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Purification and characterisation of two beta-glucosidases from termite workers *Macrotermes bellicosus* (Termitidae: Macrotermitinae)

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ABSTRACT

Objectives: The objective was to purify and characterise beta-glucosidases from termite *Macrotermes bellicosus* workers for use in glycobiotechnology and elucidate the role played by these enzymes in the degradation of plant material.

Methodology and results: Two beta-glucosidases were purified by a three-step procedure consisting of ion-exchange, size-exclusion and hydrophobic interaction chromatography techniques. Beta-Glc A and B had molecular weights estimated at 204 and 216 kDa, respectively, by SDS-PAGE; and 209 and 230 kDa, respectively, by gel filtration. Both enzymes exhibited the same temperature and pH optima for *p*-nitrophenyl-beta-D-glucopyranoside hydrolysis and were stable at 37°C. The enzymes showed a high specificity for the beta-glucosyl residue and preferred glucose-beta-(1-4) linkages to beta-(1-3), beta-(1-2) and beta-(1-6) linkages. Both beta-glucosidases were inhibited by sulfhydryl-binding reagents.

Conclusions and application of findings: The enzymes isolated in this study appear to be distinct from other known beta-glucosidases in terms of substrate specificity and low K_M value for cellobiose. The physiological role of the two beta-glucosidases in the digestive tract of the termite could be the digestion of di-and oligosaccharides derived from celluloses. The enzymes could be used as a tool in the structural analysis of D-glucose containing oligosaccharide chains of glycoproteins, glycolipids and cellulose.

Key words: beta-glucosidase, *Macrotermes bellicosus*, physiological role, termite.

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INTRODUCTION

Fungus-growing termites are distributed throughout tropical Africa and Asia (Yaovapa *et al*., 2005), where they are the dominant soil invertebrates (Wood & Thomas, 1989). These termites can have sizable nests that are capable of containing large volumes and may persist for decades. Their colonies contain millions of sterile helper individuals that are normally the offspring of a single queen (Shellman- Reeve, 1997). 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*. (Rouland *et al*., 1990; Grassé, 1992). These termites have a great impact on plant litter decomposition and carbon cycling in tropical ecosystems (Wood & Thomas, 1989; Yamada *et al*., 2005). Fungus-growing termites cultivate symbiotic fungi in their nest on a special substrate composed of dead plant material known as the fungus comb or

fungus garden (Wood & Thomas, 1989).

In most *Macrotermes* species, termite workers ingest dead plant material and deposit undigested or partially digested faeces on the top rim of the fungus comb. Thus, there is an age gradient within the fungus comb. As the fungus comb ages, decolourization from the top to the bottom of the fungus comb is observed. After a certain period, the aged part of the fungus comb is eaten by the host termites (exosymbiosis) (Wood & Thomas, 1989). 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) and microflora) (Rouland *et al*., 1990). The digestive tract of the fungus-growing termite *Macrotermes bellicosus* contains two xylanases from two different organisms: a fungal endoxylanase and a termite exoxylanase (Matoub & Rouland, 1995). The synergistic actions of both xylanases play a significant role in hydrolysis of xylan as a staple for termite nutrition.

Cellulose, the major component of plant cell walls, is the most abundant polysaccharide in nature and a virtually inexhaustible source of renewable bio-energy (Priit *et al*., 2001). Its hydrolysis primarily depends on at least three enzymes. These include endo- and exo-cellulases, beta-glucosidase or cellobiase. The former two enzymes can degrade native cellulose synergistically to generate cellobiose which is a product inhibitor for these enzymes (Bhat & Bhat, 1997). Cellobiase 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-cellulase synthesis. Furthermore, when a beta-glucosidase preparation is added to lignocellulosic materials, it plays a major role in the release of phenolic compounds, suggesting that cellulose degrading enzymes may also be involved in facilitating the breakdown of polymeric phenolic matrices (Zeng & Shetty, 2000). In this paper, we describe the procedure for purifying two beta-glucosidases from termite *Macrotermes bellicosus* workers and provide data on their enzymatic as well as elucidate the role played by these enzymes in the degradation of plant material.

MATERIALS AND METHODS

Chemicals: The substrates cellobiose, sucrose, sophorose, laminaribiose, gentiobiose, xylobiose, lactose, maltose, arabino-galactan, carboxymethylcellulose, inulin, laminarin, xylan, lichenan, starch, glucose, xylose, cellodextrins and *p*-nitrophenyl-glycopyranosides were purchased from Sigma Aldrich. DEAE–Sepharose CL-6B, Sephacryl-S200 HR, 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.

Biological material: Workers of the termite *Macrotermes bellicosus* originated from the savanna of Lamto (Côte d'Ivoire). They were collected directly from the nest and then stored frozen at -20°C.

Purification procedures: The collected termite workers (2 g) were homogenized with 15 ml 0.9% NaCl (w/v) solution in an ultra-turrax and then sonicated as previously described by Rouland *et al*. (1988). The homogenate was centrifuged at 10000 x g for 15 min. The collected supernatant constituted the crude extract. Five (10) ml of crude extract was loaded onto a DEAE-Sepharose CL-6B (2.5 x 6.7 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. Proteins were eluted using a step wise gradient with 0.2; 0.3 ; 0.4 ; 0.5 and 1 M NaCl in 20 mM acetate buffer pH 5.0. Fractions (2 ml each) were collected at a flow rate of 60 ml/h and assayed for enzyme activity. The elution with an acetate buffer 20 mM + 0.3 M NaCl (pH 5.0) provided a peak with beta-glucosidase activity (beta-Glc A). A second peak with beta-glucosidase activity (beta-Glc B) was recovered with 20 mM acetate buffer + 0.5 M NaCl (pH 5.0). Each fraction was pooled separately and saturated overnight by 80 % ammonium sulphate in a cold room. The precipitated pellets were then separed by centrifugation at 10000 x g for 30 min and dissolved in 1 ml of 20 mM acetate buffer pH 5.0. These mixtures were chromatographed separately using a Sephacryl S-200 HR column (1.6 x 65 cm) which was pre-equilibrated with the same buffer pH 5.0. Fractions of 1 ml were collected. Proteins were eluted at a flow rate of 30 ml/h using 20 mM acetate buffer pH 5.0. Elution provided a peak with beta-glucosidase activity and active fractions were pooled together. The proteins giving each peak of activity from the previous

step were pooled separately and saturated to a final concentration of 1.7 M ammonium sulphate and applied on a Phenyl-Sepharose CL-4B column (1.4 x 4.5 cm) previously equilibrated with 20 mM acetate buffer pH 5.0 containing 1.7 M ammonium sulphate. The column was washed with equilibration buffer and the proteins retained were then eluted using a stepwise gradient with 1.0; 0.5; 0.3; 0.2; 0 M ammonium sulphate in 20 mM acetate buffer pH 5.0. Fractions of 1 ml were collected at a flow rate of 60 ml/h and active fractions were pooled together. The elution with an acetate buffer 20 mM + 0.2 M NaCl (pH 5.0) provided a peak with beta-glucosidase activity (beta-Glc A). A second peak with beta-glucosidase activity (beta-Glc B) was recovered with 20 mM acetate buffer (pH 5.0). The pooled fraction was dialysed against 20 mM phosphate buffer pH 6.0 overnight in a cold room.

Determination of enzyme activity and protein concentration: Under the standard test conditions, hydrolytic activity of beta-glucosidase was determined by measuring *p*-nitrophenol released from *p*-nitrophenyl-beta-D-glucopyranoside (Yapi *et al*., 2007). An assay mixture (275 µl) consisting of a 20 mM phosphate buffer (pH 6.0), 1.25 mM *p*-nitrophenyl-beta-D-glucopyranoside and enzyme solution was incubated at 37°C for 10 min. The reference cell contained all reactants except the enzyme. Determination of other *p*-nitrophenylglycosidase activities was carried out under the same experimental conditions. The reaction was stopped by adding 2 ml of 1.0 M sodium carbonate, and the absorbance of the reaction mixture measured at 410 nm (Yapi *et al*., 2007).

The oligo-saccharidase activity was determined by measuring the amount of glucose or xylose liberated from 10 mM oligosaccharide (cellobiose, cellodextrins, laminaribiose, sophorose, gentiobiose, sucrose, lactose and maltose) after incubation at 37°C for 10 min in a 20 mM phosphate buffer (pH 6.0) (Yapi *et al*., 2007). 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 high-performance liquid chromatography (HPLC) after heating the reaction mixture at 100°C for 5 min (Yapi *et al*., 2007).

The polysaccharidase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 1 % (w/v) polysaccharide (arabino-galactan, carboxymethylcellulose, inulin, lichenan, laminarin, xylan and starch) as substrate. The enzyme (100 µl) was incubated for 30 min at 37°C with 200 µl buffer (20 mM phosphate, pH 6.0) and 100 µl polysaccharide (Yapi *et al*., 2007). The reaction was stopped by addition of 300 µl DNS and heating in boiling water for 5 min. The absorbance was read at 540 nm after cooling on ice for 5 min (Yapi *et al*., 2007).

One unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing one µmol of *p*-nitrophenol or glucose per min under the defined reaction conditions. Specific activity was expressed as units per mg of protein (U/mg of protein).

Protein was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as a standard.

Determination of homogeneity and molecular weight: To check purity and determine molecular weight, the purified enzyme was analyzed using polyacrylamide gel electrophoresis on a 12% 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 a 0.25% (w/v) Coomassie Brillant Blue R-250 solution containing 40% (v/v) methanol and 10% (v/v) acetic acid. The sample was denatured by a 5 min treatment at 100°C. Electrophoretic buffers contained sodium dodecyl sulfate (SDS) and beta-mercaptoethanol.

The native molecular weight of the enzyme was determined using gel filtration chromatography in a HPLC system. The column TSK (2.5 cm x 52 cm); QC-PAK GFC 200) equilibrated and eluted in 20 mM phosphate buffer (pH 6.0) containing sodium azide 0.5 % (w/v) was calibrated with beta-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (48.8 kDa) and cytochrome C (12.4 kDa). Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min.

Temperature and pH optima: The effect of pH on beta-glucosidase activity was determined by measuring the hydrolysis of *p*-nitrophenyl-beta-D-glucopyranoside in a series of buffers at various pH values ranging from pH 3.6 to 8.0. Acetate buffer (20 mM) from pH 3.6 - 5.6 and phosphate buffer (20 mM) from pH 5.6 - 8.0 were used. The pH values of each buffer were determined at 37°C. Beta-glucosidase activity was measured at 37°C under the conditions described above. The effect of temperature on beta-glucosidase activity was monitored in 20 mM phosphate buffer at pH 6.0 over a temperature range of 30 - 80°C using 1.25 mM *p*-nitrophenyl-beta-D-glucopyranoside as described above.

pH and temperature stabilities: The stability of beta-glucosidase was monitored over pH range of 3.6

to 8.0 in the same buffers as described the preceding section (above). After 2h incubation at 37°C, aliquots were taken and immediately assayed for residual beta-glucosidase activity. The thermal stability of the enzyme was determined at 37 and 45°C, after exposure to each temperature for a period of 10 to 60 min. The enzyme was incubated in 20 mM phosphate buffer (pH 6.0). Aliquots were drawn at intervals and immediately cooled in ice-cold water. Residual activities, determined in both cases at 37°C under the standard test conditions, are expressed as percentage activity by comparison with untreated enzyme.

Determination of kinetic parameters: The kinetic parameters (K_M, V_{max} and V_{max}/K_M) were determined in 20 mM phosphate buffer (pH 6.0) at 37°C. Hydrolysis of *p*-nitrophenyl-beta-D-glucopyranoside was quantified on the basis of released *p*-nitrophenol, similar to the standard enzyme assay. Cellobiose hydrolysis was quantified by determination of released glucose, determined by the oxidase-peroxidase method (Kunst *et al*., 1984) after heating the reaction mixture at 100°C for 5 min. K_M and V_{max} were determined from Lineweaver-Burk plot using different concentrations of *p*-nitrophenyl-beta-D-glucopyranoside (1-10 mM) and cellobiose (1-20 mM).

Effect of chemical agents: The enzyme was incubated with 1 mM or 0.1 % (w/v) of various cations in the form of chlorides for 20 min at 37ºC. After incubation, the residual activity was determined by the standard enzyme assay using *p*-nitrophenyl-beta-D-glucopyranoside as a substrate. The activity of enzyme assayed in the absence of the chemical agents was taken as 100%.

RESULTS

A summary of purification of the beta-glucosidases is shown in Table I. The purified beta-Gucosidases A and B were enriched to about 39.42 and 125.46-fold, respectively, and the yields were 11.67 and 26.53 %, respectively. The specific activities were 29.57 and 94.10 U/mg protein for beta-Glc A and B, respectively. Each enzyme showed a single protein band by SDS-PAGE. The molecular weights of purified enzymes were 204 kDa for beta-Glc A by SDS and 209 kDa by TSK (QC-PAK GFC 200) while beta-Glc B had 216 kDa by SDS and 230 kDa by TSK (QC-PAK GFC 200) (Table 2).

Beta-Glc A was most active at pH 6.0 (Table 2) and the enzyme retained more than 60% of its activity at pH range 5.0 - 6.6 (Figure 1). At 37°C, the enzyme was stable over a wide pH range of 5.6 -6.6 for up to 120 min (Table 2). The optimum temperature for the hydrolysis of *p*-nitrophenyl-beta-D-glucopyranoside was 45°C (Figure 2) but, Beta-Glc A was unstable at this temperature (Figure 3). At 37°C, the enzyme was stable for 60 min in 20 mM phosphate buffer (pH 6.0) while at 45°C, the half life of the beta-Glc A was reduced to 15 min (Figure 3). Beta-Glc A completely lost its activity after treatment for 40 min (Table 2). The value of the temperature coefficient (Q10) calculated between 30 - 40°C was 1.86, the activation energy was 38.16 KJ/mol as calculated from the Arrhenius plot (Table 2).

Table 1: Purification of beta-glucosidases A and B from termite *Macrotermes bellicosus* workers.

Purification steps	Total protein (mg)	Total activity (UI)	Specific activity (UI/mg)	Yied (%)	Purification factor
Crude extract	47.30	35.47	0.75	100	1.00
DEAE-Sepharose CL-6B					
Beta-Glc A	3.20	7.45	2.32	46.66	3.01
Beta-Glc B	2.76	15.01	5.43	42.32	7.24
Ammonium sulphate precipitation					
Beta-Glc A	2.23	6.13	2.74	17.28	3.65
Beta-Glc B	2.12	13.03	6.14	36.74	8.18
Sephacryl S-200 HR					
Beta-Glc A	0.62	5.36	8.64	15.11	11.52
Beta-Glc B	0.32	11.26	35.18	31.75	47.00
Phenyl Sepharose CL-4B					
Beta-Glc A	0.14	4.14	29.57	11.67	39.42
Beta-Glc B	0.10	9.41	94.10	26.53	125.46

Table 2: Some physicochemical characteristics of the beta-glucosidases from the termite *Macrotermes bellicosus* workers (beta-Glc A and beta-Glc B).

Physicochemical properties	beta-Glc A	beta-Glc B
Optimum pH	6.0	6.0
pH stability range	5.6-6.6	5.0-6.6
Optimum temperature (°C)	45	45
Half life at 45°C (min)	15	50
Activation energy (KJ/mol)	38.16	42.84
Temperature coefficient (Q10)	1.86	1.90
Michaelis equation	obeyed	obeyed
Molecular weight		
Mobility in SDS-PAGE (kDa)	204	216
Gel filtration (kDa)	209	230

For beta-Glc B, the maximal activity was observed around pH 6.0 (Table 2). The enzyme retained stability and more than 40% of its activity in the range pH 5.0 to 6.6, being active for more than 120 min at 37°C (Table 2). The optimum temperature for the hydrolysis of *p*-nitrophenyl-beta-D-glucopyranoside was 45°C (Table 2). At 45°C, it the half life of the beta-Glc B was 50 min and the value of the temperature coefficient (Q10) calculated between 30 - 40°C was 1.90 (Table 2). From the Arrhenius plot, the activation energy was determined to be 42.84 KJ/mol.

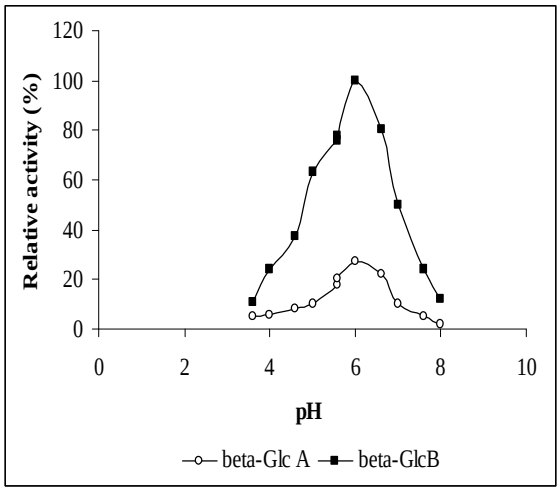

Figure 1: Effect of pH on the beta-glucosidases from the termite *Macrotermes bellicosus* workers.

Substrate specificity and kinetic parameters: Some of the *p*-nitrophenyl glycosides that were not attacked by the purified Beta-Glc A and B were alpha-glucoside, beta and alpha-galactoside, alpha and beta-mannoside, alpha and beta-xyloside, alpha and beta-L-arabinoside, beta and alpha-fucoside. Oligosaccharides not attacked included sucrose, lactose, xylobiose, maltose while polysaccharides were arabino-galactan, carboxymethylcellulose, inulin, lichenan, laminarin, xylan and starch (Table 3). Although both beta-Glc A and B acted on laminaribiose and to a lesser degree sophorose and gentiobiose, they were clearly more active on cellobiose, cellodextrins and *p*-nitrophenyl-beta-D-glucopyranoside (Table 3).

The effect of substrate concentration on enzymatic activity was studied with cellobiose and *p*-nitrophenyl-beta-D-glucopyranoside. With the two substrates, beta-Glc A and B obeyed the Michaelis-Menten equation (Table 2). The K_M, V_{max} and V_{max}/K_M values are shown in table 4. The catalytic efficiency of beta-glucosidase, given by the V_{max}/K_M ratio is much higher for the *p*-nitrophenyl-beta-D-glucopyranoside than the cellobiose (Table 4).

Effect of chemical agents on enzyme activity: The chemical agents NaCl, KCl, $CaCl_2$, $FeCl_2$, $MnCl_2$ and $BaCl_2$ had no effect on beta-Glc A and B activities (Table 5). However, Tris, DTNB (5, 5-dithio-bis (2-nitrobenzoate), *p*CMB (*p*-chloromercuribenzoate), SDS, L-cysteine and beta-mercaptoethanol had an inhibitory effect on the enzymes (Table 5).

Table 3: Activities of the beta-glucosidases from the termite *Macrotermes bellicosus* workers (beta-Glc A and beta-Glc B) on oligosaccharide, polysaccharide and synthetic chromogenic substrates.

Substrates	Relative rate of hydrolysis (%)	
Oligosaccharide and polysaccharide	beta-Glc A	beta-Glc B
Cellobiose	100	100
Cellotriose	152	122
Cellotetraose	131	143
Cellopentaose	111	97
Laminaribiose	45	32
Sophorose	9	12
Gentiobiose	4	5
Arabino-galactan	0	0
Carboxymethylcellulose	0	0
Inulin	0	0
Lichenan	0	0
Laminarin	0	0
Xylan	0	0
Starch	0	0
Synthetic chromogenic substrates	Relative rate of hydrolysis (%)	
p-nitrophenyl-beta-D-glucopyranoside	100	100
p-nitrophenyl-alpha-D-glucopyranoside	0	0
p-nitrophenyl-beta-D-galactopyranoside	0	0
p-nitrophenyl-alpha-D-galactopyranoside	0	0
p-nitrophenyl-beta-D-mannopyranoside	0	0
p-nitrophenyl-alpha-D-mannopyranoside	0	0
p-nitrophenyl-beta-D-fucopyranoside	0	0
p-nitrophenyl-alpha-D-fucopyranoside	0	0
p-nitrophenyl-beta-D-xylopyranoside	0	0
p-nitrophenyl-alpha-D-xylopyranoside	0	0
p-nitrophenyl-beta-L-arabinofuranoside	0	0
p-nitrophenyl-alpha-L-arabinofuranoside	0	0

Table 4: Kinetic parameters of the beta-glucosidases from the termite *Macrotermes bellicosus* workers (beta-Glc A and beta-Glc B) towards *p*-nitrophenyl-beta-D-glucopyranoside and cellobiose.

Substrate	beta-Glc A			beta-Glc B		
	K_M (mM)	V_{max} (U)	V_{max}/K_M (U/mM)	K_M (mM)	V_{max} (U)	V_{max}/K_M (U/mM)
p-nitrophenyl-beta-D-glucopyranoside	1.25	83.33	66.66	0.25	140.16	560.64
Cellobiose [a]	1.22	57.6	47.21	1.53	89.52	168.90

[a] For cellobiose, the value of V_{max}/K_M was calculated taking into account that one mol of cellobiose liberates two mol of glucose

DISCUSSION

Two beta-glucosidases were purified to homogeneity from workers of the termite *Macrotermes bellicosus (Termitidae, Macrotermitinae)*. The specific activities of the enzymes that were purified in this study are lower than those obtained for the beta-glycosidases purified previously from *T. molitor* larvae midgut (Ferreira *et al*., 2001), midgut of the sugarcane borer *Diatraea saccharalis* (Azevedo *et al*., 2003) and workers of the termite *M. subhyalinus*. However, the purified beta-Glc A and B have higher specific activities than the beta-glucosidases from workers of termite *M. mulleri* (Rouland, 1992), *Fusobacterium* K-60, a human intestinal anaerobic bacterium (Park *et al*., 2001) and *T. molitor* larvae lumen (Ferreira *et al*., 2003). The optimal activity temperature of 45°C

determined for the purified beta-Glc A and B is higher than that of beta-glucosidases of *Pectobacterium carotovorum* (40°C) (Hong *et al*., 2006), rye (*Secale cereale*) seedlings (25-30 °C) (Sue *et al*., 2000) and *Citrus sinensis* var. Valencia fruit (40-45°C) (Cameron *et al*., 2001).

Table 5: Effect of various chemical agents on activity of beta-glucosidases A and B purified from termite *Macrotermes bellicosus* workers.

Chemical agents		Activity (%)	
Name	Concentration in assay (mM)	beta-Glc A	beta-Glc B
Control	0 mM	100	100
NaCl	1 mM	100	100
KCl	1 mM	100	100
$CaCl_2$	1 mM	100	100
$FeCl_2$	1 mM	100	100
$MnCl_2$	1 mM	100	100
$BaCl_2$	1 mM	100	100
Tris	1 mM	80	30
SDS	0.1 %	6	11
DTNB	0.1 %	60	90
*p*CMB	0.1 %	90	80
L-cysteine	0.1 %	60	70
beta-Mercaptoethanol	0.1 %	38	45

SDS, sodium dodecyl sulphate; DTNB, 5, 5-dithio-bis (2-nitrobenzoate); *p*CMB, *para*-hydroxymercuribenzoate

The enzymes characterized in this study had a pH optimum at 6.0, which is similar to that of beta-glucosidases from *Pyrearinus termitilluminans* (Colepicolo-Neto *et al*., 1986) and *Thaumetopoea pityocampa* (Pratviel-Sosa *et al*., 1987) but higher than that of the beta-glucosidases from Abracris *flavolineata* (4.8 and 5.5) (Marana *et al*., 1995), *Locusta migratoria* (5.5) (Morgan, 1975) and *Rhodnius prolixus* (4.5) (Terra *et al*., 1988). However, beta glucosidases from *Pectobacterium carotovorum* have a much higher pH optimum of 7.0 (Hong *et al*., 2006). The similarity in the molecular weights determined by denaturing SDS-PAGE and native gel filtration suggest that beta-glucosidase is likely to be monomeric, as found in human liver (Mutoh *et al*., 1988) and *Thermus* spIB-21 (Kang *et al*., 2005) beta-glucosidases. The two enzymes were inhibited by 5, 5-dithio-bis (2-nitrobenzoate) (DTNB), *p*-chloromercuribenzoate (*p*CMB), cysteine and mercaptoethanol indicating that sulfhydryl group may be involved in both formation and maintenance of the three-dimensional protein structure, and catalytic activity in beta-glucosidases. This result is in agreement with those for the beta-glucosidases from rat kidney (Glew *et al*., 1976), *Brassica napus* (Falk & Rask, 1995) and maize (Esen, 1992).

A variety of glycosides were tested for their ability to serve as substrates for the purified enzymes. The purified beta-glucosidases A and B were inactive on high molecular mass polymers such as lichenan, xylan, carboxymethylcellulose, inulin, starch, arabino-galactan and laminarin. The purified enzymes also had no contaminating glycosidase activities such as beta-galactosidase, beta-fucosidase, beta-mannosidase, beta-arabinosidase and beta-xylosidase. The only substrates that were hydrolyzed by the enzymes were cellobiose, cellodextrins, laminaribiose, sophorose, gentiobiose and *p*-nitrophenyl-beta-D-glucopyranoside. This result suggests that the two beta-glucosidases are exo-glycosidases, and have no polysaccharidase activities. Further, these enzymes appear to have a high specificity for the beta-anomeric configuration of the glucosidic linkage, a pattern similar to that of beta-glucosidase activities from *Sclerotium rolfsii* (Shewale & Sadana, 1981) and *Aspergillus niger* (Watanabe *et al*., 1992).

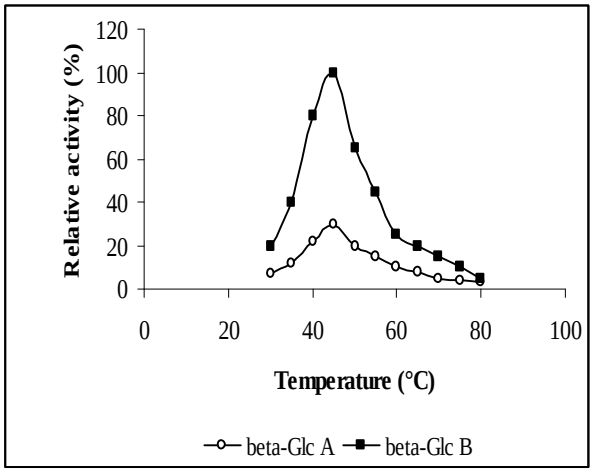

Figure 2: Effect of temperature on activity of beta-glucosidases A and B from the termite *Macrotermes bellicosus* workers.

The substrate specificity of the purified beta-glucosidases (from termites) differs from that of similar enzymes from insects and microorganism (Dion *et al*., 1999; Xiangyuan *et al*., 2001; Ferreira *et al*., 2002; Li *et al.,* 2002; Azevedo *et al*., 2003; Wallecha & Mishra, 2003 ; Kouamé *et al*., 2005). Although inability to cleave alpha-linkages is commonly reported for purified beta-glucosidases, the high substrate specificity suggests that the two beta-glucosidases could be used as a tool in the structural analysis of D-glucose containing oligosaccharide chains of glycoproteins, glycolipids, and cellulose.

Beta-glucosidase activities exhibited Michaelis Menten type kinetics and the K_M for cellobiose (a key product of cellulose hydrolysis by exo- and endo-glucanase) are much lower than those reported for other *Aspergillus* species (Workman & Day, 1982; Wase *et al.*, 1985). The low K_M value obtained, compared with other organisms (Sternberg *et al*., 1977), suggests that the purified enzymes have high affinity towards its substrate and could possibly be used for industrial saccharification.

In conclusion, the two beta-glucosidases that were purified from workers of the termite *Macrotermes bellicosus (Termitidae, Macrotermitinae* in this study appear to be distinct from other beta-glucosidases reported so far, in terms of substrate specificity and high affinity towards cellobiose. Based on our findings, we propose that the physiological role of these beta-glucosidases in the digestive tract of the termite *Macrotermes bellicosus* workers is the digestion of di-and oligosaccharides derived from plant material celluloses. The enzymes could be used as a tool in the structural analysis of D-glucose containing oligosaccharide chains of glycoproteins, glycolipids and cellulose.

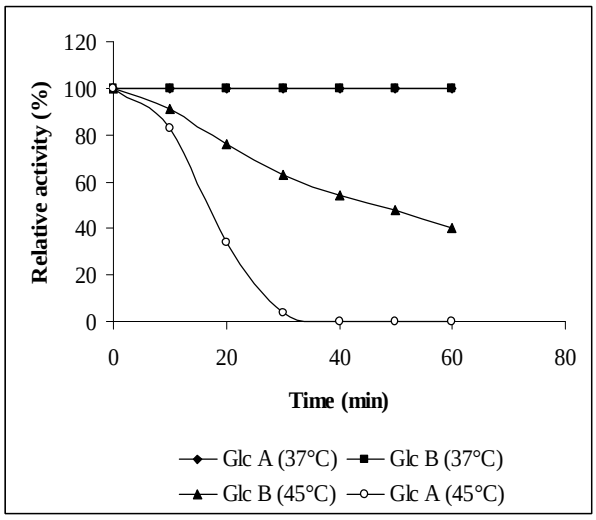

Figure 3: Thermal inactivation of the beta-glucosidases from the termite *Macrotermes bellicosus* workers.

REFERENCES

Azevedo TR, Terra WR, Ferreira C, 2003. Purification and characterization of three beta-glycosidases from midgut of the sugar cane borer, *Diatrea saccharalis*. Insect Biochemistry and Molecular Biology 33: 81-92.

Bernfeld P, 1955. Amylases, α and β. Methods in Enzymology 1: 149-158.

Bhat MK. and Bhat S, 1997. Cellulose degrading enzymes and their potential industrial applications. Biotechnology Advances 15: 583-630.

Cameron RG, Manthey JA, Baker RA, Grohmann K, 2001. Purification and Characterization of a beta-glucosidase from *Citrus sinensis* var. Valencia Fruit Tissue. Journal of Agricultural and Food Chemistry 49: 4457-4462.

Colepicolo-Neto P, Bechara EJH, Ferreira C, Terra WR, 1986. Evolutionary considerations of the spatial organization of digestion in the luminescent predaceous larvae of *Pyrearinus termitilluminans* (*Coleoptera: Elateridae*). Insect Biochemistry 16: 811-817.

Dion M, Fourage L, Hallet JN, Colas B, 1999. Cloning and expression of a beta-glycosidase gene from *Thermus thermophilus*. Sequence and biochemical characterization of the encoded enzyme. Glycoconjugate Journal 16: 27-37.

Esen A, 1992. Purification and partial characterisation of maize (*Zea mays* L.) betaglucosidase. Plant Physiology 98: 174-182.

Falk A. and Rask L, 1995. Expression of a zeatin-O-glucoside-degrading beta-glucosidase in *Brassica napus*. Plant Physiology 108: 1369-1377.

Ferreira AH, Marana SR, Terra WR, Ferreira C, 2001. Purification, molecular cloning, and properties of a beta-glycosidase isolated from midgut lumen of *Tenebrio molitor* (*Coleoptera*) larvae. Insect Biochemistry and Molecular Biology 31: 1065-1076.

Ferreira AH, Ribeiro AF, Terra WR, Ferreira C, 2002. Secretion of beta-glycosidase by middle midgut cells and its recycling in the midgut of *Tenebrio molitor* larvae. Journal of Insect Physiology 48: 113-118.

Ferreira AH, Terra WR, Ferreira C, 2003. Characterization of a beta-glycosidase highly active on disaccharides and of a beta-galactosidase from *Tenebrio molitor* midgut lumen. Insect Biochemistry and Molecular Biology 33: 253-256.

Glew RH, Peters SP, Christopher AR, 1976. Isolation and characterizatiion on of beta-glucosidase from the cytosol of rat kidney cortex. Biochimica et Biophysica Acta 422: 179-199.

Grassé PP, 1982. Termitologia tome I : Anatomie, Physiologie, Reproduction des termites. Masson, Paris. 676p.

Hong SY, An CL, Cho KM, Lee SM, Kim YH, Kim MK, Cho SJ, Lim YP, Kim H, Yun HD, 2006. Cloning and Comparison of third beta-glucoside utilization (bglEFIA) operon with two operons of *Pectobacterium carotovorum* subsp. *carotovorum* LY34. Bioscience Biotechnology and Biochemistry 70: 798-807.

Kang SR, Cho KK, Ahu JK, Bok JD, Kang SH, Woo JH, Lee HG, You SK, Choi YJ, 2005. Three forms of thermostable lactose-hydrolase from sp. IB-21: cloning expression and enzyme characterization. Biotechnology 116: 337-346.

Kouamé LP, Kouamé AF, Niamke SL, Faulet BM, Kamenan A, 2005. Biochemical and catalytic properties of two beta-glycosidases purified from workers of the termite *Macrotermes subhyalinus* (*Isoptera: Termitidae*). International Journal of Tropical Insect Science 25 : 103-113.

Kunst A, Draeger B, Ziegenhorn J, 1984. Colorimetric methods with glucose oxydase and peroxydase In: Bergmeyer HU, editors. Methods of enzymatic analysis 6 : 178-185 Verlag chemie, weinheim.

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Li YK, Chir J, Tanaka S, Chen FY, 2002. Identification of the general acid/base catalyst of a family 3 beta-glucosidase from *Flavobacterium meningosepticum*. *Biochemistry* 41 : 2751-9.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951. Protein measurement with the Folin phenol reagent. Journal of Biology and Chemistry 193: 265-275.

Marana SR, Terra WR, Ferreira C, 1995. Midgut beta-D-glucosidase from *Abracris flavolineata (Orthoptera-Acrididae)*. Physical properties, substrate specificities and function. Insect Biochemistry and Molecular Biology 25: 835-843.

Matoub M. and Rouland C, 1995. Purification and properties of the xylanases from the termite *Macrotermes bellicosus* and its symbiotic fungus *Termitomyces* sp. Comparative Biochemistry and Physiology 112B: 629-35.

Morgan MRJ, 1975. Relationship between gut cellobiase, lactase, aryl-beta-D-glucosidase and aryl-beta-galactosidase activities of *Locusta migratoria*. Insect Biochemistry 5: 609-617.

Mutoh T, Naoi M, Nagatsu T, 1988. Purification and characterization of human liver beta-galactosidase from a patient with the adult form of GM1 gangliosidosis and a normal control. Biochimica et Biophysica Acta 964 : 244-253.

Park SY, Bae EA, Sung JH, Lee SK, Kim DH, 2001. Purification and characterization of ginsenoside Rb1-metabolizing beta-glucosidase from *Fusobacterium* K-60, a human intestinal anaerobic *bacterium*. Bioscience Biotechnology and Biochemistry 65: 1163-1169.

Pratviel-Sosa F, Clement S, Percheron F, Chararas C, 1987. Studies on glycosidases and glucanases in *Thaumetopoea pityocampa* larvae II. Purification and some properties of a broad specificity beta-D-glucosidase. Comparative Biochemistry and Physiology 86B: 173-178.

Priit V, Göran P, Gunnar J, 2001. Mechanism of substrate inhibition in cellulose synergistic degradation. European Journal of Biochemistry 268: 4520-4526.

Rouland C, Civas A, Renoux J, Petek F, 1988. Purification and properties of cellulases from termite *Macrotermes mulleri* (*Termitidae, Macrotermitinae*) and its symbiotic fungus *Termitomyces* sp. Comparative Biochemistry and Physiology 91B: 449-458.

Rouland C, Brauman A, Keleke S, Labat M, Mora P, Renoux J, 1990. Endosymbiosis and exosymbiosis in the fungus-growing termites: Microbiology of poecilotherms (Ed. R. Lesel). Elsevier Science Publishers, 79p.

Rouland C, Matoub M, Mora P, Petek F, 1992. Properties of two beta-glucosidases purified from the termite *Macrotermes mulleri* and from its symbiotic fungus *Termitomyces sp*. Carbohydrates Research 233: 37-245.

Shellman-Reeve JS, 1997. Social competition and cooperation in insects and arachnids, eds. Choe, J. C. & Crespi, B. J. (Cambridge Univ. Press, Cambridge, U.K.), pp. 52–93.

Shewale JG. and Sadana JC, 1981. Purification, characterization, and properties of beta-glucosidase enzymes from *Sclerotium rolfsii*. Archives of Biochemistry and Biophysics 207: 185-196.

Sternberg D, Vijay Kumar P, Reese ET, 1977. beta-Glucosidase: microbial production and effect on enzymatic hydrolysis of cellulose. Canadian Journal of Microbiology 23: 139-147.

Sue M, Ishihara A, Iwamura H, 2000. Purification and characterization of a beta-glucosidase from rye (*Secale cereale* L.) seedlings. Plant Science. 155: 67–74.

Terra WR, Ferreira C, Garcia ES, 1988. Origin, distribution, properties and functions of the major *Rhodnius prolixus* midgut hydrolases. Insect Biochemistry 18: 423-434.

Wallecha A. and Mishra S, 2003. Purification and characterization of two beta-glucosidases from a thermo-tolerant yeast *Pichia etchellsii*. Biochimica et Biophysica Acta 1649: 74-84.

Wase DAJ, Raymahasay S, Wang CW, 1985. Production of beta-glucosidase, endo-1,4-beta-D-glucanase and D-xylanase from straw by *Aspergillus fumigatus* IMI 255091. Enzyme and Microbial Technology 7: 225 229.

Watanabe T, Sato T, Yoshioka S, Koshuima C, Kuwahara M, 1992. Purification and properties of *Aspergillus niger* beta-glucosidase. European Journal of Biochemistry 209 : 651-659.

Wood TG. and Thomas R.J, 1989. The mutualistic association between *Macrotermitinae* and *Termitomyces*, p. 69-92. In Wilding N, Collins NM, Hammond PM, Webber JF (ed.), Insect-fungus interaction. Academic Press, London, United Kingdom.

Workman WE. and Day DF, 1982. Purification and properties of beta-glucosidase from *spergillus terreus*. Applied and Environmental Microbiology 44 : 1289-1295.

Xiangyuan H, Shuheng Z, Shoujun Y, 2001. Cloning and expression of thermostable beta-glycosidase gene from *Thermus nonproteolyticus* HG102 and characterization of recombinant enzyme. Applied Biochemistry and Biotechnology 94: 243-255.

Yamada A, Inoue T, Wiwatwitaya D, Ohkuma M, Kudo T, Abe T, Sugimoto. A, 2005. Carbon mineralization by termites in tropical forests, with emphasis on fungus-combs. Ecology Research 20: 453-460.

Yaovapa T, Toru J, Yoshimasa M, Shigeharu M, Savitr T, Napavarn N, Moriya O,Toshiaki K., 2005. Symbiotic fungi produce laccases potentially involved in phenol degradation in fungus combs of fungus-growing termites in Thailand. Applied and Environmental Microbiology 71: 7696–7704.

Yapi DYA, Niamké LS, Kouamé LP, 2007. Biochemical characterization of a strictly specific beta-galactosidase from the digestive juice of the palm weevil *Rhynchophorus palmarum* larvae. Entomological Science 10: 343-352.