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Full Length Research Paper

Purification and biochemical properties of a new thermostable xylanase from symbiotic fungus, Termitomyces sp.

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A xylanase was purified from symbotic fungus, Termitomyces sp. by chromatography on columns of DEAE-Sepharose, CM-Sepharose, gel filtration and Phenyl-Sepharose. The preparation was shown to be homogenous by polyacrylamide gel electrophoresis. The purified enzyme displayed two protein bands on SDS-polyacrylamide gel electrophoresis and its molecular mass was estimated to 80-87 kDa. The xylanase exhibited maximum activity at 65-70°C and at pH 5.6, but it retained more than 80% of its activity in the pH range 5.0-6.0. The enzyme was stable for a long time-period up to 50°C and for 1 h at 60°C. Although the xylanase had a lower carboxymethylcellulase activity, it lacked activity towards substituted xylan, xylobiose, inulin, starch, polygalacturonic acid or pNP-glycosides. Kinetic parameters indicated higher efficiency in the hydrolysis of beechwood xylan and birchwood xylan. The xylanase activity was stimulated by K^+, Mn^{2+} and dithiol-reducing agents and was sensitive to Cu^{2+}, Fe^{2+}, Zn^{2+} and detergent agents. The enzymatic activity was observed in presence of urea up to a 1% (w/v) concentration. The enzyme could also be used in the presence of organic solvents such as acetone or dioxane (5%, v/v) without loss of activity.

Key words: Xylanase, Thermostable Termitomyces sp., Macrotermes subhyalinus, Termitidae.

INTRODUCTION

The Macrotermitinae termites are detrivores, feeding on dead wood, leaves or glass. Some are able to feed on living plants also; however, when they do so, they are generally considered as crop pests (Sands, 1962, 1973, 1977). The degradation of plant material by Macrotermitinae is to a great extent due to their double symbiosis: endosymbiosis and exosymbiosis with a fungus, Termitomyces sp. (Grasse, 1982; Rouland et al., 1990; Kouame et al., 2005). Termitomyces is found as mycelia in the fungus comb surface that have been called "mycotetes" (Heim, 1977). 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). Biochemical studies of the fungus-growing forest termite Macrotermes mulleri confirmed this

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hypothesis. The digestive tracts of these insects contain endoxylanase and cellulases from two different organisms: a fungal endocellulase and a termite exocellulase produced by the salivary glands (Rouland et al., 1988). Nevertheless, physiological result from savanna termites Macrotermes michaelseni (Sjöstedt) and M. subhyalinus can supply no evidence to support the acquired enzymes hypothesis (Slaytor, 1992).

The major structure of hemicellulose is xylan, which is a polymer of β-1,4-linked xyloses with arabinosyl and/or 4-O-methylglucurosyl side chains (Whistler and Richards, 1970). The enzymatic degradation of xylan to xylose requires the catalysis of both endo-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37). Xylanase occur widely in bacteria and fungi. Many reports on xylanases from Bacillus spp., Clostridium spp., Streptomyces spp., Aspergilus spp., and other microorganisms are available (Bastawde, 1992; Wong et al., 1988). Potential applications of xylanase in biotechnology include biopulping wood (Eriksson, 1985; Eriksson and Kirk, 1985), pulp bleaching (Jurasek and Paice, 1988; Kantelinen et al., 1988; Noé et al., 1986), treating animal feed to increase digestibility (Wong et al., 1988), processing food to increase clarification (Biely, 1985; Dekker, 1985), and converting lignocellulosic substances into feedstocks and fuels (Eriksson, 1985; Jeffries, 1985). It is characteristic that most xylanolytic microorganisms produce multiple xylanases with different physicochemical properties (Wong et al., 1988).

The present report deals with the purification and the biochemical properties of a new thermostable xylanase from the symbiotic fungus Termitomyces sp. of the termite Macrotermes subhyalinus (Termitidae-Macrotermitinae).

MATERIALS AND METHODS

Enzymatic source and preparation of crude extract

Termitomyces sp. were collected from Lamto (Cote d'Ivoire) directly from the nest, and then stored at -20°C. After thawing, the fungi (2 g) were washed with distilled water, then harvested by centrifugation and resuspended in 10 ml 0.9% (w/v) NaCl solution. Disruption of fungi took place in an Ultra-Turrax type T25 followed by sonication 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, 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 chemical 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 acetate buffer (pH 5.4). After washing the column with the same buffer, a 60 ml increasing discontinue gradient (0-200 mM) of NaCl dissolved in 20 mM acetate buffer (pH 5.4) was applied to the column. Proteins were eluted at a flow rate of 60 ml/h and fractions of 2 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 acetate buffer (pH 5.4). After washing the column with the same buffer, a 40 ml increasing discontinue gradient (0-200 mM) of NaCl dissolved in 20 mM acetate buffer (pH 5.4) was applied to the column. The flow rate was 56 ml/h and fractions of 2 ml were collected. The fractions containing the enzyme were pooled and extensively concentrated by adding ammonium sulphate to 80% final saturation. After centrifugation at 13 000 g for 30 min, the precipitate was dissolved in 20 mM acetate buffer (pH 5.4) and the resulting solution was passed through a Sephacryl S-200 HR column (1.6 X 65 cm) that had been equilibrated at the same buffer, at a flow rate of 30 ml/h; fractions of 1 ml were collected. The active fractions were pooled and put in a solid sodium thiosulphate 1.7 M, and then loaded onto a Phenyl-Sepharose CL-4B column (1.6 X 4.5 cm) equilibrated with 20 mM acetate buffer (pH 5.4) containing 1.7 M sodium thiosulphate. 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 discontinue gradient (1.7-0 M) of acetate buffer (pH 5.4). Finally, the active fractions were pooled, extensively dialyzed against 20 mM acetate buffer (pH 5.4) and stored at 4°C.

Enzyme assays

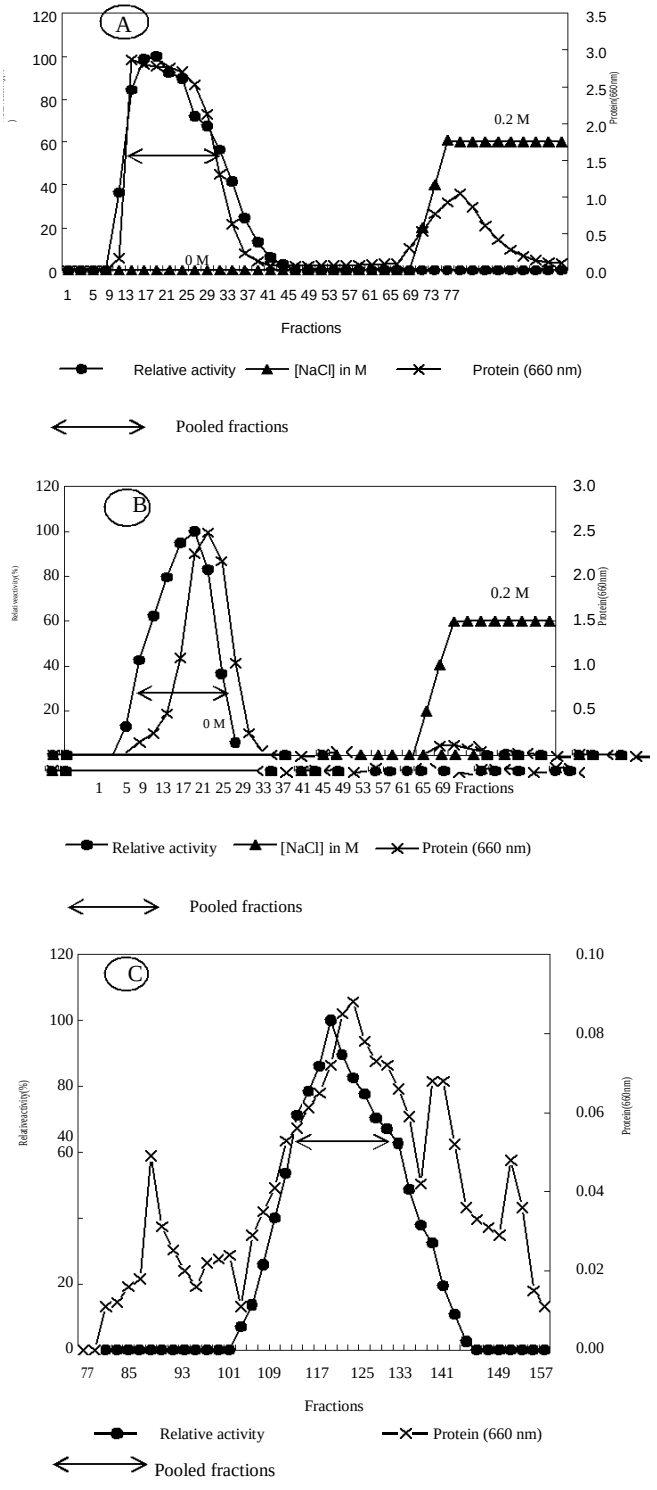
Xylanase assays were performed by incubating 250 Tl of beechwood xylan (1%, w/v) suspension in 100 mM acetate buffer (pH 5.4) with 50 Tl of enzyme solution at 60°C for 10 min. Other substrates (1% or 5 mM) were tested under the same conditions. Reactions stopped by the addition of either 300 Tl of 3,5-dinitrosalicylic acid reagent for natural substrates or 2 ml of 1 M Na_2CO_3 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 Tmol of xylose or release 1 Tmol 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 sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme sample were 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%



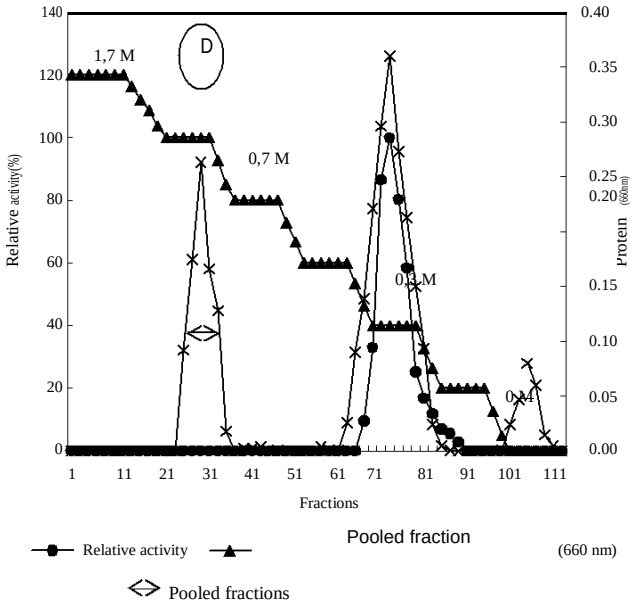


Figure 1: Purification of xylanase from symbiotic fungi Termytomyces sp. of termite Macrotermes subhyalinus. Enzyme activity was measured in acetate buffer pH 5.4 at 60 °C using xylan from beechwood as the substrate. (A) Anion-exchange chromatography on DEAE-Sepharose CL-6B column. (B) Cation-exchange chromatography on CM-Sepharose CL-4B column. (C) Gel filtration on Sephacryl S-200 HR column. (D) Hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B column. Activity (○); protein (×). For further details, see “Matérials and methods”.

(v/v) 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 Coomassie brillant blue R-250.

Electrophoresis of the native enzyme was performed using essentially the same method, but without SDS and 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 60°C under the standard test conditions. For determination of the pH optimum, 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 7.6 to 9.0 and citrate phosphate buffer (100 mM) pH 3.0 to 7.0. For the pH-stability study, the enzyme solutions were preincubated at ambient temperature for 1 h in the sodium acetate and sodium phosphate buffer at various pH values between 3.6 and 8.0. After adjusting the mixtures to pH 5.6, the residual activity was measured under the standard assay conditions.

Table 1. Purification of Termitomyces sp. xylanase.

Purification step	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification factor
Crude extract	800	384	0.5	100	1
DEAE-Sepharose CL-6B	74.3	158.9	2.1	41.4	4.4
CM-Sepharose CL-4B	62	142.5	2.3	37.1	4.8
Sephacryl S-200 HR	5	54.4	10.8	14.2	22.5
Phenyl Sepharose CL-4B	0.3	7.7	26.4	2	55

Figure 2. Native-PAGE of crude Termitomyces sp. enzyme solution (A) and purified xylanase (B).

Effect of temperature on activity and stability

For determination of the temperature optimum, the incubation was performed for 10 min in 100 mM acetate buffer (pH 5.4) at temperature ranging from 35 to 80°C. The thermal inactivation of the xylanase was studied at 37, 60 and 70°C by prewarming the enzyme solutions in 100 mM acetate buffer (pH 5.4). Aliquots were removed at different times and residual activity was measured at 60°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 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 xylanase substrate specificity was performed with polysaccharide substrates (1%) incubated at 60°C from 10 min 100 mM acetate buffer (pH 5.4) with 50 Tl 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, birchwood xylan or carboxymethyl-cellulose. 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 Tl of 100 mM acetate buffer (pH 5.4) containing the tested substrate at the indicated concentration. After prewarming the mixture for 5 min at 60°C, the reaction was initiated by 75 Tl of the enzyme solution. Aliquots (300 Tl) were withdrawn at different times. The reaction was stopped and quantified as in the standard enzyme essay.

Analysis of xylobiose degradation products

The eventual hydrolysis of xylobiose (5 mM) substrate by Termitomyces sp. xylanase was tested with 50 Tl enzyme in 100 mM acetate buffer (pH 5.4) at 60°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 Tl) was spotted onto a TLC silica gel plate 60 F_{254} (E. Merck AG, Darmstadt, Germany) and chromatographed in a solvent system containing chloroform-acetic acid-water (6:7:1, v/v/v) at room temperature. Sugars were visualized by orcinol-sulfuric acid staining as described by Brückner (1955).

RESULTS

Enzyme purification

The thermostable xylanase was purified from Termitomyces sp. crude extract by a protocol comprising four chromatographic steps (Figure 1). The pigments, which are in the crude extract, were almost completely removed during the DEAE-Sepharose CL-6B step. In spite of relatively poor purification factor, the cation-exchange chromatography over a column of CM-Sepharose CL-6B enabled Termitomyces sp. xylanase to be separated from some glycosidases that are the most abundant enzymes in the termite, Macrotermes subhyalinus, and its symbiotic fungus, Termitomyces sp., crude extract. The latter and the gel filtration step are interesting because most glycosidases, which are present in the crude extract, were retained at the top of the column and thus were eliminated from enzymatic fractions. The final step, involving a hydrophobic inter-

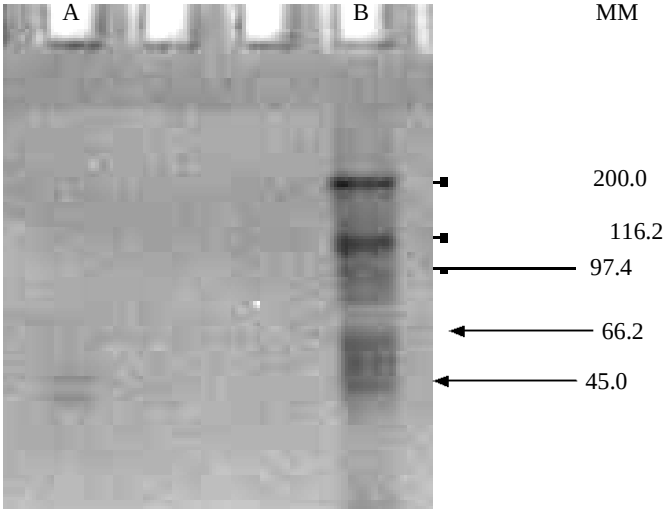


Figure 3. SDS-PAGE of purified Termitomyces sp. xylanase. Samples were analysed in a 10% polyacrylamide gel. Proteins were visualized by Coomassie brillant blue R-250. Lane A, purified enzyme; Lane B, molecular weight markers (values in kDa). The position of molecular weight markers are indicated (MM).

action chromatography over a column of phenyl-Sepharose, was crucial to separate the xylanase from another less specific glycosidase, capable of catalysing the cleavage of different glycosidasic bonds. After purification, the enzyme was enriched about 55-fold and the yield was 2% (Table 1).

Molecular properties

The enzyme showed a single protein band by polyacrylamide gel electrophoresis (Figure 2). The relative molecular mass (Mr) of the xylanase, as determined by gel filtration, was approximately 80 kDa. In SDS-PAGE, the enzyme displayed two protein bands with Mr of 41.5 and 45.5 kDa (Figure 3). Taken together, these results suggest that the enzyme possesses a dimeric structure.

pH and temperature dependence

The effect of pH on the catalytic activity of the xylanase was studied at 60°C by measuring the hydrolysis in 100 mM buffer (pH 3.0-9.0). Maximum activity was obtained at pH 5.6, but the enzyme retained more than 80% of its activity in the range pH 5.0-6.0. A maximum activity was also found at pH 5.6 in sodium phosphate and citrate phosphate buffers but, in these cases, the activity was reduced by about 10 and 20%, respectively, when compared to that obtained in sodium acetate buffer (data not shown). The dependence of the enzyme activity on the temperature was studied at pH 5.4 (Table 2) using the same substrate. For a 10 min incubation, the maximum activity was observed at 65-70°C and the value of the temperature coefficient (Q_{10}), calculated between 50 and 60°C was found to be 1.5. The latter is much lower than that observed for most enzymes (Q_{10} around 2.0). From Arrhenius plot, a value of 49.8 kJ/mol was calculated for the activation energy (Table 2).

Table 2. Physicochemical properties of purifed Termitomyces sp. xylanase.

Physicochemical properties	Termitomyces sp. xylanase
Molecular mass	
SDS-PAGE	87 kDa
Gel filtration	80 kDa
Optimum pH	5.6
Stability of pH	4.6-5.6
Optimum temperature	65-70 (°C)
Activation energy	49.8 (Kj/mol)
Michaelis-Menten equation	Obeyed

Values given are the averages of at least three experiments.

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 it was preincubated for 1 h at ambient temperature in 100 mM acetate buffer pH 5.4 (Table 2).

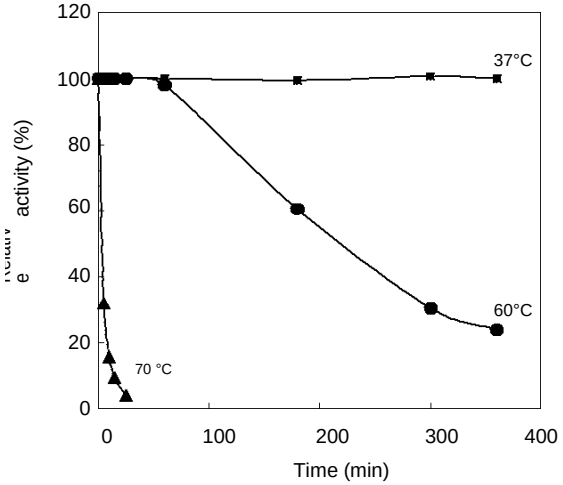


Figure 4. Thermal inactivation of Termitomyces sp. xylanase. The enzyme was preincubated at 37, 60 and 70°C in 100 mM sodium acetate buffer (pH 5.4). At the indicated times, aliquots were withdrawn and the residual enzymatic activity was measured at 60°C under the standard assay conditions, as expressed as percentage activity of zero time control of untreated enzyme.Values given are the averages of at least three experiment

Table 3. Substrate specificity of the purified fungus Termitomyces sp. xylanase.

Substrate	Concentration in assay	Activity (%)
αand β Beechwood xylan	1% (w/v)	100
αand β Birchwood xylan	1% (w/v)	103.8
αand β Carboxymethylcellulose	1% (w/v)	30.1
αand β DA-methyl Xylan	1% (w/v)	0
αand β Polygalacturonic acid	1% (w/v)	0
αand β Starch	1% (w/v)	0
αand β Inulin	1% (w/v)	0
αand β Xylobiose	5 mM	0
αand β Nitrophenyl-β-D-Xylopyranoside	5 mM	0
αand β Nitrophenyl-β-D-Glucopyranoside	5 mM	0
αand β Nitrophenyl-β-D-Fucopyranoside	5 mM	0
αand β Nitrophenyl-β-D-Galactopyranoside	5 mM	0
αand β Nitrophenyl-β-D-Cellobioside	5 mM	0
αand β Nitrophenyl-α-D-Glucopyranoside	5 mM	0
αand β Nitrophenyl-α-D-Fucopyranoside	5 mM	0
αand β Nitrophenyl-α-D-Mannopyranoside	5 mM	0
αand β Nitrophenyl-α-D-arabinopyranoside	5 mM	0

Values given are the averages of at least three experiments.

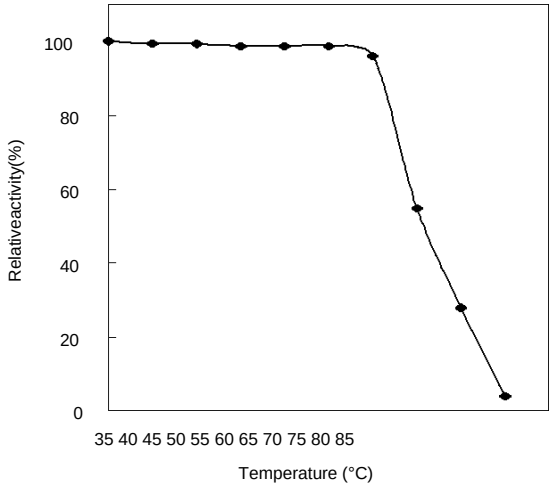


Figure 5. Thermal denaturation of Termitomyces sp. xylanase. The enzyme was maintained for 10 min at the indicated temperatures in 100 mM sodium acetate buffer (pH 5.4). The residual activity was then measured at 60°C under the standard assay conditions. Values given are the averages of at least three experiments.

Thermal stability

Figure 4 shows the thermal inactivation kinetics performed at 37, 60 and 70°C. After 6 h incubation at 60°C, the enzyme is fully stable for 1 h. The half-life of the xylanase at 60°C was more than 3 h. The xylanase is rapidly inactivated at 70°C. At this temperature, the half-life of the enzyme was 4 min and it completely lost its activity after treatment for 30 min (Figure 4). The effect of temperature on the enzyme stability was also investigated by preincubating enzyme solutions for 15 min at different temperatures (Figure 5). The enzyme retained 100% of its activity up to 65°C. At higher temperature, thermostability decreased rapidly.

Effect of metal ions

The activator or inhibitor effects of mono- and divalent cations on the enzyme activity were studied (Table 5). K^{+} and Mn^{2+} slightly activated the enzyme. Cu^{2+}, Zn^{2+} and Fe^{2+} strongly inhibited the activity, whereas Na^{+}, Ca^{2+} and Ba^{2+} had much more limited inhibitory effect.

Substrate specificity and kinetic parameters

A variety of glycosides were tested for their ability to serve as substrates. The xylanase has a high specificity for unsubstitute xylan. It catalyzes the hydrolysis of beechwood xylan and birchwood xylan, but not DA-methyl xylan. The enzyme can also hydrolyse carboxymethylcellulose but at a much lower rate. However, neither xylobiose (data not shown), acid polygalacturonic, starch, inulin, nor p-nitrophenyl-glycosides activity was detected (Table 3). For purposes of comparison, the kinetic parameters were determined, in particular, for three substrates: Beechwood xylan, Birchwood xylan and carboxymethylcellulose. With the three substrates, the enzyme obeyed the Michaelis-Menten equation. Examination of Table 4 shows that the

Table 4. Kinetic parameters of purified Termitomyces sp. xylanase.

Substrate	K_M(mg/ml)	Vmax (UI/mg of protein)	Vmax/K_M
Beechwood xylan	4.3	35.1	8.2
Birchwood xylan	1.6	13.3	8.5
Carboxymethylcellulose	5.6	9.1	1.6

Values given are the averages of at least three experiments.

Table 5. Effect of metal ions on the activity of purified Termitomyces sp. xylanase.

Metal ion (1 mM)	Xylanolytic activity (% of control)
None	100
Na^+	100 ± 2
K^+	118 ± 1
Mn^{2+}	108 ± 3
Ca^{2+}	95.9 ± 2
Ba^{2+}	90.3 ± 4
Zn^{2+}	56.2 ± 3
Fe^{2+}	51.6 ± 3
Cu^{2+}	11.5 ± 1

Values given are the averages of at least three experiments.

enzyme specificity is largely dependent on the nature of the substrate tested. The K_M and V_{max} values are reported in Table 4. Although the value of V_{max} is in favor of beechwood, the V_{max} / K_M ratio, known to be more significant parameter with respect to catalytic efficiency, is much higher for birchwood than for beechwood and carboxymethylcellulose (Table 4).

Effect of of dithio-reducing agents, detergents and organic solvents

The scission of disulfide bonds by performic acid oxidation is a classic technique in protein chemistry. This method leads not only to transformations of tryptophan residues but also may oxidize, under drastic experimental conditions, phenolic groups and the hydroxyl functions of serine and threonine residues (Hirs, 1967). As a consequence, procedures which involve reductive cleavage and subsequent blocking of the thiol groups must be preferred for quantitative digestion by the xylanase of polysaccharide or glycoprotein substrates containing disulfide bonds. -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 fungus xylanase. At a concentration of 1% (v/v) in the reaction mixture, the -mercaptoethanol activated the enzyme activity by about 90%. The other reductive agents such as cysteine, dithiothreitol and DTNB displayed behaviors identical to that of -mercaptoethanol (Table 6).

The effect of ionic and nonionic detergents currently used for denaturing of glycoproteins was tested on the xylanase activity. The result reported in Table 6 shows that all of them, except Nonidet P40, are inhibitors of Termitomyces sp. xylanase but to different degrees. As for urea, it can be present in the reaction mixture up to concentration of 1% without loss of the xylanase activity (Table 6).

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 xylanase activity. For up to 5% concentrations of all the tested organic solvents, the xylanase retained more than 80% of its initial activity. An activation of the enzyme was even observed with acetone (Figure 6).

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; Kouame et al., 2001 and 2005). However, no or few enzymatic works concerning symbiotic fungus Termitolyces sp. of termites Macrotermes subhyalinus have been so far reported. The xylanase from the crude extract of Termitomyces sp. was purified to homogeneity using standard techniques i.e. anion-exchange DEAE-Sepharose CL-6B chromatography, cation-exchange CM-Sepharose CL-6B chromatography, Sephacryl S-200 HR gel filtration chromatography and Phenyl Sepharose CL-4B hydrophobic interaction chromatography. The latter chromatography was crucial to separate the xylanase from another glycosidase of the crude extract which is less specific. A similar result concerning Phenyl-Sepharose gel has been reported for the purification of the specific endopeptidase Thr-N from Archachatina ventricosa digestive juice (Niamké et al., 1999). The final purification factor was higher than that obtained for the xylanase from Staphylococcus sp. SG-13 (12-fold) (Gupta et al., 2000) but it was very lower compared with

Table 6. Effect of detergents and reducing agents on the activity of purified Termitomyces sp. xylanase.

Reagent	Conc.	Xylanolytic activity (% of control)
None		100
Nonidet P 40	1% (w/v)	104.6 ±1
Triton X-100	1% (v/v)	95.5 ± 4
Tween 80	1% (v/v)	97.2 ± 3
Lubrol Wx	1% (w/v)	97.9 ± 3
Urea	1% (w/v)	101.8 ± 2
Sodium dodecyl sulfate (SDS)	1% (w/v)	36.9 ± 2
L-cysteine	1% (w/v)	200.9 ±1
-mercaptoethanol	1% (v/v)	191.2 ± 2
DL-dithiothreitol	1% (w/v)	114.9 ± 1
DTNB	0.5% (w/v)	115.7 ± 2
pCMB	0.5% (w/v)	95.3 ± 2

DTNB = dithionitrobenzoate; pCMB = para-chloromercurybenzoate
Values given are the averages of at least three experiments.

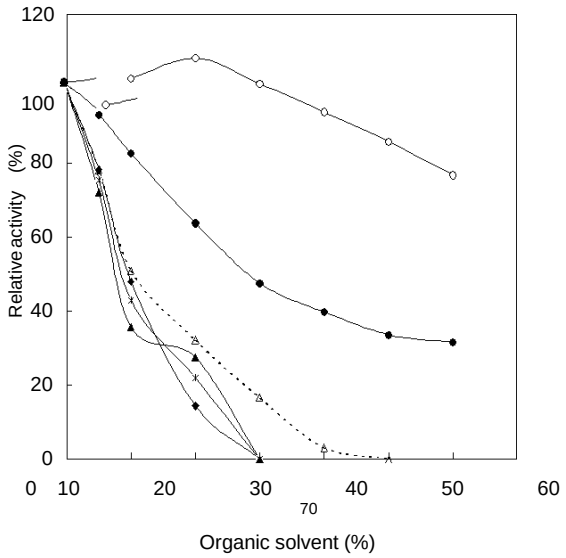


Figure 6. Effect of organic solvents on the activity of Termitomyces sp. xylanase. The enzyme was incubated at 60°C in 100 mM sodium acetate buffer (pH 5.4). The enzyme activity was measured in the presence of methanol (▲), ethanol (⋆), butanol (♦), acetone (○), acetonitrile (Δ) and dioxane (d). Values given are the averages of at least three experiments.

that reported for xylanase from Alcaligenes sp. (292-fold) (Araki et al., 1998).The purified enzyme is dimeric with a relative molecular mass estimated to 80-87 kDa. This Mr value of this enzyme is similar to those of the group of xylanases which has been assigned to the category of high-Mr, acidic xylanases, in contrast to low-Mr, basic xylanases (Wong et al., 1988).

Since only relatively large oligosaccharides would be liberated by the action of the xylanase 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 the xylanase to be optimized. The activity of the enzyme is maximal at pH 5.6 but it displays a better stability at pH 4.6-5.6. So, a pH of 5.4 is a good compromise between the activity and stability of the enzyme to perform the specific hydrolysis of xylan over a long time-period. This optimum pH is similar to fungus xylanases from Aspergilus sp. (Camacho and Aguilar, 2003), Arthrographis sp. strain F4 (Okeke and Obi, 1995) but different from xylanases of Aureobasidium pullulans Y-2311-1 (pH 4.8) (Li et al., 1993) and a marine fungus (pH 8.5) (Raghukumar et al., 2004). Like the xylanases characterized from different microorganisms, the pH stability of xylanase from the symbiotic fungus of the termite Macrotermes subhyalinus 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 enzyme activity being higher in a sodium acetate buffer than in a citrate phosphate or sodium phosphate buffers. The xylanase was optimally active at 65-70°C. The optimum temperature is lower than those reported for xylanases from the hyperthermophilic crenarchaeon sulfolobus solfataricus strain MT4 (90°C) (Cannio et al., 2004), the mutant Trichoderma reesei DB1 (82°C) (Jänis et al., 2004), but it higher than those obtained for the family 11 xylanase from Phanaerochaete chrysosporium cloned and expressed in Aspergillus niger (60°C) (Decelle et al., 2004) and the xylanase from Staphylococcus sp. SG-13 (Gupta et al., 2000). Termitomyces sp. xylanase exhibited a maximum activity at 37°C for a long time-period, for 1 h at 60°C and the half-lifes of the xylanase at 60 and 70°C were approximately 220 and 4 min,

respectively, at its 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 Termitomyces sp. xylanase in biobleaching of pulps. The xylanase is sensitive to Zn^{2+}, Fe^{2+}, Cu^{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 stimulation of activity by the reductive agents suggests that disulfide bonds play an essential role in the native conformation of Termitomyces sp. xylanase. The stimulation of fungus xylanase activity in the presence of Mn^{2+}, K^{+} and dithiol-reducing agents has previously been reported (Cesar and Mrsa, 1996; Fialho and Carmona, 2004) and is in accordance with the present study. 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 active 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 active in the presence of urea suggests that hydrogen bonds play a limited role in the stabilization of the glycosidase. The enzyme shows good stability in the presence of organic solvents, an activation even being observed with acetone but not with dioxane. The latter is particularly valuable to render soluble a certain number of macromolecules like, for example, the vegetable proteins (Colas et al., 1993).

Termitomyces sp. xylanase is specific for hydrolyzing natural xylan (nonsubstitute) and has a lower cellulase activity, which are desirable properties for biobleaching of pulps. Some xylanases have both xylanase and cellulase activities (Shareck et al., 1991). No para-nitrophenol is released by the effect of the enzyme on synthetic substrates. Also, no free xylose is produced from xylobiose by this enzyme. On the basis of these results, we suggest that Termitomyces sp. xylanase is an endo-β-1,4-xylanase. In spite of the narrow high specific activity of this enzyme toward natural xylan, the K_M of this enzyme is similar to that of xylanases from other sources (Camacho and Aguilar, 2003; Araki et al., 1998; Gupta et al., 2000).

Finally, the present study showed that the xylanase from the symbiotic fungus Termitomyces sp. of the termite Macrotermes subhyalinus is active in acid conditions, thermostable and showed high hydrolytically affinity for natural xylans and only a lower activity for CMC. Hence, it is 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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