International Journal of Biomolecules and Biomedicine (IJBB)
ISSN: 2221-1063 (Print) 2222-503X (Online)
Vol. 2, No. 3, p. 8-14, 2012
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Characterization of the dual activity of an endo-beta-D-glycosidase from salivary glands of *Macrotermes subhyalinus* little soldier against carboxymethylcellulose and xylan

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Received: 25 October 2012
Revised: 16 November 2012
Accepted: 17 November 2012

Key words: Bifunctional enzyme, carboxymethylcellulase, endo-beta-D-glycosidase, termite little soldier, *Macrotermes subhyalinus*, xylanase.

Abstract

This work reports the characterization of endo-beta-D-glycosidase from salivary gland of little soldier of *Macrotermes subhyalinus*. Based on thin-layer chromatographic analysis of the degradation products, the carboxymethylcellulase activity produced glucose, cellobiose and cellodextrins from carboxymethylcellulose as the substrate. When xylan from Birchwood was used, end products were xylobiose and xylodextrins. The presence of metal ions such as NaCl, $MgCl_2$ and NH_4Cl positively influenced the activity of β-glucosidase but the activity was inhibited in presence of $CuCl_2$, $ZnCl_2$, SDS.

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Introduction

Hemicellulose is a general term for noncellulosic polysaccharides in plant cell walls, and includes xylan, mannan, galactan and arabinan. Among these, xylan is the principal type of hemicellulose (Polizeli *et al.,* 2005). So, termites can digest 74-99% of cellulose and xylan in wood which are respectively linear homopolymers of glucose linked by β-1,4 glycosidic bonds and Xylose linked by β-1,4 glycosidic bonds (Breznak and Brune, 1994 ; Tseng *et al.,* 2002).

Cellulases responsible for the hydrolysis of the β-1,4-glucosidic bonds in cellulose are subdivided into either non-processive cellulases (endocellulases) or processive cellulases (including different exocellulases and some new processive endocellulases) (Barr *et al.*, 1996 ; Reverbel-Leroy, 1997). During the degradation of cellulose, non-processive cellulases and processive cellulases have been found to work synergistically (Irwin *et al.*, 1993 ; Li-Jung *et al.,* 2010). It has been generally accepted that effective biological hydrolysis of cellulose into glucose requires synergistic actions of three enzymes including endo-β-1,4-glucanase (EC 3.2.1.4, EG, randomly cleaving internal linkages), cellobiohydrolase (EC 3.2.1.91, CBH, specifically hydrolyzing cellobiosyl units from non-reducing ends), and β-*D*-glucosidase (EC 3.2.1.21, hydrolyzing glucosyl units from cellooligosaccharides) (Perez *et al.,* 2002).

Many studies have revealed that salivary glands of termites contribute to cellulose and xylan digestion (Watanabe *et al.,* 1997, 1998 ; Tokuda *et al.*, 1999; Bléi *et al.*, 2010). In this respect, Nakashima *et al.* (2002) have found that salivary glands of the termite *Coptotermes formosanus* workers was essential to hemicelluloses degradation. A new endo-beta-D-glycosidase from salivary glands of Macrotermes subhyalinus little soldier with a dual activity against carboxymethylcellulose and xylan has also been isolated and partially described by fagbohoun *et al.*, 2012.

The purpose of this study is to complete the description of this new enzymatic activity

Materials and methods

Enzymatic source and preparation of crude extract

Little soldiers of the termite *Macrotermes subhyalinus* were from the savannah of Lamto (Toumodi, Côte d'Ivoire). They were collected directly from their nests and then stored frozen at -20°C. Salivary glands (10 g) were dissected and homogenized with 20 mL 0.9 % NaCl (w/v) solution using a blender (Ultra-Turrax) and then sonicated as previously described by Rouland *et al.* (1988a). The homogenate was centrifuged at 20,000 x g for 15 min. The collected supernatant constituted the crude extract. After freezing at -180°C in liquid nitrogen, the crude extract was stored at -20°C (Kouamé *et al.,* 2005).

Chemicals

Oligosaccharides and *p*-nitrophenylglycopyranosides were purchased from Sigma Aldrich. Bovine serum albumin (BSA) was from Bio-Rad. All other chemicals and reagents were of analytical grade.

Enzyme assays

Under the standard test conditions, xylanase or cellulase activity was assayed spectrophotometrically by measuring the release of reducing sugars from Birchwood xylan or carboxymethylcellulose (CMC). The reaction mixture (0.38 ml) contained 0.2 ml of 0.5% xylan or CMC (w/v) dissolved in 20 mM acetate buffer (pH 5.0) and 0.1 ml enzyme solution. Determination of other polysaccharidase activities was carried out under the same experimental conditions. The reference cell contained all reactants except the enzyme. After 30 min of incubation at 45°C, the reaction was terminated by adding 0.3 ml of dinitrosalicylic acid solution (Bernfeld, 1955) followed by 5 min incubation in a boiling water bath. The tubes were cooled to room temperature for 10 min and 2 ml

of distilled water was added. The product was analysed by measuring the optical density at 540 nm.

The disaccharidase activity was determined by measuring the amount of glucose or xylose liberated from disaccharide by incubation at 45°C for 30 min in a 20 mM acetate (pH 5.0), containing 10 mM disaccharide. The reference cell contained all reactants except the enzyme. The amount of glucose was determined by the glucose oxidase-peroxidase method (Kunst *et al.,* 1984) after heating the reaction mixture at 100°C for 5 min. The hydrolysis of xylobiose was assayed by withdrawing aliquots (100 µl) which were heated at 100°C for 5 min. After filtration through a 0.45 µm hydrophilic Durapore membrane (millipore), the reaction mixture (20 µl) was analysed quantitatively by HPLC at room temperature. Chromatographic separation of sugars (xylobiose and xylose) were performed on a Supelcosyl LC-NH2 (5 µm) column (0.46 x 25 cm) from Supelco® using acetonitrile/water (75: 25; v/v) as the eluent, and monitored by refractometric detection. The flow rate was maintained at 0.75 ml min-1 (Kouamé *et al.,* 2005).

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

One unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing one µmol of reducing sugar per min under the defined reaction conditions. Specific activity was expressed as units per mg of protein (U/mg of protein). Protein concentrations were determined spectrophotometrically at 660 nm by method of Lowry et al. (1951) using bovine serum albumin as a standard.

Effect of some chemical agents

To determine the effect of various compounds (cations, detergents, sulfhydryl specific and reducing agents) as possible activators or inhibitors, the purified enzyme was preincubated at 37°C for 1 hour with the compounds and the activity was assayed under the enzyme assay conditions. Residual activities were expressed as percentage referred to control without chemical agents.

Determination of kinetic parameters

The kinetic parameters (KM, Vmax and kcat/KM) were determined in 20 mM acetate buffer (pH 5.0) at 45°C. Hydrolysis of xylans (Birchwood and Beechwood) or carboxymethylcellulose was quantified on the basis of released reducing sugars similarly as in the standard enzyme assay. KM and Vmax were determined from Lineweaver-Burk plot using different concentrations of xylan (2.0 - 10.50 mg/ml, w/v) and carboxymethylcellulose (2.0 - 10.50 mg/ml,w/v).

Thin-layer chromatography analysis of hydrolysates

The reaction mixture consisting of 0.1 mL of carboxymethylcellulose or xylan from Birchwood (0.5%, w/v) in 20 mM acetate buffer (pH 5.0) and 0.1 mL of enzyme was incubated at 45°C for up to 24 h. Aliquots (50 µL) were withdraw at regular time intervals and the reaction was stopped by heating at 100°C for 5 min. Hydrolytic products were analyzed by applying samples (5 µL) to TLC plates (silica 60 F_{254}) using butanol/ethanol/water (3:5:2, v/v/v) as the mobile phase system. The sugar plots were developed with 3% (w/v) phenol in sulphuric acid/ ethanol (5:95, v/v) and visualized at 110°C for 5 min.

Results

Substrate specificity and kinetic parameter

The purified enzyme was assayed for hydrolytic activity against a variety of natural and synthetic substrates (Table 1). After 30 min of incubation, this glycosidase could not release reducing sugar from inulin, starch, polygalacturonic acid, sigmacel 50 cellulose and cellulose microcristallin. It's also did not hydrolyzed cellobiose, xylobiose and all synthetic substrates. Although the enzyme degraded CMC and xylans (Birchwood and Beechwood) (Table 1).

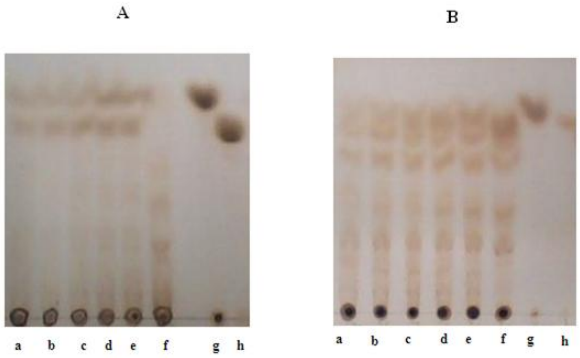

Fig. 1. Time course of end products from (A) carboxymethylcellulose or (B) xylan from Birchwood hydrolysis for a carboxymethylcellulase/xylanase from little soldier salivary glands of the termite Macrotermes subhyalinus. (A): a=30 min; b= 1h; c= 2h; d= 4h; e= 6h; f= 24h; g= xylose h= xylobiose. (B): a=30 min; b= 1h; c= 2h; d= 4h; e= 6h; f= 24h; g= glucose; h= cellobiose.

Table 2 summarizes the data obtained from Lineweaver-Burk plots for the purified enzyme on three types of substrates, CMC and xylans. The K_M, V_{max} and V_{max}/ K_M values are reported in Table . The catalytic efficiency of the enzyme, given by the V_{max}/ K_M ratio is much higher for xylans than CMC (Table 2).

Thin-layer chromatography analysis of hydrolysate

To test whether the purified enzyme is an endoglycosidase, Beechwood xylan and carboxyméthylcellulose hydrolyzed by the purified enzyme. The hydrolysis products were analyzed TLC (Figure 1A, 1B). Within the first 24 H of the reaction, carboxymethylcellulose and Beechwood xylan produced the expected oligosaccharides, disaccharides.

Table 1. Substrate specificity of purified carboxymethylcellulase and xylanase from little soldier salivary glands of the termite *Macrotermes subhyalinus*. Values given are the averages of at least three experiment.

Substrat	Concentration	Activity hydrolysis (%)
Carboxymethylcellulose	2.6 mg/ml	100
Xylan (Beechwood xylan)	2.6 mg/ml	120.56±3.76
Xylan (Birchwood xylan)	2.6 mg/ml	124.61±5.29
Cellulose microcristalline	2.6 mg/ml	0.00
Sigmacel 50-Cellulose	2.6 mg/ml	0.00
Polygalacturonic acid	2.6 mg/ml	0.00
Starch	2.6 mg/ml	0.00
Inulin	2.6 mg/ml	0.00
Cellobiose	2.6 mg/ml	0.00
Xylobiose	5 mM	0.00
p-NP-β-D-Xylopyranoside	5 mM	0.00
p-NP-β-D-Glucopyranoside	5 mM	0.00
p-NP-β-N-Acetylglucopyranoside	5 mM	0.00
p-NP-β-D-Fucopyranoside	5 mM	0.00
p-NP-β-D-Galactopyranoside	5 mM	0.00
p-NP-β-D-Galacturonide	5 mM	0.00
p-NP-N-Acetylgalacturonide	5 mM	0.00
p-NP-β-D-Cellobioside	5 mM	0.00
p-NP-α-D-Glucopyranoside	5 mM	0.00
p-NP-α-D-Fucopyranoside	5 mM	0.00
p-NP-α-L-Fucopyranoside	5 mM	0.00
p-NP-α-D-Xylopyranoside	5 mM	0.00
p-NP-α-D-Mannopyranoside	5 mM	0.00
p-NP-α-D-Arabinopyranoside	5 mM	0.00

Effect of chemical agents on enzyme activity

Chemical agents $CuCl_2$, $ZnCl_2$, SDS, showed an inhibitory effect on cellulase and xylanase activities of the purified enzyme. However, the other entire chemical tested had no effect on the same activities (Table 3).

Table 2. Kinetic parameters of purified carboxymethylcellulase and xylanase from little soldier salivary glands of the termite *Macrotermes subhyalinus* towards carboxymethylcellulose, xylan from Birchwood and xylan from Beechwood. The Michaelis constants (KM) and the maximum velocities (Vmax) are expressed as mM and units/mg protein, respectively.)

Substrate	K_M	V_{max}	V_{max}/K_M
Carboxylmethylcellulose	0.77	14.22	18.47
Xylan Birchwood	0.38	11.62	30.57
Xylan Beechwood	0.47	11.21	23.85

Table 3. Effect of chemical agents on the carboxymethylcellulase and xylanase from little soldier salivary glands of the termite *Macrotermes subhyalinus*.

Reagent	Concentration (mM)	Relative Activity (%)	
		Carboxymethylcellulase activity	Xylanase activity
Témoin	0	100	100
NaCl	0.1	155.69±1.21	158±2.56
	1	172.42±3.08	180.77±3.29
$SrCl_2$	0.1	113.25±4.77	102.05±2.05
	1	91.05±3.65	106.52±3.17
$BaCl_2$	0.1	101.30±3.46	102±1.09
	1	116±5.22	98.72±5.54
$CuCl_2$	0.1	66.66±1.37	46.48±4.21
	1	21.78±2.14	30.57±2.27
$ZnCl_2$	0.1	103.47±5.27	111.28±6.32
	1	87.17±3.07	90.84±8.21
$MgCl_2$	0.1	99.07±4.27	105.09±3.16
	1	100±3.04	103.40±2.70
NH_4Cl	0.1	107.91±4.68	108.33±2.13
	1	101±1.07	104.21±2.42
[a]**EDTA** (%, W /v)	0.1	100.59±2.54	101.22±5.39
	1	98.16±4.38	94.55±2.35
[b]***p*CMB** (%, W /v)	0.1	95.45±2.34	90.92±2.12
	1	98.48±1.58	99.34±2.47
[c]**SDS** (%, W /v)	0.1	43.33±1.70	52.67±2.28
		23.54±1.11	15.45±1.08
[d]**DTNB** (%, W /v)	0.1	100.21±1.38	99.67±1.85
	1	98.03±1.65	97.44±2.02

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

Discussion

A new enzyme with dual activity (carboxyméthylcellulase and xylanase) from little soldier salivary glands of the termite *M. Subhyalinus* was purified. A variety of natural and synthetic of glycosides were tested for their ability to serve as substrates for the purified enzyme. This enzyme as no contaminating glycosidase activities such as glucosidase, xylosidase, fucosidase, galactosidase and mannosidase. It was also inactive on other high molecular mass polymers such as inulin, starch, polygalacturonic acid, sigmacel 50 cellulose and cellulose microcristallin. Only xylans and carboxymethylcellulose were hydrolysed by the purified enzyme. From these result it appears that the purified enzyme is a bifunctional polysaccharidase.

Similar observations have been reported for the bifunctional polysaccharidase from *Aspergillus niger* (Ferderick *et al* 1985), *Fibrobacter succinogenes* 585 (Matte *et al*., 1992), *Trichoderma konigüse* ATCC 26113 (Kim *et al*., 1993) and symbiotique fungus *Termitomyces* sp of the termite *M. Subhyalinus* (Faulet *et al*., 2006 b).

Twenty four (24) hours hydrolysis of carboxyméthylcellulose and xylan from Birchwood produced oligosaccharides, disaccharides, and monosaccharides. These result indicated that the purified enzyme randomly cleaved internal beta-1,4 glucosidic and beta-1,4 xylosidic. After 24 h, no evident producing of glucose, cellobiose was shown, suggesting the presence of glycosyl transfer reaction in the cellulase active site.

The purified enzyme was sensitive to $CuCl_2$ and SDS so that these chemicals must be eliminated after treatment of the oligosaccharides or glycoprotein substrate when this needs to be denatured, before its hydrolysis by the enzyme. A similar result was reported for xylanases (Faulet et al., 2006a) and cellulase (Séa *et al.,* 2006) from worker of the termite *M. Subhyalinus*. Furthermore, the purified enzyme showed very high level of xylanase and cellulase activities in presence of NaCl. These result suggested that sodium ions not only plays important role in interaction of enzyme with substrates but also enhance its catalytic efficiency (Badhan *et al.,* 2008).

The presence of this bifunctional enzyme in the salivary gland of the little soldier of this termite reveals its role in the digestion process of woody resources. However the weakness of this activity could presage the presence of inhibitory substances. The worker of the termite *M. subhyalinus* could regurgitate its own salivary glands secretions to supplement the digestive needs of the soldier of the same insect that live in close association with him. It is possible that this natural phenomenon is done essentially to complete the soldier enzymatic activities in its digestive tract. The next stages of the studies will be focused on the identification of substances responsible for inhibition of the enzymatic activities in the salivary secretions.

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