6B	32.52	3.9	0.12	23	1.8
Sephacryl-S 200 HR	1.86	1.12	0.6	7	9.1
CM-Sepharose CL-6B	0.3	0.45	1.5	3	22.8
Phenyl-Sepharose CL-4B	0.014	0.11	8.02	1	114.6

Figure 1. Native-PAGE analysis of the termite *M. subhyalinus* worker xylanase. The samples were loaded onto a 10 % gel. Lane 1, crude extract of termite worker; lane 2, purified xylanase.

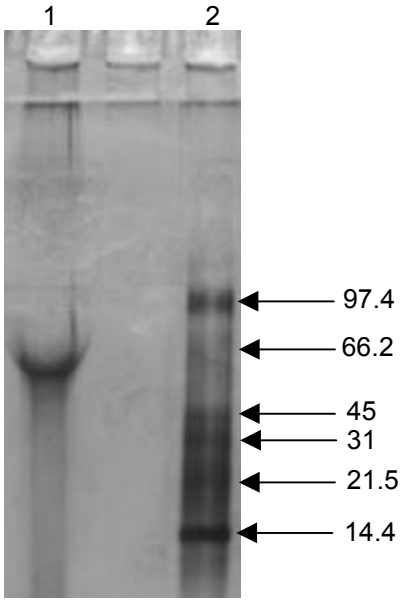

Figure 2. SDS-PAGE analysis of the termite *M. subhyalinus* worker xylanase. The samples were loaded onto a 10 % gel. Lane 1, purified xylanase and lane 2, molecular weights of standard proteins (values in kDa are indicated at the right).

pH and temperature dependence of the activity of the termite worker xylanase

The effect of pH on the catalytic activity of the xylanase was studied at 50 °C by measuring the activity of the enzyme in 100 mM buffer (pH 3.0-9.0). Maximum activity was obtained at pH 5.0 – 5.6, but the enzyme retained more than 90 % of its activity in the

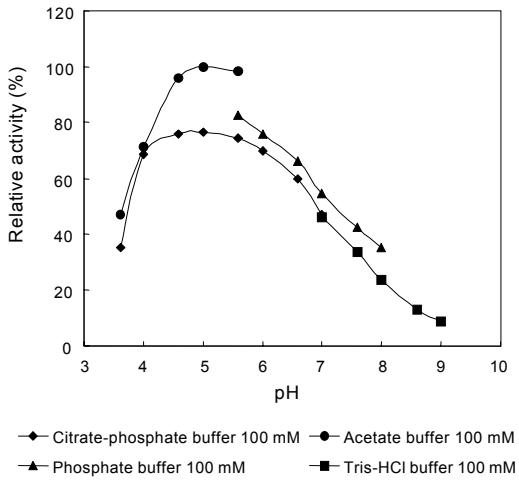

Figure 3. Effect of pH on the termite *M. subhyalinus* worker xylanase activity. The experiments were carried out at 50 °C with beechwood xylan as substrate in a serie of buffers at various pH ranging from 3.6 to 9.0. The buffers used were citrate-phosphate buffer (100 mM) from pH 3.6 to 7.0; sodium acetate buffer (100 mM) from pH 3.6 to 5.6; sodium phosphate buffer (100 mM) from pH 5.6 to 8.0 and Tris-HCl buffer (100 mM) from pH 7.0 to 9.0. Xylanase activity was measured at 50 °C under the standard test conditions. Values given are the averages of at the least three experiments.

pH ranging 4.4 – 5.6 (figure 3). A maximum of activity for this enzyme was also found at the same range of pH in citrate phosphate and sodium phosphate buffers but, in these cases, the activity was reduced by about 24.6 and 16 %, respectively when compared to that obtained in sodium acetate buffer (figure 3). The dependence of the enzyme activity on the temperature was studied at pH 5.0. For 20 min incubation, the maximum activity was observed at 60 °C (figure 4**)**. The value of the temperature coefficient (Q_{10}), calculated between 45 and 55 °C was found to be 1.4. The latter is much lower than that observed for most enzymes (Q_{10} around 2.0). From Arrhenius plot, a value of 26.7 kJ/mol was calculated for the activation energy (table 2).

pH stability

The pH dependence of the stability of the enzyme showed a maximal stability at pH range of 4.6 to 5.6 when the xylanase was preincubated for 1 h at ambient temperature in 100 mM sodium acetate and sodium phosphate buffers (table 2).

Thermal stability

The figure 5 shows the thermal inactivation kinetics performed at 37, 50, 60 and 65 °C. After 6 h preincubation at 37 and 50 °C, the xylanase remained fully stable. It retained 100 % of its initial activity. At its optimum temperature (60 °C), the enzyme was less stable, however it retained fully activity for 30 min. The half-life of the xylanase at 60 °C was more than 2 h. At this temperature, it lost completely its activity after treatment

for 6 h. The enzyme was rapidly inactivated at 65 °C (figure 5). The effect of temperature on the xylanase stability was also investigated by preincubating enzyme solutions for 15 min at different temperatures (figure 6). The xylanase retained 100 % of its activity up to 60 °C. At higher temperature, the thermostability decreased rapidly.

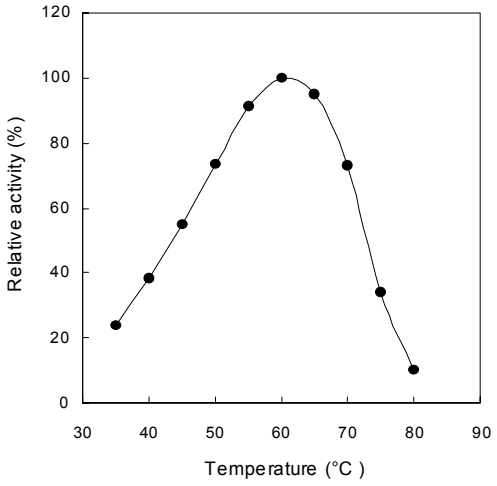

Figure 4. Effect of temperature on the termite *M. subhyalinus* worker xylanase. The experiments were carried out at the indicated temperatures for 20 min in 100 mM sodium acetate buffer (pH 5.0) using beechwood xylan as substrate under the standard test conditions. Values given are the averages of at least three experiments.

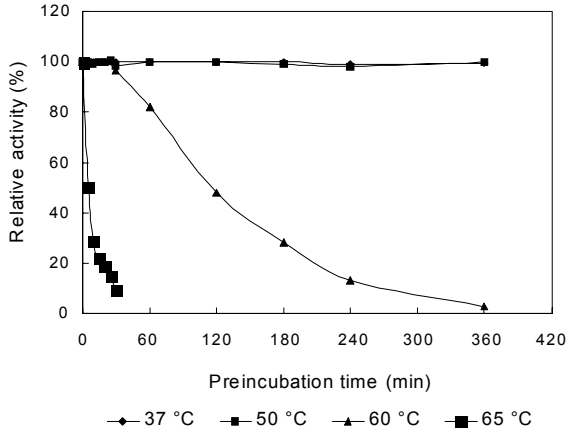

Figure 5. Thermal inactivation of the termite *M. subhyalinus* worker xylanase. The enzyme was preincubated at each temperature in 100 mM sodium acetate buffer (pH 5.0). At the temperature indicated times, aliquots were withdrawn and the residual activity was measured at 50 °C under the standard assay conditions. Residual activity is expressed as percentage activity of zero-time control of untreated enzyme. Values given are the averages of at least three experiments.

Table 2. Some physicochemical characteristics of the termite *M. subhyalinus* worker xylanase. Values given are the averages of at least three experiments.

Physicochemical properties	Values Termite worker
Optimum temperature (°C)	60
Optimum pH	5.0-5.6
pH stability	4.6-5.6
Molecular weight (kDa) SDS-PAGE	60.1
Molecular weight (kDa) Gel filtration	61.2
Q_{10}	1.4
Activation energy (kJ/mol)	26.7
Michaelis Menten equation	Obeyed

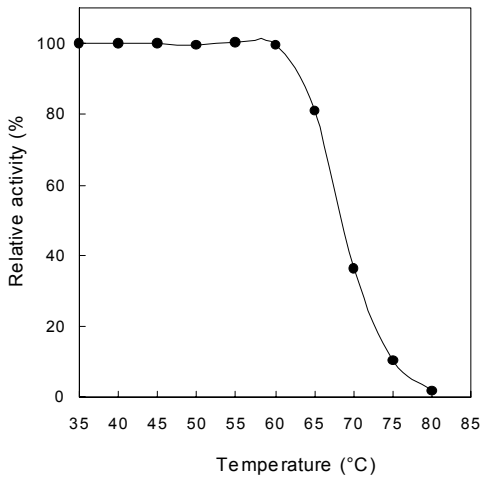

Figure 6. Thermal denaturation of the termite *M. subhyalinus* worker xylanase. The enzyme were preincubated at each temperature for 15 min. The remaining activity was measured at 50 °C under the standard assay conditions. Values given are the averages of at least three experiments.

Effect of metal ions on activity of the termite worker xylanase

The activator or inhibitor effects of mono- and divalent cations on the enzyme activity were studied (table 3). The xylanase showed different comportments in the presence of these ions. Na^{+}, Mn^{2+}, Sr^{2+}, Ca^{2+} and Mg^{2+} slightly activated the enzyme. Cu^{2+} and Fe^{2+} had a strongly inhibitory effect at 5 mM whereas the other ions had much more limited inhibitory effect or none.

Substrate specificity and kinetic parameters

The purified xylanase was assayed for hydrolytic activity against a variety of natural and synthetic substrates. birchwood xylan and beechwood xylan were hydrolyzed more and less in similar rates at 50 °C and pH 5.0. Under these conditions, no detectable activity towards carboxymethylcellulose, cellulose, cellobiose, starch, polygalacturonic acid, inulin, xylobiose or *p*-nitrophenyl-glycosides was observed (table 4).

A Lineweaver-Burk plot of the activity over a broad range of beechwood xylan and birchwood xylan con-

Table 3. Effect of metal ions on the termite *M. subhyalinus* worker xylanase. Values given are the averages of at least three experiments.

Reagent	Concentration (mM)	Relative activity (% of control)
None	0	100.0
Na^+	1	107.6
	5	110.6
K^+	1	100.0
	5	97.7
Mn^{2+}	1	125.0
	5	151.1
Mg^{2+}	1	100.0
	5	107.6
Sr^{2+}	1	100.0
	5	112.8
Fe^{2+}	1	90.9
	5	64.4
Ca^{2+}	1	103.0
	5	106.6
Ba^{2+}	1	100.0
	5	90.1
Cu^{2+}	1	82.6
	5	45.4
Zn^{2+}	1	100.0
	5	94.0
Pb^{2+}	1	89.0
	5	72.0

Table 4. Activities of the purified termite *M. subhyalinus* worker xylanase on natural and synthetic substrates. Values given are the averages of at least three experiments. Substrates were incubated with the enzyme solution at 50 °C for 20 min. (nd = not degraded).

Substrates	Xylanasic activity (%)
Beechwood xylan	100
Birchwood xylan	92.25
Carboxymethylcellulose	nd
DA-methyl xylan	nd
Polygalacturonic acid	nd
Cellulose	nd
Starch	nd
Inulin	nd
Cellobiose	nd
Xylobiose	nd
*p*Nitrophenyl-β-D-Xylopyranoside	nd
*p*Nitrophenyl-β-D-Glucopyranoside	nd
*p*Nitrophenyl-β-D-Fucopyranoside	nd
*p*Nitrophenyl-β-D-Galactopyranoside	nd
*p*Nitrophenyl-β-D-Cellobioside	nd
*p*Nitrophenyl-α-D-Glucopyranoside	nd
*p*Nitrophenyl-α-D-Fucopyranoside	nd
*p*Nitrophenyl-α-D-Mannoyranoside	nd
*p*Nitrophenyl-α-D-Arabinopyranoside	nd

centrations (0.12 to 1%) showed K_M and V_{max} values of the xylanase in table 5. Although the value of V_{max} is favour of beechwood xylan, the V_{max} / K_M ratio, known

Table 5. Kinetic parameters of the termite *M. subhyalinus* worker xylanase towards beechwood xylan and birchwood xylan.Values given are the averages of at least three experiments. The Michaelis constants (K_M) and the maximum velocities (V_{max}) are expressed as mg/ml and units/mg of protein, respectively.

Substrates	Xylanase		
	K_M	V_{max}	V_{max}/K_M
Beechwood xylan	13.0	93.5	7.2
Birchwood xylan	3.1	31.3	9.9

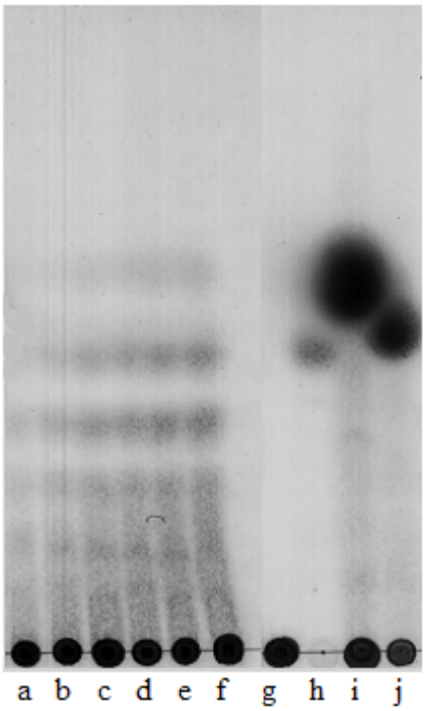

Figure 7. Thin-layer chromatogram analysis of xylan hydrolysis products released by the purified termite worker xylanase. The samples (3 µl each) for each time interval were chromatographed on a silicate gel plate. Lane a to f: degradation of xylan (10 min to 60 min); lane g to j: standards [xylan (g), xylobiose (h), xylose (i), arabinose (j)].

to be more significant parameter with respect to catalytic efficiency, is much higher for birchwood xylan than for beechwood xylan (table 5).

Hydrolysis products released by the purified xylanase from xylan were separated by TLC (figure 7). The predominant end products from xylan hydrolysis were xylobiose and xylose, even though a small chain of xylooligosaccharide (xylotriose) was initially produced. This suggested that xylotriose was produced as an intermediate that was eventually cleaved to xylobiose and xylose. It appeared that other xylooligosaccharides were also produced from xylan, and these products did not disappear with increasing time. No arabinose residue was detected among the hydrolysis products of the xylan (figure 7). Also, no hydrolysis product of xylobiose was observed (data not shown).

Table 6. Effect of reducing agents on the termite *M. subhyalinus* worker xylanase. Values given are the averages of at least three experiments.

Reducing agents	Concentration	Relative activity (% of control)
None	0	100.0
DL-dithiothreitol	0.1 % (w/v)	102.3
	1 % (w/v)	107.5
L-cysteine	0.1 % (w/v)	167.2
	1.0 % (w/v)	226.4
β-mercaptoethanol	0.1 % (v/v)	151.2
	1 % (v/v)	200.5
*p*CMB*	0.1 % (w/v)	71.5
	1 % (w/v)	36.3
DTNB**	0.1 % (w/v)	81.6
	1 % (w/v)	39.1

**p*CMB: *para*-Hydroxy-mercuribenzoic acid
***DTNB: 5,5 –dithio-bis (2-nitrobenzoic acid)

Table 7. Effect of detergents and urea on the termite *M. subhyalinus* worker xylanase. Values given are the averages of at least three experiments.

Reducing agents	Concentration	Relative activity (% of control)
None	0	100.0
Anionic		
SDS	0.1 % (w/v)	10.9
Taurocholic acid sodium salt	1 % (w/v)	89.7
Polyoxyethylene-9-lauryl ether	1 % (w/v)	100.3
Nonionic		
Triton X-100	1 % (v/v)	88.5
Noinidet P-40	1 % (v/v)	87.9
Tween 80	1 % (v/v)	89.2
Cationic		
Tetradecyl Trimethyl Ammonium Bromide	1 % (w/v)	78.1
Hexadecyl Trimethyl Ammonium Bromide	1 % (w/v)	67.6
Urea	0.1 % (w/v)	95.9
	1 % (w/v)	89.1

Behavior of the termite worker xylanase in the presence of dithiol-reducing agents, detergents and organic solvents

Dithiol-reducing agents

As a consequence, procedures which involve reductive cleavage and subsequent blocking of the thiol groups must be preferred for quantitative digestion by the termite worker xylanase of polysaccharide or glycoprotein substrates containing disulfide bonds than the scission of disulfide bonds by performic acid oxidation. β-mercaptoethanol is one of reagents most widely used for the reduction of disulfide bonds. For this reason, its effect was tested on the activity of the termite worker xylanase. At concentrations of 0.1 and 1 % (v/v) in the reaction mixture, the β-mercaptoethanol activated the enzyme activity by about 51.3 and 100.5 %, respectively. The other reductive agents such as cysteine and dithiothreitol displayed behaviors identical to that of β-mercaptoethanol (table 6). However, the enzyme activity was strongly inhibited by *p*CMB and DTNB at high concentration (1 %).

Detergents and urea

The effect of ionic and nonionic detergents currently use for denaturing of glycoproteins was tested on the termite worker xylanase activity. The result reported in table 7 shows that all of them, except Polyoxyethylene-9-lauryl ether (no effect), are inhibitors of the enzyme activity but to different degrees. However, SDS inhibits strongly the xylanase activity at 0.1 %. As for urea, it can be present in the reaction mixture up to concentration of 1 % without loss more than 10 % of the xylanase activity (table 7).

Organic solvents

One of the main advantages of carrying out enzyme reactions in organic media is avoiding the problems of solubility of hydrophobic substrates in water. This is why we have examined the effect of various organic solvents at different concentrations on the termite worker xylanase activity. For up to 10 % concentrations of all the tested organic solvents, except the alcohols, the enzymatic activity of the xylanase was preserved. An activation of the enzyme was even observed with acetone and dioxane (figure 8).

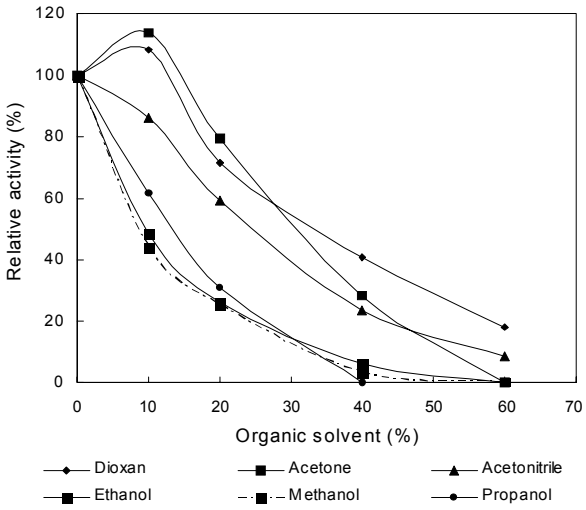

Figure 8. Effect of organic solvents on the termite *M. subhyalinus* worker xylanase. The enzyme was incubated at 50 °C for 20 min in 100 mM sodium acetate buffer (pH 5.0). The enzyme activity was measured in the presence of dioxane, acetone, acetonitrile, methanol, ethanol and propanol. Values given are the averages of at least three experiments.

Discussion

Glycosidases from termite have been largely studied with regard to their hydrolytic and transglycosylation activities in order to understand the symbiotic relationship with bacteria and the fungus *Termitomyces* sp. which grows on structures (fungus comb) built by termite workers (Rouland *et al.*, 1990; Matoub, 1993; Kouamé *et al.*, 2001 and 2005). However, no or few enzymatic works concerning workers of termites *M. subhyalinus* have been so far reported. A new xylanase from the crude extract of termite *M. subhyalinus* workers was purified to homogeneity using standard techniques i.e anion-exchange DEAE-Sepharose CL-6B chromatography, Sephacryl S-200 HR gel filtration chromatography, cation-exchange CM-Sepharose CL-6B chromatography and Phenyl-Sepharose CL-4B hydrophobic interaction chromatography. The pigments, which are in the crude extract, were almost completely removed during the DEAE-Sepharose CL-6B step. The gel filtration chromatography over a Sephacryl S-200 column enabled the xylanase to be separated from other glycosidases that are the most abundant enzymes in the termite *M. subhyalinus* workers crude extract (Rouland *et al.*, 1988; Kouamé *et al.*, 2005). The cation-exchange chromatography on CM-Sepharose CL-6B gel and hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B gel were crucial to separate the xylanase from the other proteins and impurities of the crude extract. A similar result concerning Phenyl-Sepharose gel has been reported for the purification of the specific endopeptidase Thr-N from *Archachatina ventricosa* digestif juice (Niamké *et al.*, 1999).

The purified enzyme is monomeric with a relative molecular mass (M_r) value similar to those of the group of xylanases which has been assigned to the category of high-M_r, acidic xylanases, in contrast to low-M_r, basic xylanases (Wong *et al.*, 1988).

Since only mono- and oligo-xylosaccharides would be liberated by the action of the xylanase of termite *M. subhyalinus* workers from polysaccharides, this enzyme is expected to be of limited importance in the nutrition of the organism, but it may be useful for applications requiring the selective removal of hemicelluloses in the pulp and paper industry. In this context, the characterization of physicochemical properties enables the experimental conditions for the digestion of natural xylans by termite *M. subhyalinus* worker xylanase to be optimized. The activity of the enzyme is maximal at pH 5.0-5.6 but it displays a better stability at pH ranging 4.6-5.6. So, a pH of 5.0 is a good compromise between the activity and stability of the enzyme to perform the specific hydrolysis of xylan over a long time-period. The pH and Temperature for optimal enzyme activity of this enzyme are in the range of those reported for xylanases from other mesophilic fungi and bacteria (Wong *et al.*, 1988). Like the xylanases characterized from different microorganisms, the pH stability of the xylanase from the termite *M. subhyalinus* worker is in range of those reported for xylanases from other fungi and bacteria (4.5-10.6) (Subramaniyan and Prema, 2000). The importance of the nature of the buffer should also be noted, the activity of the enzyme being higher in a sodium acetate buffer than in a citrate phosphate or sodium phosphate buffers. The xylanase exhibited a total activity at 37 and 50 °C for a long time-period (more than 6 h), for 30 min at 60 °C and the half-life of the enzyme at the latter was approximately 2 h at this pH stability range. The pH stability and thermostability, a prerequisite in pulp and paper industry, proved to be favourable factors for the application of the termite *M. subhyalinus* worker xylanase in biobleaching of pulps. The xylanase is sensitive to Cu^{2+}, Fe^{2+} and detergent agents so that these chemicals, in particular the latter, must be eliminated after treatment of the oligosaccharide, polysaccharide or glycoprotein substrate when this needs to be denatured before its hydrolysis by the enzyme. A similar result has been reported for xylanase from *Staphylococcus* sp. SG-13 (Gupta *et al.*, 2000), *Aspergillus nidulans* KK-99 (Taneja *et al.*, 2002) and for the specific endoproteinase Thr-N from *Archachatina ventricosa* (Niamké *et al.*, 2003). The effect on activity by the reductive agents suggests that disulfide bonds play an essential role in the native conformation of the termite worker xylanase. The stimulation of the activity of xylanase in the presence of Mn^{2+}, Na^{+} and dithiol-reducing agents has previously been reported (Cesar and Mrsa, 1996; Fialho and Carmona, 2004) and is in accordance with the present study. The behaviour of the enzyme in the presence of *p*CMB and DTNB has also been reported from Bacillus sp. strain SPS-0 (Bataillon *et al.*, 2000). Most native macromolecules are rapidly unfolded by urea and tend to remain in this state even when the macromolecules solution is later diluted with respect to urea (Bennett, 1967). Thus, it is of particular interest to find that the xylanase is slightly inactivated in urea. Digestion under these conditions can be attempted for macromolecules that are not readily attacked by the xylanase under nondenaturing conditions. Thus, the fact that the xylanase is slightly inactivated in the presence of urea suggests that hydrogen bonds play a limited role in the stabilization of the glycosidase. The xylanase of the termite *M. subhyalinus* worker shows good stability in the presence of organic solvents, an activation even being observed with acetone and dioxane. The first is often used to precipitate, and the latter is particularly valuable to render soluble a certain number of macromolecules like, for example, the vegetable proteins (Colas *et al.*, 1993).

The xylanase is specific for hydrolyzing natural xylan and is free of cellulase activity, which are desirable properties for biobleaching of pulps. Some xylanases have both xylanase and cellulase activities (Shareck *et al.*, 1991), but not the one from termite *M. subhyalinus* worker. This enzyme is a cellulase-free xylanase. Xylobiose and xylose are produced as end products, while higher xylooligosaccharides appear to be produced only as intermediates of xylan hydrolysis by this enzyme. No *para*-nitrophenol is released by the effect of the enzymes on synthetic substrates and no free arabinose is produced fom xylan by this enzyme. This xylanase cannot also hydrolyse xylobiose to xylose. On the basis of these results, it is safe to say that this enzyme is a typical endo-β-1,4-xylanase. In spite of the extremely high

specific activity of this enzyme toward xylan, the K_M of this enzyme is similar to that of xylanases from other sources (Nakamura *et al.*, 1993; Lin *et al.*, 1999; Archana and Satyanarayana, 2003).

Finally, the present study showed that the xylanase from the termite *M. subhyalinus* worker was active in acid conditions, thermostable, cellulase-free and showed high hydrolytically affinity for only natural xylans. Hence, it qualified for use in biotechnological applications and, all its properties make it a useful tool for biobleaching in pulp and paper industry.

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